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ATP shows more potential as a urinary biomarker than acetylcholine and PGE_2, but its concentration in urine is not a simple function of dilution

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Abstract

Aims: To determine whether the amount of ATP, prostaglandin E_2 (PGE_2), and acetylcholine (ACh) in voided urine are influenced enough by that released within the lower urinary tract (LUT) for them to be useful biomarkers of bladder function.

Methods: Participants without LUT symptoms collected total urine voids at 15, 30, 60, and 120 min (20 males/23 females) and 240 min (18 males/26 females) following the previous void. Aliquots of urine were immediately frozen at −20°C and later used to measure ATP (luciferin-luciferase), PGE_2 (enzyme-linked immunosorbent assay), ACh (mass spectrometry), creatinine (colorimetric), and lactose dehydrogenase (colorimetric).

Results: The amount of ATP in voided urine correlated strongly with the rate of urine production, suggesting that the majority, if not all, the ATP in voided urine has an LUT, and likely bladder, origin. In contrast, there appeared to be no significant net LUTs release of creatinine or ACh into the urine. PGE_2 was intermediate with an LUT component that increased with urine production rate and contributed about 25% of the total at 1 ml/min in women but a smaller fraction in men.

Conclusion: Whereas the majority of the ATP measured within the voided urine originates in the LUT, ACh reflects that extracted from the plasma in the kidneys and PGE_2 is a mixture of both sources. ATP has the most potential as a biomarker of benign bladder disorders. Expressing urinary ATP concentration relative to creatinine concentration is questioned in light of these results.

KEYWORDS
creatinine, lower urinary tract

1 | INTRODUCTION

Measuring the concentration of transmitters within the urine as possible biomarkers of benign bladder disorders is increasingly relevant to investigate pathological origins.[1,2] ATP, prostaglandin E2 (PGE_2), and acetylcholine (ACh) are all potential candidates[3,4] but to be useful as a urinary biomarker a significant fraction of that measurable in the urine must have a lower urinary tract (LUT) origin.

ATP is released in both the kidneys[5] and the ureters,[6–9] but high levels of ectoATPases activity[5,8,9] limit its half-life to only 3–4 min in these areas.[5] This contrasts to 30–60 min[5,10] within the bladder lumen where ectoATPases activity is lower, particularly on the apical membrane of the umbrella cells[11,12] reflecting the lower permeability of the bladder urothelium.[13–15] Thus, it is possible that the LUT and bladder, in particular, may contribute a significant fraction of the ATP found in urine.

There are only a very limited number of studies measuring urinary ACh. This is partially due to the high levels of urinary choline that reduces the sensitivity and accuracy of ACh detection methods, especially as ACh itself is hydrolyzed to choline. Using mass spectrometry, however, Kirsch et al.[3] found that urinary ACh levels were significantly higher than those in the plasma and that they correlated strongly with plasma choline levels in females suggesting that urine ACh levels could be primarily determined by the amount of choline present in the body.

Although PGE_2 is known to be synthesized throughout the urinary tract in response to stretch, sympathetic nerve stimulation, tissue damage, or inflammatory mediators,[16] it is unclear what fraction of the total PGE_2 in voided urine this represents. Studies in rats where urine was sampled in the left ureter and the bladder suggested that about half the PGE_2 found in the voided urine in rats has a bladder origin,[16] but this could be different in humans.

A significant problem with measuring the concentrations of any urinary biomarkers is the large variability in the rate of urine production. Creatinine concentration is often used to adjust for this, with the concentrations of other substances being expressed as a proportion. However, this presumes that other substances are handled by the urinary tract in a similar way to creatinine, that is, that there is little or no net secretion or reabsorption in the urinary tract, which cannot be assumed, especially for potential markers of LUT dysfunction.

The purpose of this study was to investigate the extent to which secretion or reabsorption of potential biomarkers across the LUT contributes to the amount of ATP, ACh, and PGE_2 within the voided urine and how this is affected by the rate of urine production. This has implications for how best to compare the levels in urine samples from different human subjects and between samples from the same individual and whether expressing their concentration relative to that of creatinine is useful.

2 | MATERIALS AND METHODS

This study received ethical approval from the King's College Ethics Research Committee (HR-16/17-3859). Participants were students or staff members at King's College London and all gave informed written consent for the study. They had not taken any medication or vitamin or mineral supplements within the previous 48 h and all had normal bladder function as assessed using the overactive bladder symptom bother short-form questionnaire (OAB-q).[17]

2.1 | Urine sample collection

Participants were asked to collect the entire void at designated time intervals of 15, 30, 60, 120, and 240 min while eating and drinking normally. Initially, participants were asked to give samples at all five time points (7F/4M), but then to reduce their burden and aid recruitment, they were only asked to provide samples at the four shorter time points (16F/16M) and an additional group gave a single 240 min sample (19F/14M). Where subjects gave more than one sample, they were assigned a randomized order. The first void of the day was not used for any samples.

Samples were excluded if they were ≤5 ml ($n = 5$, samples), there was confusion over voided volumes ($n = 4$ samples), the urine production rate was very low (≤0.25 ml/min, $n = 5$ samples), or lactose dehydrogenase (LDH) production rates were very high, indicative of possible renal or urinary tract tissue damage (>7 mU/min, $n = 4$ participants) plus one participant with very high creatinine concentrations (1300 ± 200 mg/dl, mean ± *SD*). This left a total of 208 urine samples from 76 participants, 42 females (F, 27 ± 1 years), and 34 males (M, 28 ± 2 years).

Total void volumes were recorded and then 1 ml aliquots were frozen within 10 min and stored at −20°C for later analysis. The rate of urine production was calculated by dividing sample volume by the time between voids, assuming a constant production rate.

2.2 | Measurement of ATP

ATP concentration was measured at room temperature in undiluted urine samples using a luciferin-luciferase assay (FL-AAM; Sigma). Urine samples were spun at 2000*g* for 5 min and a 20 μl sample added to 20 μl of assay mix diluted 10-fold. Luminescence was measured immediately and ATP concentration was calculated from a standard curve constructed for each experiment using ATP standards (GloMax 20/20; Promega). All samples were measured in triplicate and average values were used. Measurements were all made well within the 3-month storage window identified from repeated sampling before any loss of ATP from the samples was detected.

2.3 | Measurement of ACh

ACh was measured using mass spectrometry-based on the methods of Kirsch et al. [3] using a Thermo LTQ XL mass spectrometer. Each urine sample was thawed and 10 μl added to 490 μl water and 500 μl of 10 ng/ml internal ACh standard (ACh-D4), giving a final 100-fold dilution. The samples were run on a Thermo Hypersil Gold aQ 150 × 2.1 mm 3 μm column at room temperature. The flow rate was 200 μl/min with a partial loop injection mode and injection volume of 10 μl. The solvents were 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Gradient elution was initiated at 5% B (95% A) for 0.5 min, rising to 25% B at 3.0 min and again to 95% B at 3.5 min, maintained at 95% B until 5.5 min, ramped down to 5% B at 5.8 min, and held at this composition for 6.2 min giving a 12 min total run time. Positive electrospray ionization mode with a capillary voltage of 14.0 V and desolvation gas (N_2) temperature of 350°C and retention time of ~2.5 min were used to elude ACh peaks. ACh concentrations were calculated by reference to the internal standard in each sample and known ACh concentrations of 0–2500 ng/ml run at the start of each experiment.

2.4 | Measurement of PGE_2

PGE_2 was measured using a Prostaglandin E_2 Express ELISA Kit (500141; Cayman Chemicals) in which PGE_2 in the sample competes for limited PGE_2-monoclonal antibody bound to a 96-well plate. Urine samples were fully defrosted, spun at 2000*g* for 5 min, and diluted sufficiently to fall into the 15.6–2000 pg/ml range of the kit. Absorbance was measured at 405 nm and PGE_2 concentration calculated using standards run in the same plate.

2.5 | Measurement of creatinine

Creatinine concentration was measured using a colorimetric assay kit (500701; Cayman Chemicals) run on a 96-well plate. Urine samples were fully defrosted and spun at 2000*g* for 5 min and a sample of the supernatant diluted 5–50-fold such that the creatinine concentration fell within the 0–15 mg/dl range of the kit. Absorbance was measured at 490 nm and creatinine concentration calculated using standards run in the same plate.

2.6 | Measurement of lactose dehydrogenase LDH

LDH was measured using an LDH kit (MAK066; Sigma) run in a 96-well plate according to the manufacturer's instructions. LDH activity was determined by the amount of NADH produced from the reduction of NAD and measured with a colorimetric assay by the absorbance at 450 nm, against a standard curve of known NADH concentrations (2.5–12.5 nmol/well) run on the same plate. LDH activity was then calculated from the rate of production of NADH.

2.7 | Data analysis

Statistical tests (Spearman's Rank correlation and Mann–Whitney *U* test) were performed as indicated in the text or figure legends using GraphPad Prism. The null hypothesis was rejected at $p < .05$.

3 | RESULTS

The rate of LDH production in urine was estimated from one sample from each participant (mean: 2.3 ± 1.5 mU/min, mean ± *SD*, $n = 76$). Four participants who had values >7 mU/min were identified as outliers and because this may indicate increased leakiness of the bladder wall, all of their samples were excluded.

The concentrations of creatinine, ATP, ACh, and PGE_2 were measured for each urine sample and plotted as a function of the time to produce each microliter of urine, the inverse of urine production rate (Figure 1). Significant positive correlations were found for creatinine, ACh, and PGE_2 in both women (red circles) and men (blue triangles). However, no significant positive correlation was observed between ATP concentration and time to produce each microliter of urine from either women or men, indicating that ATP concentration in the urine varies in a

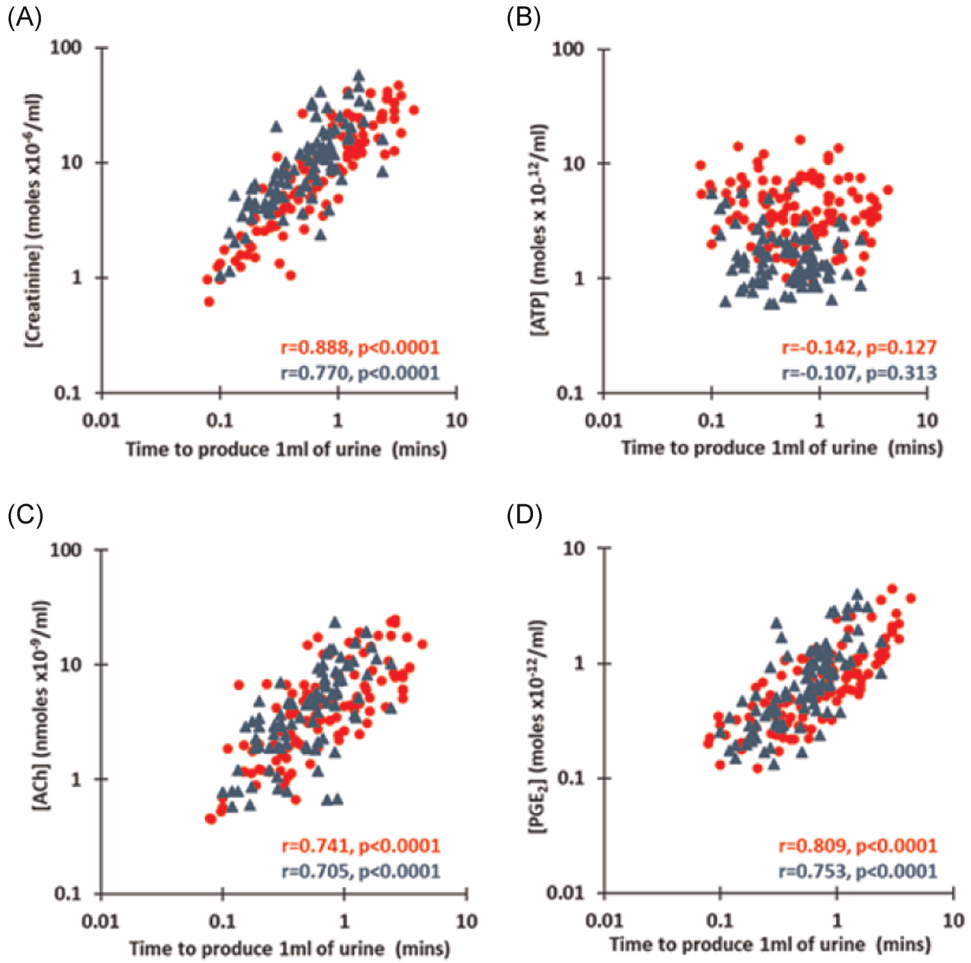

FIGURE 1 Creatinine, ATP, ACh, and PGE_2 concentration as a function of the time to produce each microliter of urine. Creatinine (A), ATP (B), ACh (C), and PGE_2 (D) concentration is plotted as a function of the average time in minutes for each microliter of urine to collect (inverse of the rate of urine production). Red circles (females, $n = 117$ samples), and blue triangles (men, $n = 91$ samples). Spearman's rank correlation r and p values are shown. ACh, acetylcholine; PGE_2, prostaglandin E_2

fundamentally different way to these other three substances.

Furthermore, ATP concentration was significantly higher in women than men [median (interquartile range): women ($n = 117$), 3.6 nmol/l (2.5–5.9); men ($n = 91$), 1.3 nmol/l (1.0–2.1); $p < .001$], which was not the case for creatinine, ACh, or PGE_2. In view of the gender-dependent differences in ATP concentrations, the analysis below is shown separately for women and men.

The independence of ATP concentration from the rate of urine production was investigated further by measuring the amount of each substance as a function of voided volume when collected over a single time period, 120 min, in female participants (Figure 2). The amount of creatinine measured in the urine, 120 min after the previous void was independent of the void volume (Figure 2A, $r = .048$, $p > .05$, $n = 23$), whereas the amount of ATP showed a strong correlation with void volume both across samples from multiple participants (Figure 2B, $r = .751$, $p < .0001$, $n = 23$, circles) and across samples from a single individual (one of the authors) (Figure 2B, $r = .864$, $p < .0001$, $n = 31$, diamonds). ACh and PGE_2 both behaved more like creatinine than ATP in agreement with Figure 1 (ACh: $r = .280$, $p > .05$, PGE_2: $r = .296$, $p > .05$, $n = 23$).

The analysis of Figure 2 using a fixed time interval between voids can be extended to data derived from different intervoid intervals by plotting the rate at which a particular substance is added to the urine as a function of the urine flow rate itself (Figure 3): note the units of the relationships in both figures have the same units (mol/ml). Values from samples at the five different void time intervals are shown in different colors to allow comparison.

If there is a constant rate of extraction of a substance from the plasma into the urine in the kidneys with no net secretion or absorption within the LUT, the plot in Figure 3 should form a horizontal line with zero

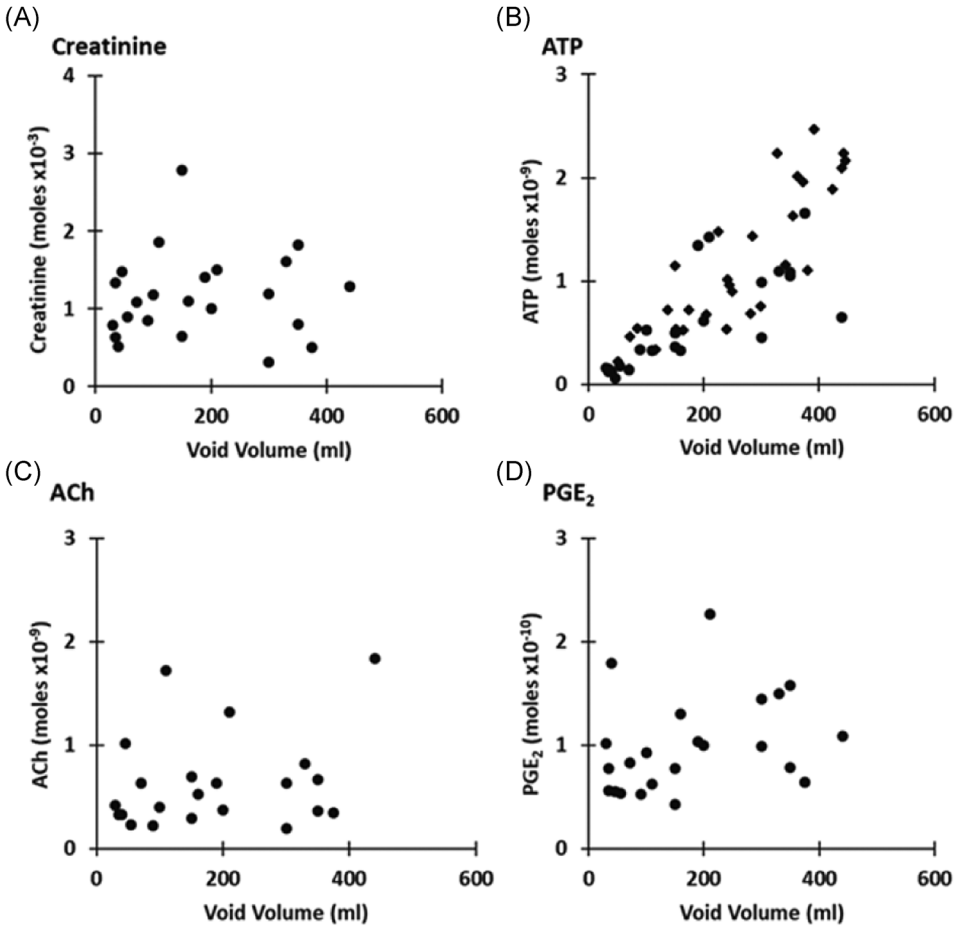

FIGURE 2 The effect of void volume on the amount of creatinine, ATP, ACh, and PGE_2 within the voided urine of women. Total (A) creatinine ($n = 23$), (B) ATP ($n = 23$, circles) (additional 31 samples from one of the authors shown with diamond symbols), (C) ACh ($n = 23$), and (D) PGE_2 ($n = 23$) as a function of the void volume only for voids 120 min after previously passing urine. The void interval of 120 min was chosen for this analysis as a longer time interval favored a larger spread of void volumes than a shorter one and there was a smaller time error on the 120 min time interval than the 240 min one due to being part of a set of samples (mean ± *SD*, 122 ± 5 min, $n = 23$, 241 ± 14 min, $n = 26$). ACh, acetylcholine; PGE_2, prostaglandin E_2

gradients and the y-intercept representing the rate of entry of the substance into the urine in the kidneys. Any dependency on urine production rate, that is, a nonzero slope would indicate release within the LUT.

The rate of creatinine production (Figure 3A) showed no dependency on the rate of urine production consistent with a constant rate of release of creatinine in the kidney and no net secretion or absorption within the LUT as described above. In contrast, ATP (Figure 3B) showed a strong dependency on urine production rate with an intercept indistinguishable from zero. ACh (Figure 3C) was similar to creatinine. However, the PGE_2 (Figure 3D) in the voided urine came from a mixture of that released in the kidneys (nonzero intercept) and some released in the LUT as indicated by a nonzero slope, the contribution of which rose with urine production rate. Estimates from linear fits to these data suggested that about 25% of the total PGE_2 in voided urine may come from the LUT at urine production rates of 1 ml/min, but this would be closer to 60% if urine production increased to 5 ml/min.

Corresponding plots for male participants are shown in Figure 4. The results were very similar to those in women, except PGE_2 dependency on urine production rate was less obvious, suggesting a smaller component of LUT release of PGE_2 in men than women.

The concentrations of substances in urine are often expressed relative to the concentration of creatinine to adjust for differences in the rate of urine production. Figure 5 shows scatter plots for the ACh, PGE_2, and ATP concentration of each sample as a function of its creatinine concentration. There was a strong positive correlation between ACh and PGE_2 concentrations and creatinine concentration in both women and men but not between ATP concentration and creatinine concentration, as predicted from the observations above. This severely questions the practice of expressing

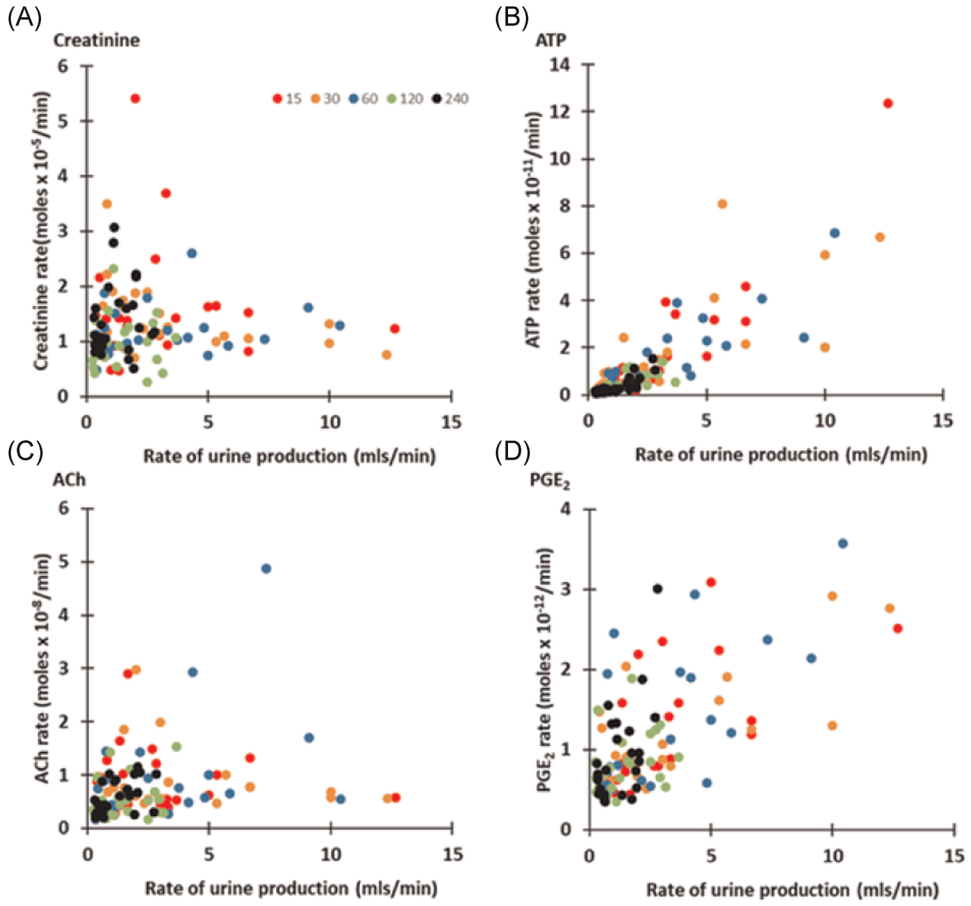

FIGURE 3 Rate of addition to the voided urine of creatinine, ATP, ACh, and PGE_2 as a function of rate of urine production in female participants. (A) Creatinine, (B) ATP, (C) ACh, and (D) PGE_2. Urine samples at different time intervals between voids are shown as different colored symbols, 15 min (red), 30 min (orange), 60 min (blue), 120 min (green), and 240 min (black). Mean slopes (±*SD*) from best fit linear fits were (mol/min) creatinine, -0.02 ± 0.04; ATP, $\mathbf{0.5 \pm 0.2}$; ACh, 0.1 ± 0.1; and PGE_2 $\mathbf{0.2 \pm 0.1}$, intercepts were (mol/min) creatinine, $\mathbf{1.4 \pm 0.3}$; ATP, -0.1 ± 0.4; ACh, $\mathbf{0.6 \pm 0.3}$; and PGE_2, $\mathbf{0.6 \pm 0.1}$. Bold values were significantly nonzero. ACh, acetylcholine; PGE_2, prostaglandin E_2

urinary ATP concentration in terms of urinary creatinine concentration.

4 | DISCUSSION

This is the first study to look closely at how the levels of ATP, ACh, and PGE_2 vary with urine production rate. A possible explanation for the different dependencies of the above substances on urine production could come from their origins. For a substance derived from the glomerular filtrate, and hence the plasma, which is neither secreted nor absorbed from the renal tubules and urinary tract, the *amount* accumulating in the bladder will be constant for a fixed period of time and the *concentration* will vary according to the urine production rate. In contrast, for a substance that is virtually absent from the initial glomerular filtrate or which is released but subsequently broken down, but then has a net secretion into the tubular fluid or the urinary tract lumen, the *amount* could vary with an increase in the final volume. In the latter case, it is proposed that the increase in volume per se will increase the secretion but be diluted in a greater volume of urine such that the *concentration* may remain reasonably constant. For other substances, it is possible that a mixture of delivery from the glomerular filtrate and some net secretion by the urinary tract may occur.

The results presented here suggest that whereas all the creatinine and ACh measured in the voided urine is already present when the urine enters the LUT, release of ATP within the LUT and most likely the bladder contributes the majority if not all the ATP present. PGE_2 was intermediate in that there were significant levels already present in the urine entering the LUT, but additional release contributed an increasing amount as urine production rate increased, at least in women.

It is likely that the majority of the ATP that enters the urine in the bladder comes from the urothelium, which

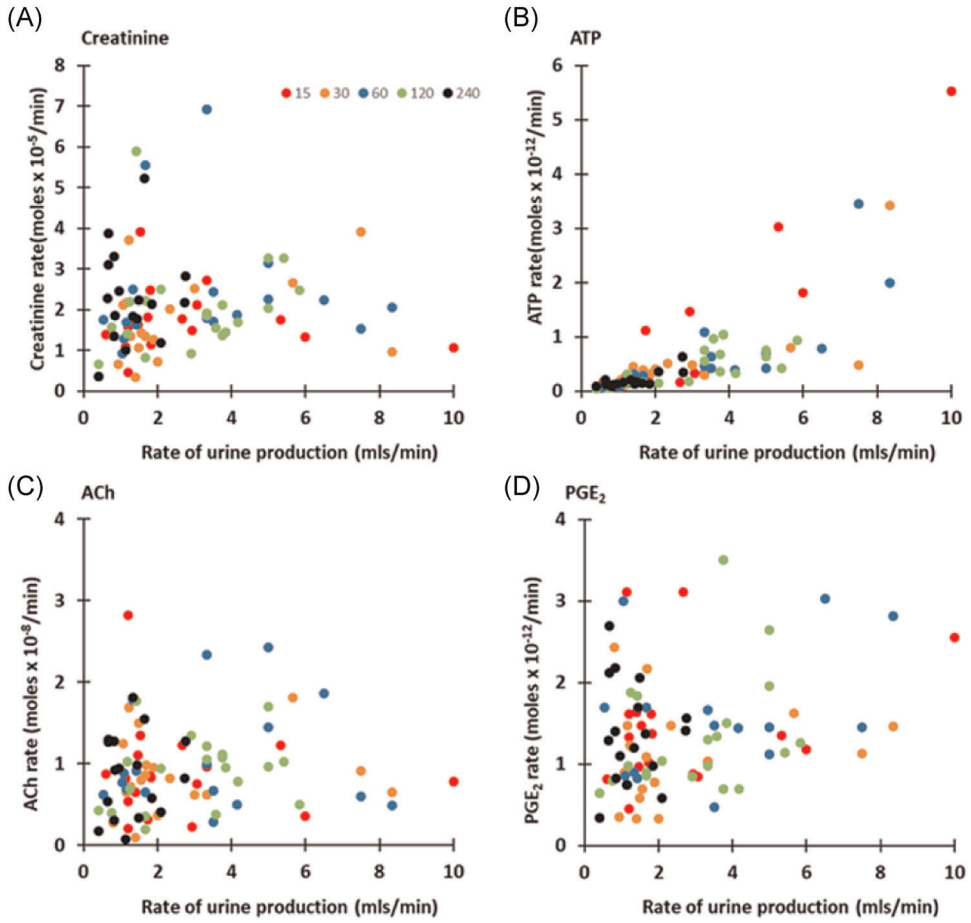

FIGURE 4 Rate of addition to the voided urine of creatinine, ATP, ACh, and PGE2 as a function of rate of urine production in male participants. (A) Creatinine, (B) ATP, (C) ACh, and (D) PGE2. Urine samples at different time intervals between voids are shown as different colored symbols, 15 min (red), 30 min (orange), 60 min (blue), 120 min (green), and 240 min (black). Mean slopes (±*SD*) from best fit linear fits were (mol/min) creatinine, 0.06 ± 0.08; ATP, **0.3 ± 0.2**; ACh, 0.04 ± 0.04; and PGE_2, 0.06 ± 0.08, intercepts were (mol/min) creatinine, **2.0 ± 0.3**; ATP, −0.2 ± 0.3; ACh, **0.8 ± 0.1**; and PGE_2, **1.3 ± 0.2.** Bold values were significantly nonzero. ACh, acetylcholine; PGE_2, prostaglandin E_2

itself constitutes about 60%–75% of the total amount of ATP released in the bladder.[18] ATP concentration was found to be very dependent on the rate of urine production, which we assume relates to the rate of bladder stretch. This fits well with observations from in vitro experiments where bladder strips have been shown to release significantly more ATP following stretching than in a resting state.[19] Indeed, the amount of urothelial ATP release has been closely linked to the speed, magnitude, and direction of stretch.[11,20] The increased ATP production at higher rates of urine production has also been seen by others in patients who gave two urine samples, one after water-loading.[4] Also, in cystoscopy studies that measure whole bladder release directly, Cheng et al.[21] found ATP levels up to 60 nM, 5–10 times higher than those seen physiologically, when using a very high bladder filling rate (75 ml/min).

The lack of correlation of ATP concentration with that of creatinine is not unexpected given the observation that creatinine is released at a constant rate, but ATP is dependent on the amount of bladder stretch. A lack of correlation has also been reported by others.[22] This does, however, question the value of expressing urinary ATP concentrations in terms of creatinine concentration or any other marker of dilution rather than the rate of urine production.

Urinary ACh appeared to be predominantly determined by the amount of ACh extracted from the plasma in the kidneys. This fits well with reports of a high level of correlation between urinary ACh and plasma choline levels in females, suggesting that urine ACh levels could be influenced primarily by the amount of choline present in the body.[3]

Our results suggest that although there is some LUT component to the total voided PGE_2, it is less than that of ATP. We estimate that LUT release is unlikely to contribute more than about a third of the total at normal filling rates, although the percentage will increase with greater

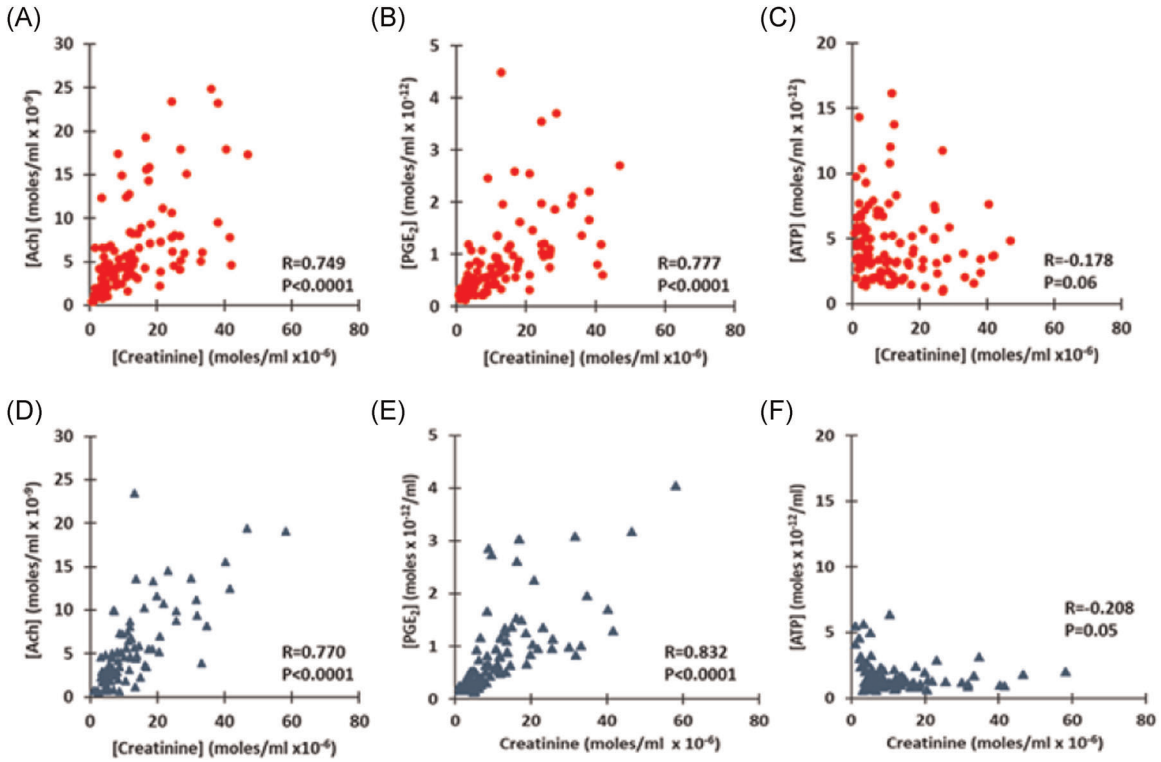

FIGURE 5 ACh, PGE_2, and ATP concentration as a function of creatinine concentration. (A–C) Females ($n = 117$), (D–F) males ($n = 91$). Spearman's rank correlation r and p values are shown for each graph. There was a significant positive correlation between ACh and PGE_2 and creatinine concentrations but not ATP. ACh, acetylcholine; PGE_2, prostaglandin E_2

hydration. It is likely that, as in the case of ATP, more of the PGE_2 released by the urothelium than other layers will reach the urine. It is unclear why this was only seen in the females but may reflect an effect not seen with the slightly smaller number of male samples, a proportionally larger constant rate of release in the males or a difference in bladder properties. Given that the bladder ATP release was also significantly lower in men than women, it could be a genuine difference in stretching properties of the bladder, although despite male bladders being shown to have a thicker detrusor than those of women,[23] gender differences in contractility have not been seen.[24]

5 | LIMITATIONS

The biggest problem in this study was that participants tended to drink more when doing the shorter interval samples than the longer ones. This initially led us to think that ATP levels were higher at shorter times between voids,[25] but although a small effect of time between voids cannot be ruled out, we now believe that this difference can be fully explained by the difference in the rate of urine production.

Participants were asked to collect the whole void and then to tip a sample from that into a tube for analysis. Collecting the whole void meant that in smaller samples, there was a risk of contamination. We did not, however, want to risk asking participants to collect a mid-stream sample while filling the jug as this may have compromised the accuracy of the volume measured if the sample was lost, spilt, or the tube content was missed from the total, for example. To reduce the risks of contamination, only samples of 5 ml or more were included.

6 | CONCLUSIONS

The LUT contributes the majority of the ATP found in the voided urine and up to about a third of the PGE_2 in women but little or none of the creatinine and ACh. ATP represents a better potential biomarker of bladder function than ACh and PGE_2 and sensitivity could be improved by water loading. The commonly used practice of expressing urine ATP concentration as a function of creatinine concentration is questioned in the light of these results.

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CONFLICT OF INTERESTS

Dr. Dasgupta reports other from Chief Scientific Officer, Proximie, other from Chief Medical Officer, Mystery Vibe, other from Mentor and Advisor, Jiva.ai, outside the submitted work. Remaining authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Study concept and design: L. M. McLatchie and P. Dasgupta. *Acquisition of data*: L. M. McLatchie and A. Caldwell. *Analysis and interpretation of data*: L. M. McLatchie and C.H. Fry. *Drafting of the manuscript*: L. M. McLatchie. *Critical revision of the manuscript for important intellectual content*: L. M. McLatchie, A. Sahai, A. Caldwell, P. Dasgupta, C.H. Fry. *Obtaining funding*: C.H. Fry and A. Sahai.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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