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Investigating the associations of mucosal P2Y6 receptor expression and urinary ATP and ADP concentrations, with symptoms of overactive bladder

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Author contributions

The Authors contributed to the study as follows:

Conception and design: SF, LA, JT, FA, SN, SJ, BJ, SEML, CHF, JSY

Acquisition of data: SF, JT, JSY

Analysis and interpretation of data: SF, JSY

Drafting the manuscript: SF, JSY

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The authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Abstract

Aims. To characterise purinergic signalling in overactive bladder (OAB).

Methods. Mucosal biopsies were taken by flexible cystoscopy from patients with storage symptoms referred to Urology Departments of collaborating hospitals. Immunohistochemistry (n=12) and western blotting (n=28) were used to establish the qualitative and quantitative expression profile of P2Y6 in human mucosa. Participants from the general population provided a mid-stream urine sample. Bioluminescent assays were used to quantify ATP (n=66) and ADP (n=60) concentrations, which were normalised to creatinine (Cr) concentration. All participants completed a questionnaire (ICIQ-OAB) to score urinary symptoms of OAB.

Results. P2Y6 immunoreactivity, more prominent in the urothelium (co-localised with the uroepithelial marker pan-cytokeratin), was more greatly expressed in OAB compared to age- and gender-matched controls (BPH) without OAB symptoms. Mucosal P2Y6 was positively correlated only with incontinence (p=0.009). Both urinary ATP and its hydrolysis product, ADP, an agonist to P2Y6, were positively correlated with total OAB symptom score (p=0.010 and p=0.042, respectively).

Conclusions. The positive correlation of P2Y6 only with incontinence may indicate a different phenotype in OAB wet and warrants further investigation. Positive correlations of ATP and ADP with total OAB symptom score demonstrate upregulation in purinergic signalling in OAB; shown previously only in animal models. Further research is required to validate whether purinoceptors are indeed new therapeutic targets for this highly prevalent symptom complex.

Keywords: Urinary Bladder; Overactive Bladder; Purinoceptor; ATP; ADP.

Introduction

Overactive bladder (OAB) is a symptom complex characterised by bothersome symptoms of urinary urgency, increased frequency, nocturia, with or without urge incontinence; in the absence of proven infection or other obvious pathology [1]. Despite being a highly prevalent condition, present in 9% to 43% of females and 7% to 27% of males [2], the underlying mechanisms of idiopathic OAB are not fully understood. Given that symptoms are associated with urine storage, research has focussed on mechanisms underlying the perception of bladder fullness. Distension of the urothelium during filling elicits non-neuronal release of ATP [3] which activates P2X3 and P2X2/3 purinoceptors on sub-urothelial nerve afferents [4]. Upregulation of ATP release has been demonstrated in *in vitro* preparations from patients with idiopathic OAB with detrusor overactivity (DO) [5] and manifests in increased urinary ATP concentration [6].

Release of ATP is itself likely to be modulated by P2Y purinoceptors; although the majority of the evidence for this comes from animal studies. P2Y agonists evoke ATP release [7], increase spontaneous contractions of the detrusor [8] and increase the frequency of voiding [9], to the point of evoking DO [10]. Underpinning these studies is the observation of P2Y-receptor mediated ATP release, suggesting an autocrine and / or paracrine feedback mechanism to enhance further release of ATP from the urothelium [7]; therefore, changes in the expression of P2Y receptors and / or molecules involved in its signalling pathway may amplify the bladder's sensory responses seen in bladder conditions such as OAB. P2Y6 is expressed in the human bladder mucosa and its involvement in release of ATP from the urothelium has been demonstrated [11][12]. Therefore, any changes in P2Y6 expression may contribute to the symptoms of OAB.

The aim of this study was to characterise purinergic signalling in OAB; focussing on expression patterns of P2Y6 receptors in human mucosa layer, and investigating the associations of P2Y6 receptor expression and urothelial-derived signalling mediator, ATP and its hydrolysis product, ADP, with characteristic symptoms of OAB.

Subjects and Methods

Participant recruitment and methods performed on data and samples are together summarised in Figure 1.

Human tissue and data collection

Participants for P2Y6 quantification analysis

Between 2010 and 2012, thirty-two participants were recruited from patients with storage symptoms referred to a specialist Urology Department at the Royal Surrey County Hospital, UK, for cystoscopic assessment. It is routine practise in the UK for small biopsies of mucosa to be taken by a flexible cystoscope for histological assessment. Ethical approval was given (REC: 10/H1109/60) for an additional biopsy of mucosa to be taken by flexible cystoscope for the purposes of this study. Participants completed the ICIQ-OAB questionnaire to record their urinary symptoms and associated bother. Of the 32 participants, four biopsy samples were used for method optimisation and the remaining samples (n=28) were used for P2Y6 quantification via Western blotting.

Participants for mucosal P2Y6 expression pattern analysis

Between 2015 and 2016, fourteen participants were recruited from patients with storage symptoms referred to a specialist Urology Department at the Shohada-e-Tajrish Hospital, Iran, for cystoscopic assessment. All participants completed a questionnaire (ICIQ-OAB) to score urinary symptoms of OAB. As in the UK, histological assessment of a small mucosal biopsy is routine and ethical approval was given (REC: 13/SC/0501) for an additional mucosal biopsy to be taken. During clinical examination, ten participants were diagnosed as having OAB and two were diagnosed as having benign prostatic hyperplasia and two participants were excluded due to missing clinical information. The P2Y6 expression of the mucosal biopsies from those twelve participants were characterised by immunohistochemistry.

Participants for urinary ATP and ADP concentration analyses

One hundred and thirteen volunteer participants were recruited (2014-2016, REC: 13/SC/0501) according to the inclusion and exclusion criteria (see below) to this study.

Consented participants were asked to complete the ICIQ-OAB questionnaire and to provide a fresh midstream urine sample. Microscopic examination, dipstick urinalysis and chromogenic urinary tract infection medium test were immediately performed on a small proportion of each collected urine sample. According to the performed tests, 10 participants were diagnosed as having with yeast / bacterial infection or haematuria and four withdrew consent without reason, and therefore these participants were excluded from the study. The remaining urine samples (n=95) were utilised for ATP and ADP analyses.

Inclusion Criteria: Male or female participants aged ≥18 and able to give informed consent for participation in the study.

Exclusion Criteria: Male or female participants aged ≤18; taking any medication for OAB; unable to give informed consent; diagnosed with neurologic disease (stroke, MS, Parkinson's disease, spinal cord injury); have a history of uterine, cervical, vaginal or urethral cancer; history of cyclophosphamide use or any type of chemical cystitis; history of benign or malignant bladder tumours; have had Botulinum toxin injections into the bladder, neuromodulation or augmentation cystoplasty.

Mucosal P2Y6 expression analysis

Bladder biopsies were immediately washed in PBS and fixed with 4% paraformaldehyde in PBS (sc-281692, Santa Cruz biotechnology, US) overnight at 4°C. Biopsy samples were then washed three times in PBS for 10 minutes at room temperature (RT), then stored in PBS with 0.02% sodium azide (sc-296028, Santa Cruz biotechnology, US) at 4°C and transferred to the University of Portsmouth (UK) for further processing and analysis. Biopsy samples were incubated in 30% sucrose solution overnight at 4°C, then embedded in OCT mounting medium (361603E, VWR, UK) and frozen. Cryosections (10 µm; 2-3 per slide) were mounted on Superfrost Plus™ microscope slides (J1800AMNZ, ThermoFisher Scientific, UK) and stored at −20°C until use. Cryosections were incubated with a blocking solution (2.5% normal horse serum; S-2012, Vector laboratories, UK) for 1 h at RT followed by incubation in mixture of primary antibodies, rabbit Anti-P2Y6 antibody (ab92504, 1:100, Abcam, UK) and mouse anti-pan cytokeratin antibody (ab86734, 1:166, Abcam, UK) diluted in Tris-buffered saline containing 0.3% Triton-X100 (TBS-Tx) at RT for 2 h. Sections were then washed three times in TBS-Tx and incubated in a mixture of appropriate secondary antibodies conjugated with Alexa

Fluor 647 (donkey anti-rabbit IgG, 711-605-152, Jackson ImmunoResearch Laboratories, US) and Alexa Fluor 555 (goat anti-mouse IgG, ab150118, Abcam, UK) for 1 h at RT. The sections were then washed three times with TBS-Tx for 10 min, air dried and mounted with Vectashield® mounting medium with DAPI (H-1200, Vector Laboratories, UK). Slides were viewed at ×40 magnification using a confocal laser-scanning microscope (LSM710, Zeiss, Germany). Images were acquired using ZEN 9000 software and analysed by Image-J software. Presented immunofluorescence staining figures are representative images. A whole blot is shown in Supplementary Figure 1.

P2Y6 quantification analysis

Biopsies were immediately immersed in either Krebs physiological solution (120mM sodium chloride (NaCl), 15.5mM sodium hydrogen carbonate ($NaHCO_3$), 2.5mM magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 5.9mM potassium chloride (KCl), 1.2mM potassium dihydrogen orthophosphate (KH_2PO_4)) and were transferred to the University of Portsmouth. Samples were immediately snap-frozen in liquid nitrogen and were lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor (RIPA; Pierce 89900, Thermo Scientific, UK) using a mortar and pestle and were stored at -80 ^{o}C freezer until use. Protein concentrations of lysate samples were determined using bicinchoninic acid protein assay kit (Pierce 23227, ThermoFisher Scientific, UK), according to the manufacturer's instructions. Ten micrograms of protein from each sample was separated using 10% SDS-PAGE and were wet transferred onto polyvinylidene difluoride membranes (overnight, 4°C). Membranes were then blocked with 5% milk-phosphate buffered saline (milk-PBS) for 1 h at RT. The membranes were then incubated with the mixture of primary antibodies, rabbit anti-P2Y6 antibody (ab92504, 1:1000, Abcam, UK) and anti-beta Actin (β-actin) antibody (ab8227, 1:1000, Abcam, UK) diluted into 3% milk-PBS for 2 h, following which they were washed three times for 10 minutes in PBS-Tween (0.1%). Membranes were then incubated in an appropriate mixture of secondary antibodies conjugated with horseradish peroxidase including Goat Anti-Rabbit IgG (170-6515, Bio-Rad, UK) and Goat Anti-Mouse IgG (170-6516, Bio-Rad, UK) diluted in 5% Milk-PBS for 1 hr. The membranes were then washed three times for 10 minutes in PBS-Tween (0.1%). Immuno-reactive bands were detected via an enhanced chemiluminescence reagent (Clarity™, 1705061, Bio-Rad, UK / Luminata Forte, WBLUF0100, Merck Millipore, UK) and

were viewed using a Syngene / Bio-Rad chemiluminescent camera. The densities of the immunoreactive bands on the western blot images were quantified using ImageJ software and were standardised to their relative β-actin (i.e. P2Y6/β-actin) expression levels. The included western blot figure in this study is a representative blot. The researchers were blinded to participants' ICIQ-OAB questionnaire information whilst running tests on the biopsy samples. Any participant who failed to complete part of the ICIQ-OAB questionnaire was excluded from the corresponding correlation analysis.

Urinary ATP and ADP concentration analyses

Microscopic examination, dipstick urinalysis and urine culture with chromogenic urinary tract infection test (PO0794A, Thermo Scientific, UK) were immediately performed on a small proportion of each collected urine sample. Details of the participants that this excluded are shown in Figure 1. The remainder of each eligible participant's urine sample was centrifuged (at 4000 rpm, 10 mins, at 4°C), separated into cell pellet and supernatant and stored at -80°C until use. The urinary (cell-free) concentrations of ATP and ADP were measured in duplicate using ENLITEN® ATP assay system bioluminescence detection kit (FF2000, Promega, UK) and ADP assay kit (MAK133, Sigma-Aldrich, UK), respectively. The researchers were blinded to participants' ICIQ-OAB questionnaire information whilst running tests on the urine samples. Any participant who failed to complete part of the ICIQ-OAB questionnaire or with a urinary ATP or ADP value outside the detection limit (standard curve) of the assays, was excluded from the corresponding correlation analysis.

Data and Statistical Analysis

D'Agostino-Pearson normality test was performed on all the generated data. Pearson product-moment correlation coefficient (parametric) or Spearman's rank correlation coefficient (non-parametric) were used for correlation analyses on untransformed data. GraphPad Prism 8.0.0 software was used for all the analyses and the preparation of lin-log correlation graphs. Statistical significance was observed when the p-value was ≤0.05.

Results

Immunolocalisation of P2Y6 receptors in human bladder mucosa biopsies

The presence of P2Y6 receptors in human bladder mucosa biopsies (n=12) was confirmed by immunofluorescence confocal microscopy. P2Y6 labelling was observed in both layers of human mucosa, i.e. urothelium and sub-urothelium layers (Figure 2). Analysis focussed on P2Y6 expression in mucosa at the two extremes of the ICIQ-OAB scores: a male participant diagnosed with OAB (ICIQ-OAB score: 13) and an age-matched male control with BPH (ICIQ-OAB score: 2) (Figure 2). The immunoreactivity for P2Y6 was more prominent in the urothelium layer of the OAB biopsy (Figure 2B) compared to the control biopsy (Figure 2A), where higher co-localisations of P2Y6 receptors with urothelial cells were observed in the OAB biopsy sample (Figure 2B; yellow in the merged images).

Correlations between the expression levels of P2Y6 receptors in human bladder mucosa biopsy samples and OAB-associated clinical characteristics

The expression levels of P2Y6 receptors were studied in 28 human bladder mucosa biopsy samples and were correlated with participants' OAB-associated clinical characteristics measured using the ICIQ-OAB questionnaire. These characteristics included individual and total OAB symptom scores and age. Specific immunoreactivity for P2Y6 protein in human mucosa samples was observed at the expected molecular weight of 42 KDa (Figure 3Ai). P2Y6 expression, normalised to β-actin (i.e. P2Y6/β-actin), positively correlated with incontinence severity (Table 2). Expression did not correlate with total ICIQ-OAB symptom score (Figure 3Aii), nor frequency, nocturia, urgency scores or age (Table 2).

Correlations of the urinary levels of ATP and ADP with OAB-associated clinical characteristics

The urinary concentrations of ATP and its hydrolysis product, ADP, were measured in 95 human urine samples. Measured urinary ATP and ADP levels were standardised to their

corresponding urinary creatinine concentrations (ATP/Cr; ADP/Cr) and their relationships with participants' OAB-associated clinical characteristics and age were investigated (Figure 3B,C).

Significant positive correlations were observed between the urinary levels of [ATP]/[Cr] and the total ICIQ-OAB symptom score (Figure 2B) and the frequency score (Table 2). Urinary [ATP]/[Cr] did not correlate with nocturia, urgency, incontinence scores or age (Table 2).

A significant positive correlation was observed between the urinary levels of [ADP]/[Cr] and the total ICIQ-OAB symptom score (Figure 2C). Urinary [ADP]/[Cr] did not correlate with individual symptoms or age (Table 2).

Discussion

The lack of understanding of the pathophysiological mechanisms underlying the development of OAB and its phenotypes has led to misdiagnosis, underdiagnosis and delayed treatment. Better symptom specific characterisation may help to better identify phenotypes and eventually lead to better treatment selection. Therefore, the aim of this study was to characterise the relationship between purinergic signalling and a spectrum of OAB-associated clinical characteristics.

We investigated the expression of P2Y6 purinoceptors in the mucosa layer of the human bladder, given compelling evidence from animal studies of modulation of non-neuronal ATP release by P2Y6 [9][10]. P2Y6 immunofluorescence was observed in urothelial and sub-urothelial layers of human mucosa (Figure 2). Its expression was elevated in the mucosa of OAB patients compared to control patients (BPH) not exhibiting OAB symptoms; with a previous study having shown that urothelial P2Y6 is unaltered in BPH compared to controls [12]. The qualitative difference in expression with OAB is illustrated in Figure 2, which compares expression in an OAB patient (ICIQ-OAB score of 13) versus an age-matched asymptomatic (ICIQ-OAB score of 2) control.

To further investigate this observation and the idea that P2Y6 may be a therapeutic target for OAB, P2Y6 expression was quantified and correlated with total OAB symptom severity and the severity of individual symptoms, given a number of symptom combinations (OAB phenotypes) observed clinically [13]. A significant positive correlation was observed between the mucosal expression levels of P2Y6, normalised to β-actin (i.e. P2Y6/β-actin), and the severity of incontinence (Table 2). There was, however, no correlation between P2Y6/β-actin and the total ICIQ-OAB symptom score (Figure 3Aii), nor frequency, nocturia, urgency scores or age (Table 2).

A positive correlation between P2Y6/β-actin and, of all symptoms, only incontinence requires interpretation. As recruitment of participants did not include urodynamics, we therefore cannot definitely rule out a contribution of SUI; however, given that the majority of participants in this part of the analysis were male (18/28), its contribution is likely to be

insignificant. Rather, as many OAB patients do not initially present with incontinence ('OAB dry'), this symptom may, itself, may be seen as further development of the symptom complex [14] and thus P2Y6 expression a phenotype of this development; or 'OAB wet' is in itself an OAB phenotype, with a different pathophysiological basis that includes altered P2Y6 expression. Future studies should further investigate the relationship between P2Y6 expression in OAB-dry and OAB-wet, and with larger participant numbers.

Previous studies have shown that the activation of P2Y6 receptors modulates bladder urodynamics in anaesthetized rats; increasing voiding frequency [9] and inducing detrusor overactivity [10]. These effects were associated with an increase in urothelial ATP release, prompting our investigation into OAB symptom-associated changes associated with urinary ATP. For this, we chose to recruit from the general population, in order to give a broader spectrum of OAB symptom severity. ATP concentration, normalised to creatinine (i.e. [ATP]/[Cr]), was positively correlated with frequency and total symptom severity (Figure 2B, Table 2). While others have shown elevated [ATP]/[Cr] with DO [6] and elevated stretch-evoked ATP release from the mucosa of DO patients [5], our study is the first to our knowledge to treat OAB symptom severity as a continuum. Participants in our study had, on average, relatively lower OAB symptom score severity (Table 1) compared to previous studies, however, the observation of significant correlations with the total ICIQ-OAB symptom scores suggests that ATP is elevated even with mild OAB; an observation that adds weight to the argument that it could be useful diagnostic biomarker. However, studies have shown that ATP is elevated in pyuria [15]; interstitial cystitis [16]; and BPH [17], so a lack of specificity may limit its usefulness. Future studies may want to include additional measures (such as more detailed questionnaires of urinary symptoms) to be certain of excluding these groups when recruiting from the general population.

In addition to the issue of whether ATP is specific to OAB is the observation that ATP is hydrolysed into ADP through ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) present in the mucosa layer. Little is known about the role and expression levels of these ectoenzymes in the human bladder in health and disease. Carneiro et al. 2014 reported that dephosphorylation of ATP in rat predominantly occurs in the urothelium layer and to a lesser extent in the sub-urothelium and detrusor layers [9]. So urinary ADP may be a

more suitable indicator of OAB progression than ATP. ADP is, in itself, a ligand for P2Y6 [18] and we have shown its effects in an animal model of neurogenic OAB [8]. Therefore, the urinary levels of ADP and its association with OAB symptom severity was also investigated. Similar to ATP, a significant positive correlation was observed between the urinary levels of [ADP]/[Cr] and the total ICIQ-OAB symptom score (Figure 2C). Urinary [ADP]/[Cr] did not correlate with individual symptoms or age (Table 2).

Limitations

Samples from the same participants would allow multivariate analysis, but due to the sequential nature of this study, this was not possible; perhaps explaining the disparity in correlations between P2Y6/β-actin; [ATP]/[Cr]; [ADP]/[Cr] and expression with OAB-associated clinical characteristics.

In order to more fully address the hypothesis that OAB is characterised by an upregulation in purinergic signalling, the study would have benefitted from additional purines to ADP which act on P2Y6; specifically, UDP, 5-bromo-UPT and UTP [18]. Limitation in the methods used to measure these chemicals need to be overcome in order to allow a more complete study.

The lack of urodynamic characterisation of recruited participants, such as to identify stress or mixed urinary incontinence, is a limitation of our study. It is not routine practise in the UK to perform urodynamics for uncomplicated storage symptoms and this is reflected in guidelines, such as the AUA/SUFU 'diagnosis and treatment of overactive bladder (non-neurogenic) in adults.

The collection sites for the different experimental tests did not control for the age and gender of the subjects which vary significantly across the sites. Future studies should address this shortcoming.

We cannot rule out the possibility that OAB was secondary to outflow obstruction. Future studies should therefore either involve participants with broad range of OAB symptoms in order to allow the identification of further OAB phenotypes, and should be extended to include older adults and those with mixed urinary incontinence; or include more rigorous recruitment criteria.

Conclusions

The positive correlation of P2Y6 only with incontinence may indicate a different phenotype in OAB wet and warrants further investigation. Positive correlations of ATP and ADP with total OAB symptom score demonstrate upregulation in purinergic signalling in OAB; shown previously only in animal models. Further research is required to validate whether purinoceptors are indeed new therapeutic targets for this highly prevalent symptom complex.

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Data Availability

The data that were generated and / or analysed for this study are available from the corresponding author upon request.

Conflict of Interest

The authors declare no conflicts of interest.

List of abbreviations

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate

β-actin: Beta actin

DO: Detrusor overactivity

E-NTPDase: Ectonucleoside triphosphate diphosphohydrolases

ICIQ-OAB: International consultation on incontinence questionnaire - overactive bladder

NRES: National research ethics service

OAB: Overactive bladder

RT: Room temperature

TBS-Tx: Tris-buffered saline containing 0.3% Triton-X100

Figure Legends

Figure 1. Flow diagram of participants recruitment, selection and subsequent tests.

OAB: overactive bladder; BPH: benign prostatic hyperplasia; ICIQ-OAB = International consultation on incontinence questionnaire - overactive bladder; [a] = see Methods and Materials section for inclusion and exclusion criteria.

Figure 2. Representative images of immunolocalisation of P2Y6 receptors in human bladder mucosa biopsies from control (BPH) and OAB individuals.

Cryosections (10µm) of human bladder biopsies were labelled with antibodies to P2Y6 (green); urothelium layer is labelled with pan-cytokeratin antibodies (red); nuclei are labelled with DAPI (blue). White arrow: denotes co-localisation (yellow in the merged images) of P2Y6 receptors with urothelial cells; images were acquired using confocal microscopy, magnification: 40X, scale bar=20µm; BPH: benign prostatic hyperplasia; OAB: overactive bladder.

Figure 3. Associations of mucosal P2Y6 receptor expression and urinary ATP and ADP with total ICIQ-OAB score. (**A**) **i.** Representative Western blots of P2Y6 (42 KDa, expected band size) and β-actin (47 KDa, expected band size) expressions in human bladder mucosa biopsy samples; + control: human bronchial epithelium lysate; - control: human bronchial epithelium lysate with no P2Y6 primary antibody. **ii.** Correlation between the expression levels of P2Y6 (normalised to β-actin) and participants' total ICIQ-OAB severity scores, shown on a linear graph. (**B**) Correlation between the urinary levels of ATP normalised to creatinine (ATP/Cr) and participants' total ICIQ-OAB severity scores, shown on a lin-log plot. (**C**) Correlation between the urinary levels of ADP normalised to creatinine (ADP/Cr) and participants' total ICIQ-OAB severity scores, shown on a lin-log plot. *p*: p-value; *r*: Spearman / Pearson r value; Bold value: significant p-value of ≤ 0.05.

Table 1. Characteristics of study participants used for analyses.

Participant characteristics	Mucosal P2Y6 expression analysis	P2Y6 quantification analysis	Urinary ATP and ADP concentration analyses	
			ATP	ADP
n	10[a]	28	66	60
Age, mean (range) (yrs)	61 (46-82)	69 (37-92)	52 (21-93)	54 (21-93)
Gender				
Female	7	10	49	36
Male	3	18	17	24
ICIQ-OAB characteristics				
Frequency[b], mean (SD)	0.77 (±0.42)	0.52 (±0.33)	0.37 (±0.31)	0.36 (±0.32)
Nocturia[b], mean (SD)	0.73 (±0.34)	0.56 (±0.27)	0.15 (±0.18)	0.17 (±0.19)
Urgency[b], mean (SD)	0.75 (±0.31)	0.36 (±0.23)	0.17 (±0.18)	0.20 (±0.20)
Incontinence[b], mean (SD)	0.74 (±0.31)	0.18 (±0.20)	0.09 (±0.17)	0.10 (±0.18)
Total ICIQ-OAB symptom score[b], mean (SD)	0.74 (±0.28)	0.39 (±0.16)	0.18 (±0.13)	0.19 (±0.14)

[a] Participants diagnosed as having OAB (for further information see Figure 1); [b] Symptoms scores were range standardised on a 0 to 1 scale.

Table 2. Correlations between mucosal P2Y6 receptor expression and urinary ATP and ADP concentrations with overactive bladder characteristic symptom severity and age. Urinary concentrations of ATP and ADP were normalised to urinary creatinine concentration. Significant correlations (i.e. p-value of ≤ 0.05) are highlighted in bold.

Correlations		Mucosal P2Y6 expression	Urinary [ATP]	Urinary [ADP]
Frequency severity score	**p-value**	0.629	**0.000**	0.277
	r	0.096	0.460	0.143
Nocturia severity score	**p-value**	0.134	0.996	0.384
	r	0.291	0.001	0.115
Urgency severity score	**p-value**	0.864	0.157	0.165
	r	0.034	0.176	0.182
Incontinence severity score	**p-value**	**0.009**	0.315	0.306
	r	0.487	0.126	0.134
Total ICIQ-OAB score	**p-value**	0.107	**0.010**	**0.042**
	r	0.311	0.315	0.264
Age	**p-value**	0.507	0.077	0.995
	r	0.131	0.219	-0.001

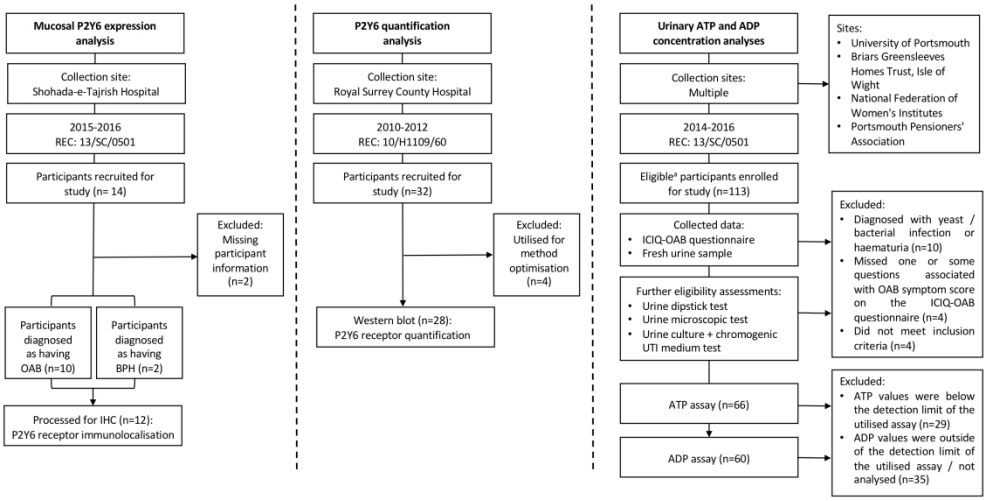

Figure 1. Flow diagram of participants recruitment, selection and subsequent tests.
OAB: overactive bladder; BPH: benign prostatic hyperplasia; ICIQ-OAB = International consultation on incontinence questionnaire - overactive bladder; a = see Methods and Materials section for inclusion and exclusion criteria.

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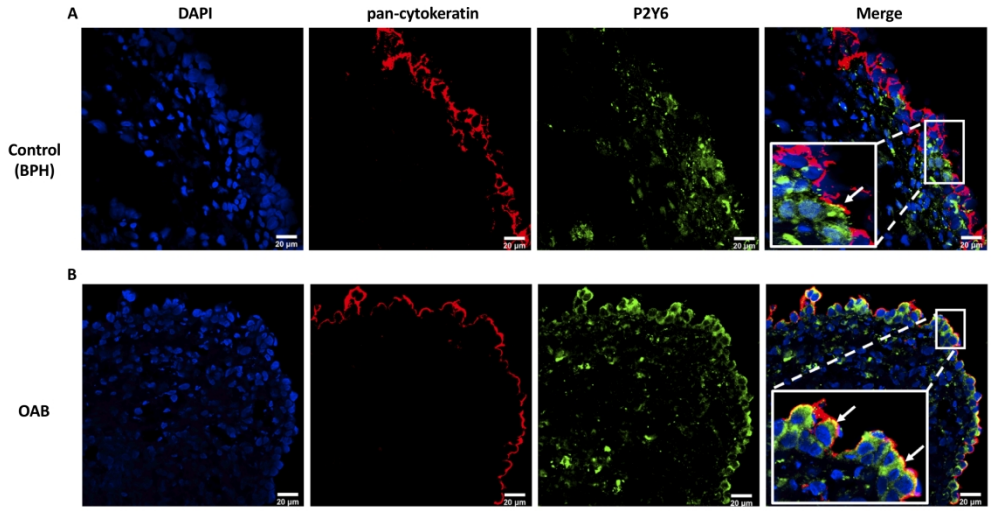

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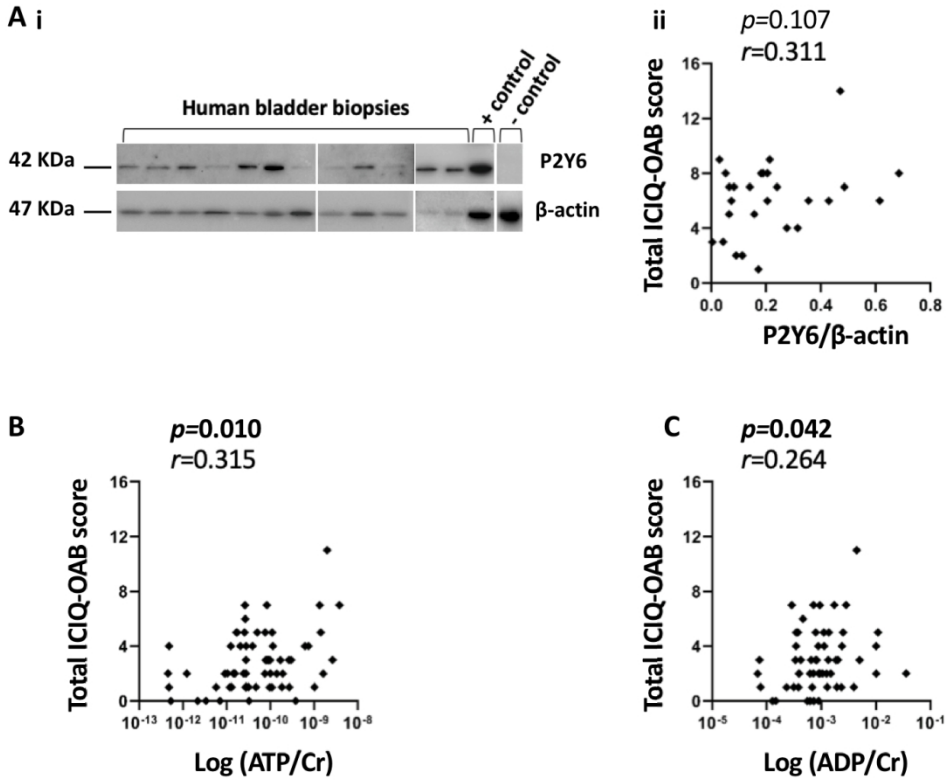

Figure 3. Associations of mucosal P2Y6 receptor expression and urinary ATP and ADP with total ICIQ-OAB score. (A) i. Representative Western blots of P2Y6 (42 KDa, expected band size) and β-actin (47 KDa, expected band size) expressions in human bladder mucosa biopsy samples; + control: human bronchial epithelium lysate; - control: human bronchial epithelium lysate with no P2Y6 primary antibody. ii. Correlation between the expression levels of P2Y6 (normalised to β-actin) and participants' total ICIQ-OAB severity scores, shown on a linear graph. (B) Correlation between the urinary levels of ATP normalised to creatinine (ATP/Cr) and participants' total ICIQ-OAB severity scores, shown on a lin-log plot. (C) Correlation between the urinary levels of ADP normalised to creatinine (ADP/Cr) and participants' total ICIQ-OAB severity scores, shown on a lin-log plot. p: p-value; r: Spearman / Pearson r value; Bold value: significant p-value of ≤ 0.05.

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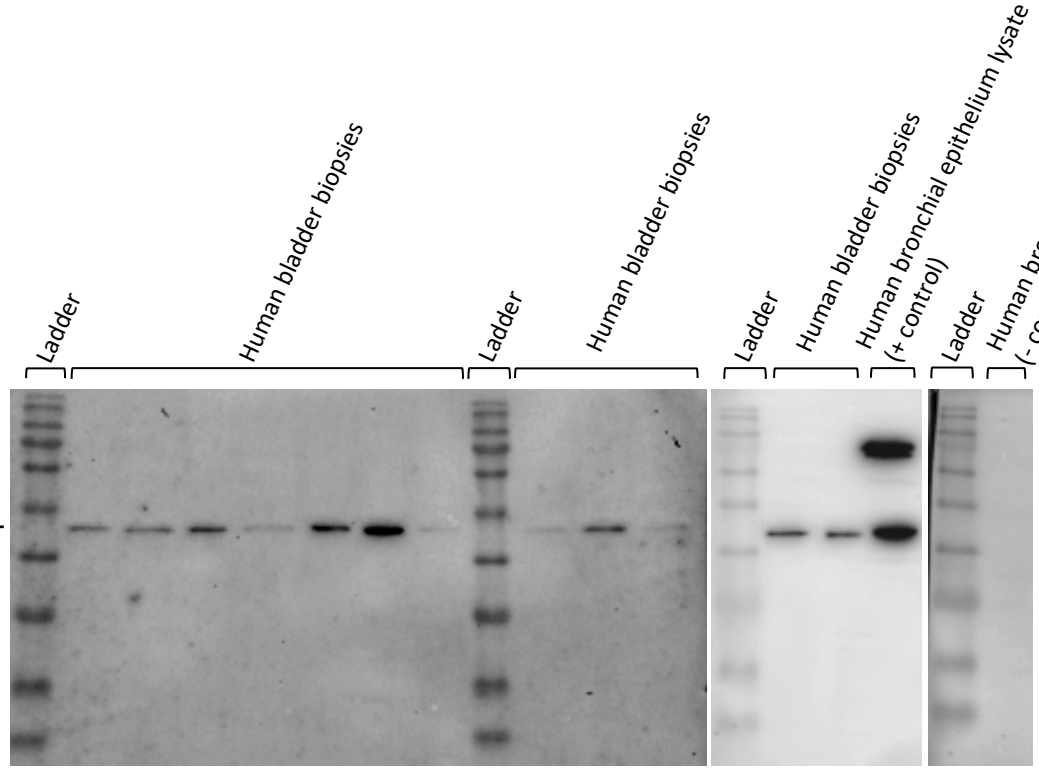
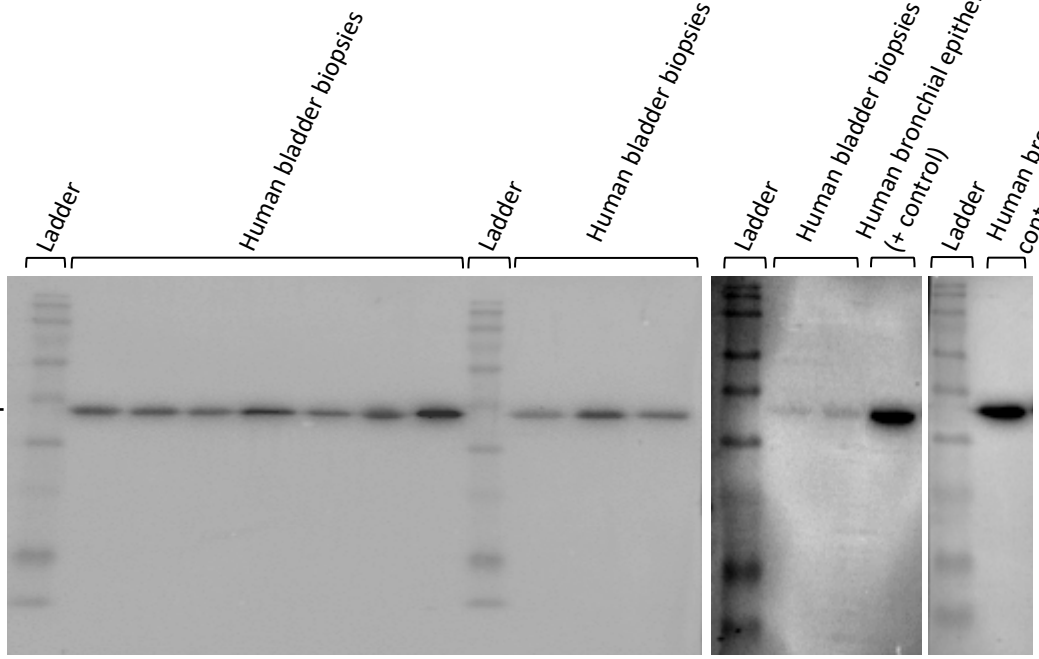


P2Y6, Abcam 92504, expected band size : 42 KDa



β-actin**,** Abcam 8227, expected band size: 47 KDa