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Metabolomics strategy for diagnosing urinary tract infections

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Metabolomics has emerged as a mainstream approach for investigating complex metabolic phenotypes but has yet to be integrated into routine clinical diagnostics. Metabolomics-based diagnosis of urinary tract infections (UTIs) is a logical application of this technology since microbial waste products are concentrated in the bladder and thus could be suitable markers of infection. We conducted an untargeted metabolomics screen of clinical specimens from patients with suspected UTIs and identified two metabolites, agmatine, and N6-methyladenine, that are predictive of culture-positive samples. We developed a 3.2-min LC-MS assay to quantify these metabolites and showed that agmatine and N6-methyladenine correctly identify UTIs caused by 13 *Enterobacterales* species and 3 non-*Enterobacterales* species, accounting for over 90% of infections (agmatine AUC > 0.95; N6-methyladenine AUC > 0.89). These markers were robust predictors across two blinded cohorts totaling 1629 patient samples. These findings demonstrate the potential utility of metabolomics in clinical diagnostics for rapidly detecting UTIs.

Infections are caused by a wide range of microbes that, like all organisms, consume nutrients and secrete metabolic waste products. These microbial metabolites can be highly abundant at the site of infection[1] and thus, could potentially serve as targets for a metabolism-based diagnostic strategy. Urinary tract infections (UTIs) are particularly amenable to this metabolic diagnostic approach because microbial metabolites are concentrated in the bladder and can be easily detected by liquid chromatography-mass spectrometry (LC-MS) analysis of patient urine. *Escherichia coli*, the most common UTI pathogen, produces a variety of diamines, polyamines, and acylated conjugates of these molecules that are not typically found in human urine. These molecules have been reported as potential UTI biomarkers for over three decades[2,3], with more recent work suggesting that these amine-linked molecules may play a role in resistance to nitrosative stress in the bladder[4,5].

Although microbial metabolites have been recognized as potential diagnostic targets, they have yet to be translated into a clinical diagnostic tool. The reason for this is threefold: (1) concentrations of the microbial polyamines are quite variable in urine[6] and are thus of borderline utility for diagnostic purposes, (2) only biomarkers of *E. coli* have been reported thus far, which is problematic because it is only one of many UTI-causing organisms[7], and (3) LC-MS has only recently become sufficiently robust to serve as a practical diagnostic platform. The primary objective of this study was to conduct a systematic analysis of microbial metabolites found in urine from symptomatic UTI patients to identify any molecules with sufficient predictive power to enable rapid, metabolic-based diagnostics. Using an untargeted LC-MS metabolomics approach, we identified two novel biomarkers that report on a wide transect of UTI pathogens. We then assessed the potential utility of metabolomics-based UTI screening in two blinded cohorts totaling 1629 urine samples.

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Results

UTI biomarker discovery

UTIs are diagnosed based on patient symptoms supported by laboratory diagnostic tests. These clinical tests include culturing urine specimens for 18 h to identify patients with significant bacteriuria (defined by Alberta Precision Laboratories guidelines as specimens with $>10^7$ CFU/L)[8]. Microbial catabolites present in the urine could potentially provide a culture-independent mechanism for rapidly identifying these positive samples. To identify potential biomarkers in these urine specimens, untargeted metabolomics analyses (Fig. 1a and Supplementary Table 1) of 77 culture-positive urine samples and 33 culture-negative samples ($<10^7$ CFU/L)—all collected from symptomatic patients—were conducted using LC-MS. The most diagnostic features in this dataset, as judged by area under the curve (AUC) of the receiver operating characteristic curve, were *m/z* 114.1025 (AUC = 0.98), *m/z* 131.1289 (AUC = 0.89), and *m/z* 150.0772 (AUC = 0.91; Fig. 1b). The first two of these untargeted signals were putatively assigned to agmatine, a product of arginine metabolism[9], and the third was assigned to N6-methyladenine, a modified nucleobase found in prokaryotes[10]. These assignments were validated by LC-MS/MS fragmentation patterns as well as co-elution of the target signal with analytical standards (Fig. 1c, d).

Validation of agmatine as a UTI biomarker

Although both agmatine and N6-methyladenine were predictive of positive urine cultures, the levels of these metabolites were not uniform across UTI-causing pathogens. Infections caused by the most prominent UTI-causing *Enterobacterales* species, such as *E. coli*, were associated with elevated agmatine, whereas N6-methyladenine levels were elevated in infections caused by a small number of non-*Enterobacterales* species (Supplementary Table 1). To better understand these species-specific phenotypes, the performance of agmatine was

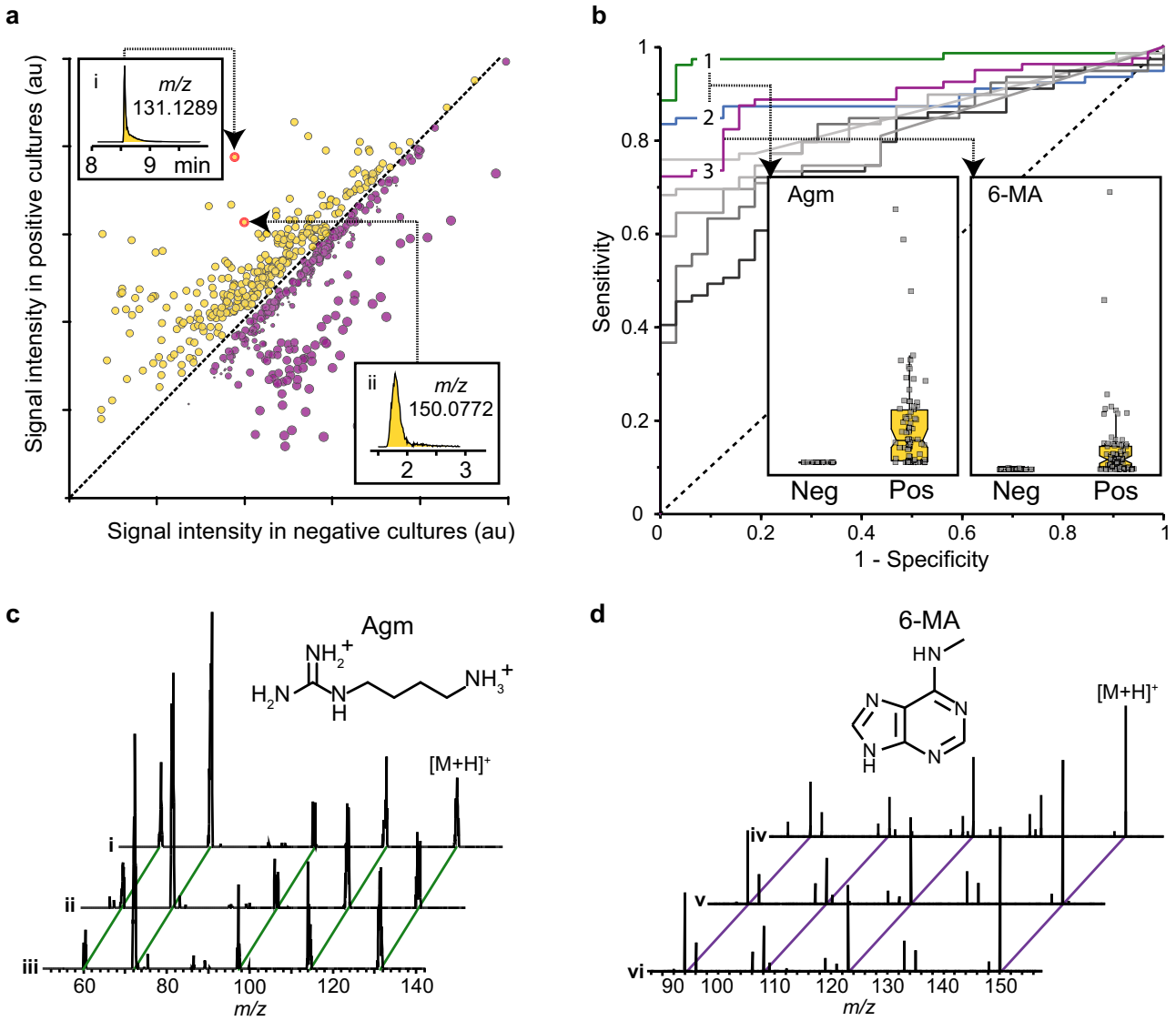

Fig. 1 | Discovery of agmatine and N6-methyladenine as UTI biomarkers. a LC-MS data were acquired from metabolites predictive of culture-positive (yellow points) and culture-negative (purple points) urine samples. Potential UTI biomarkers were identified (*m/z* 131.1289 and *m/z* 150.0772 shown as examples; see inset for chromatogram). **b** Predictive UTI biomarkers were ranked according to receiver operating characteristic curves. Signal 1 (*m/z* 114.1025) and signal 2 (*m/z* 131.1289) were assigned to agmatine, and signal 3 (*m/z* 150.0771) was assigned to N6-methyladenine. **c** Agmatine and **d** N6-methyladenine assignments were verified by tandem LC-MS/MS fragmentation patterns observed in a culture-positive urine sample (i, iv), an analytical standard of the target molecule (ii; 250 nM, v; 50 μM), and a standard added to a culture-negative urine sample at the same concentration (iii, vi). Agm agmatine, 6-MA N6-methyladenine, Neg growth-negative urine, Pos growth-positive urine.

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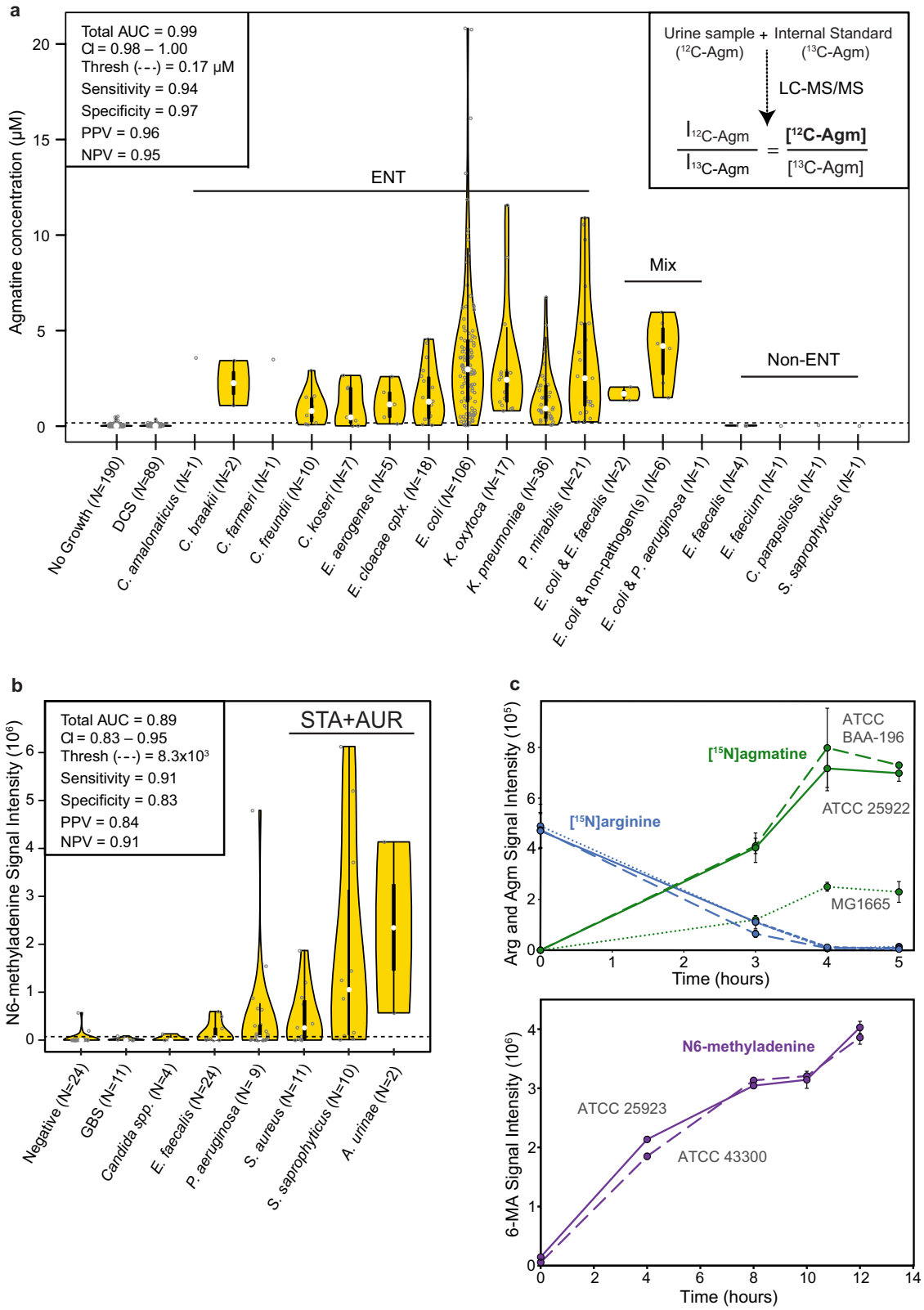

assessed using 519 urine cultures that were taken directly from a clinical diagnostic pipeline. Stable isotope labeled [U-^{13}C]agmatine was spiked into samples, samples were purified using solid phase extraction (SPE) on a silica column, and agmatine and [U-^{13}C]agmatine levels were measured using a targeted mass spectrometry method. Agmatine concentrations were thus quantifiable based on the signal ratio between isotope-labeled and native species. As expected, urinary agmatine levels were closely correlated with the presence of uropathogenic *Enterobacterales*, whereas culture-negative urine samples contained no detectable agmatine (AUC of 0.99, CI: 0.98–1.00; Fig. 2a and Supplementary Table 2). Moreover, we found that a diagnostic threshold of 174 nM agmatine was predictive of infections caused by *E. coli*, *Citrobacter* species, *Enterobacter* species, *Klebsiella* species, and *Proteus mirabilis* (sensitivity, 94%; specificity, 97%; PPV, 0.96; NPV,

Fig. 2 | Microbial metabolite signals differentiate *Enterobacterales* and certain non-*Enterobacterales* positive urines from controls. a Violin plot of agmatine concentrations in urine samples determined by spike-in of stable isotope-labeled internal standard following SPE (see inset). White dots indicate median values, thick black bars indicate interquartile ranges, and thin black lines indicate 3× interquartile hinge points. **b** Violin plot of N6-methyladenine signal intensities from a prospectively collected non-*Enterobacterales* cohort of patient urine samples. White dots indicate median values, thick black bars indicate interquartile ranges, and thin black lines indicate 3× interquartile hinge points. **c** *E. coli* (three different strains, indicated respectively by solid, dashed, and dotted lines) were grown in sterile urine (top panel) spiked with [$^{15}N_2$]arginine (blue) for 8 h and were monitored for [$^{15}N_2$]agmatine production (green). *S. aureus* (two different strains, indicated respectively by solid and dashed lines) were grown in sterile urine (bottom) for 12 h and were monitored for N6-methyladenine production (purple). In both graphs, data are presented as mean values, +/− standard deviation of technical replicates (*E. coli* = 3; *S. aureus* = 2) taken from each separate culture. Agm agmatine, AUC area under curve, CI confidence interval, Thresh threshold, PPV positive predictive value, NPV negative predictive value, DCS doubtful clinical significance, Non-ENT non-*Enterobacterales*, ENT *Enterobacterales*, STA *Staphylococcus* species, AUR *Aerococcus urinae*, Arg arginine, 6MA N6-methyladenine, GBS group B streptococcus. Source data are provided in the Source Data file.

0.95; Fig. 2a), all of which showed significantly higher agmatine levels than urine from culture-negative patients ($p = 2.15 \times 10^{-45}$, $p = 1.43 \times 10^{-49}$, $p = 2.22 \times 10^{-52}$, $p = 3.85 \times 10^{-40}$, $p = 5.91 \times 10^{-47}$, respectively via two-tail unequal variance Welch *T*-test relative to culture-negative urine samples; Supplementary Table 2).

Validation of N6-methyladenine as a UTI biomarker

Although the agmatine-based screening approach captures most UTI infections caused by *Enterobacterales* species—which collectively account for >83% of UTIs[7]—it did not capture those caused by enterococci, staphylococci, or other less common species (e.g group B streptococcus, *Pseudomonas aeruginosa*, *Aerococcus urinae*, *Candida albicans*). Since these pathogens account for a small percentage of all UTIs, we prospectively collected a targeted cohort of 71 non-*Enterobacterales* UTI specimens along with 24 negative controls. LC-MS analyses showed that N6-methyladenine signals correctly differentiated culture-negative urine samples from this selected cohort of infections (sensitivity, 91%; specificity, 83%; PPV, 0.84; NPV, 0.91; Fig. 2b and Supplementary Table 3). However, there were clear species-to-species differences in the quality of these metabolic diagnostics, with *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Aerococcus urinae* showing the most significant differences after Bonferroni correction from multiple hypothesis testing ($p = 1.26 \times 10^{-3}$, $p = 3.22 \times 10^{-4}$, $p = 3.72 \times 10^{-6}$, respectively via two-tail unequal variance Welch *T*-test relative to culture-negative urine samples; Fig. 2b and Supplementary Table 4). N6-methyladenine levels were predictive of these three non-*Enterobacterales* species (AUC of 0.80; CI: 0.69–0.92; Supplementary Table 3). N6-methyladenine is part of a wider family of adenine derivatives, including 1-methyladenine and adenine, which were elevated in the urine of these UTI patients (Supplementary Table 4). However, our LC-MS analyses showed that the diagnostic performance of these additional markers was lower, and these compounds were linked to fewer species than N6-methyladenine.

Validation of microbial metabolic capacity

Agmatine and N6-methyladenine in the urine of UTI patients could potentially originate from microbial or human metabolism. To better understand the origin of these molecules, filter-sterilized urine was taken from healthy controls and was inoculated with *E. coli* and *S. aureus* isolates. In the case of the *E. coli* culture, 20 μM of ^{15}N-labeled arginine (a physiologically relevant level approximating the concentration of native urinary arginine) was added to the urine at the start of the incubation. Throughout the incubation, isotope-labeled arginine levels progressively decreased while isotope-labeled agmatine was produced (Fig. 2c). The appearance of isotope-labeled agmatine is expected under these conditions due to the microbial arginine decarboxylase activity of *E. coli*[9]. Likewise, we screened the *S. aureus* cultures over 12 h via LC-MS and observed that N6-methyladenine was produced by these microbes when grown in filter-sterilized urine (Fig. 2c). In summary, the most diagnostic biomarkers observed in UTI specimens are readily produced by UTI pathogens grown in filter-sterilized urine, indicating that these metabolites are naturally produced through microbial metabolic activity.

Urine specimens for UTI diagnostics are generally collected in outpatient clinics and delivered to centralized microbiology testing labs. Sample transport logistics and other delays in analysis could therefore complicate microbial diagnostics if microbes were allowed to grow. To control this, urine specimens are collected in boric acid tubes, which inhibit microbial growth. To verify that microbes stored in boric acid tubes will not produce our target metabolites, which could cause false positive results in specimens with sub-clinical bacterial levels (<10^7 CFU/L), we screened for agmatine production in three reference strains of *E. coli* and four clinical isolates, in boric acid over 24 h via LC-MS. In the absence of boric acid preservatives, agmatine levels reached a peak intensity after 7 h and maintained elevated levels over the next 17 h (Supplementary Fig. 1a). In contrast, isolates stored in boric acid preservatives did not produce agmatine over the 24-h period (Supplementary Fig. 1b).

Clinical cohort 1

Although untargeted metabolomics is currently not used for clinical diagnostics, mass spectrometry is a routine component of many diagnostic labs where it serves as a platform for quantifying a range of biomolecules including steroid hormones, drugs of abuse, and metabolites that are linked to inborn errors of metabolism[11]. Consequently, much of the requisite infrastructure necessary for diagnosing UTIs via metabolic profiles can be found in diagnostic laboratories. However, the instrumentation used for untargeted metabolomics analyses is quite different from that employed in clinical settings. To demonstrate the feasibility of adapting our metabolomics approach to routine clinical diagnostics, we developed a targeted diagnostics workflow using a triple quadrupole LC-MS instrument that is commonly used for clinical mass spectrometry.

To determine the performance of this clinically-adapted UTI diagnostics approach, we conducted a head-to-head performance evaluation of our LC-MS method vs results obtained via the traditional clinical microbiology approach[12]. This cohort consisted of 587 urine specimens submitted to Alberta Precision Laboratories (South Hub) over a 48-h period. The demographics of these specimens have been described previously[7]. Briefly, these samples were taken from the greater Calgary metropolitan area where 74% of urine cultures are submitted from ambulatory out-patient visits, 18% from hospitalized patients, and 9% from nursing home residents. Females have a 6-fold higher incidence rate of UTIs than males in this population. Importantly, Alberta follows the "Choosing Wisely Canada" guidelines wherein physicians are instructed to only submit urine cultures from symptomatic patients[13]. This head-to-head trial confirmed our previous results: urine agmatine levels correctly predicted *Enterobacterales* infections (AUC of 0.96, CI: 0.94–0.98; Fig. 3). Moreover, at the previously determined agmatine threshold of 174 nM, LC-MS diagnostics had a 95% sensitivity and 86% specificity for predicting *Enterobacterales* infections (Fig. 3 and Supplementary Table 5). As expected, our culture-negative samples, those classified as being of doubtful

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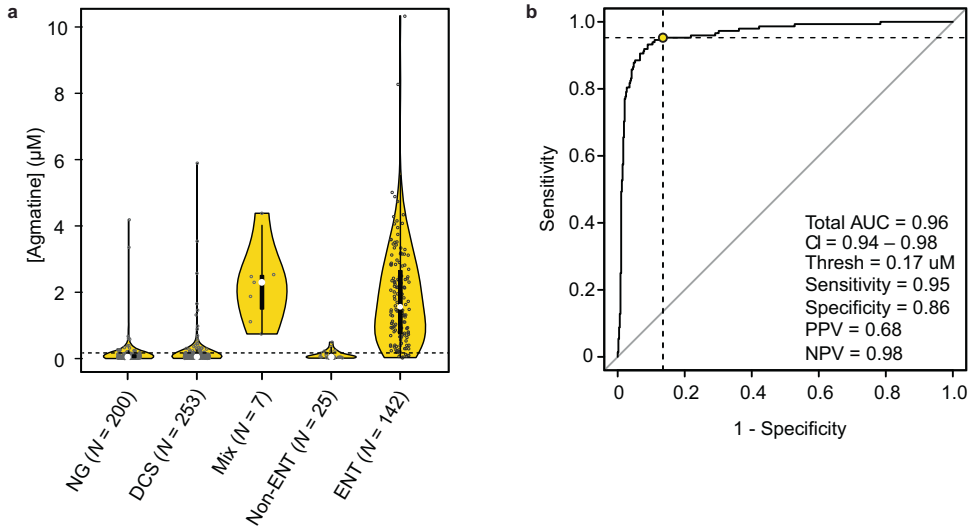

Fig. 3 | Blinded performance trial of clinically adapted metabolomics-based UTI diagnostics. a Agmatine concentrations in a blinded cohort of clinical urine samples. Categories on the *x*-axis represent calls from traditional microbiology analysis performed at a regional diagnostic lab. White dots indicate median values, thick black bars indicate interquartile ranges, and thin black lines indicate 3× interquartile hinge points. **b** Receiver operating characteristic curve demonstrating the performance of agmatine as a diagnostic marker for *Enterobacterales* in a blinded, prospective trial. Sensitivity and specificity for 0.17 μM threshold is represented by the yellow dot. NG no growth, DCS doubtful clinical significance, non-ENT non-*Enterobacterales*, ENT *Enterobacterales*, Mix polymicrobial cultures containing at least one *Enterobacterales* member, AUC area under curve, CI confidence interval, Thresh threshold, PPV positive predictive value, NPV negative predictive value. Source data are provided in the Source Data file.

clinical significance (DCS; <10^7 CFU/L), and a small number of urine samples (<5%) containing non-*Enterobacterales* microbes all had agmatine levels below the limit of detection.

Clinical cohort 2

One limitation of our original targeted method is that it was specific to agmatine and thus could not be expanded to the analysis of other urinary metabolites, including creatinine, a standard marker of urinary dilution[14,15]. To address this limitation, we developed a "dilute and shoot" sample preparation workflow coupled with a 3.2-min LC-MS targeted assay for agmatine, N6-methyladenine, and creatinine. The analytical characteristics of this assay are detailed in Supplementary Figs. 3 and 4 and Supplementary Tables 8–13. To enable accurate quantification of agmatine, N6-methyladenine and creatinine, which are present at substantially different concentrations in urine (nM vs mM), we used a higher dilution (1:200 final) than in the previous trial. Using this assay, we analyzed 1042 urine specimens from suspect UTI cases. In this cohort, we again showed that urinary agmatine concentrations over 174 nM are an accurate predictor of *Enterobacterales* infections (sensitivity, 93%; specificity, 90%; PPV, 0.67; NPV, 0.98; Fig. 4 and Supplementary Table 6). Moreover, we showed that creatinine normalization had a slight negative impact on the performance of the assay (AUC of 0.95, CI: 0.93–0.97 vs AUC of 0.92, CI: 0.89–0.95; for agmatine alone vs creatinine-normalized agmatine levels, respectively; Fig. 4, Supplementary Table 6). N6-methyladenine levels were also found to be predictive of UTIs (AUC of 0.71, CI: 0.69–0.74; inclusive of all species; Supplementary Table 6). As expected, the performance of N6-methyladenine was improved when analyses were restricted to the three known N6-methyladenine-producing species (AUC of 0.89, CI: 0.75–1.0; Supplementary Table 6 and Supplementary Fig. 2). In this restricted sub-cohort, a N6-methyladenine concentration of 217 nM had a sensitivity of 0.78 and specificity of 0.79 for differentiating infections with *S. aureus*, *S. saprophyticus*, and *A. urinae* vs infections with all other species and negative specimens (Supplementary Fig. 2 and Supplementary Table 6). We also tested the performance of a composite model using both agmatine and N6-methyladenine levels to predict infections. This combined model performed slightly worse than the agmatine-only model as a generalized tool for detecting UTIs (inclusive of all species, AUC of 0.78 vs 0.85 for the composite vs agmatine-only models, respectively; Supplementary Table 6).

Discussion

UTIs are one of the most common bacterial infections and are responsible for over eight million healthcare visits per year in the U.S. alone[16,17]. As a result, urine cultures are one of the most common tests performed by microbiology reference labs (24–40% of all cultures analyzed)[18]. The current diagnostic workflow used to process these samples follows a multi-day procedure that includes streaking urines on agar plates, incubation for 18 h, colony counting, microbial identification via matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, and antibiotic susceptibility testing[12]. This long diagnostic workflow (24–48 h) encourages clinicians to prescribe antibiotics before lab results are available. Given that up to 70% of suspected UTI cases collected by clinical testing facilities are ultimately found to be culture negative, the current empiric antimicrobial prescribing practice leads to overprescribing and may contribute to the spread of antimicrobial resistance[19–21]. Therefore, rapid diagnostic tools that can quickly identify patients with UTIs could enable more precise antimicrobial prescribing practices[22–24].

Herein, we used untargeted metabolomics to identify native biomarkers present in uncultured urine that robustly differentiate between culture-negative and culture-positive patients. Using these untargeted data, we identified two predictive metabolites (agmatine and N6-methyladenine) that are elevated in uncultured urine from patients who were later confirmed to have UTIs. We show that these biomarkers are produced by UTI pathogens when they are cultured in urine and that an agmatine concentration of 174 nM and N6-methyladenine concentration of 217 nM in urine corresponds with the clinical definition of bacteriuria (>10^7 CFU/L)[8]. We developed a clinical LC-MS workflow to detect these molecules and demonstrated that these biomarkers are robust predictors of UTIs in two independent cohorts consisting of 1629 clinical specimens in total (AUC > 0.95 and 0.89 for 13 *Enterobacterales* and 3 non-*Enterobacterales* species,

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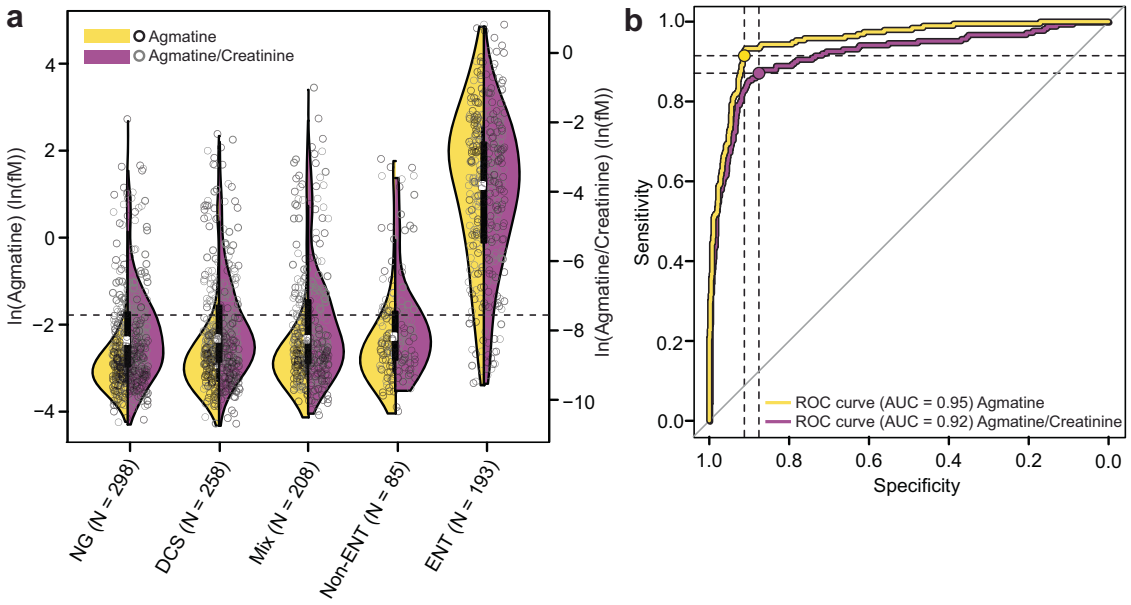

Fig. 4 | Creatinine normalized agmatine concentrations in a blinded cohort of urine specimens. a Violin plot of agmatine concentrations compared to creatinine normalized agmatine concentrations. White dots indicate median values, thick black bars indicate interquartile ranges, and thin black lines indicate 3× interquartile hinge points. **b** Receiver operating characteristic curves demonstrating the equivalence between detected agmatine levels and creatinine normalized agmatine levels. Sensitivity and specificity were calculated for the 0.17 µM threshold. NG no growth, DCS doubtful clinical significance, non-ENT non-*Enterobacterales*, ENT *Enterobacterales*, Mix polymicrobial cultures containing at least one *Enterobacterales* member, ROC receiver operating characteristic, AUC area under curve. Source data are provided in the Source Data file.

respectively). Collectively, these data show that metabolite levels present in uncultured urine can be used to accurately reproduce the results generated by traditional urine culture and, therefore, could potentially enable novel rapid UTI testing approaches.

The multiple independent trials we conducted over the course of this study provide some insights into leveraging metabolite biomarkers of UTIs. Firstly, our creatinine normalization study showed that correcting metabolite levels to account for variability in urinary dilution does not improve their diagnostic performance—if anything these corrections make things worse (Fig. 4 and Supplementary Table 6). This observation is consistent with our microbial culturing experiments, which showed that agmatine and N6-methyladenine are normal microbial catabolites produced when UTI pathogens are grown in urine. Thus, if agmatine and N6-methyladenine levels in patient samples primarily result from microbial metabolism within the urinary tract, we would expect both the biomarker levels and planktonic bacterial density in the urine (in CFU/L) to be diluted in parallel. Given that the clinical definition of bacteriuria is based on bacterial density[8] it is unsurprising that the microbially-produced catabolites we identified were most diagnostically robust when their levels were not corrected for urinary dilution. This observation has practical implications: quantifying the abundant creatinine necessitated a higher dilution than is necessary for quantifying the low-abundance agmatine and N6-methyladenine. This over-dilution diminished the performance of our target markers in our final clinical cohort, as their signals were closer to the LC-MS noise threshold. Thus, omitting creatinine normalization improves the performance of our assay.

Another practical observation from our final clinical cohort is that a mixed agmatine/N6-methyladenine model for predicting infection performed slightly worse than an agmatine-only model (all-cause UTI; mixed AUC of 0.78, CI: 0.74–0.81; agmatine-only AUC of 0.85, CI: 0.82–0.88; Supplementary Table 6). The reason for this is that only 9 out of the 1042 urine specimens (9 out of 277 positive samples; 3.2%) in the final cohort were from infections with N6-methyladenine-producing organisms. This prevalence is consistent with previous studies in the same region (3.2% vs 2.1%)[7]. Thus, screening samples via N6-methyladenine levels added false discovery, but minimal additional sensitivity to the assay. This is a common problem in screening for rare events[25] and suggests that an agmatine-only screening approach might be preferable.

One important consideration about the generalizability of our findings is that the mix of species observed in clinical specimens will have a direct impact on the clinical performance of our biomarker-based diagnostic approach. The proportions of species we observed are largely consistent with those reported elsewhere with the exception of *S. saprophyticus* (an N6-methyladenine-producer), which was underrepresented relative to the North American average (1% vs 5%)[26,27]. Therefore, N6-methyladenine could potentially be of greater diagnostic value in other health jurisdictions—especially in jurisdictions with a greater proportion of out-patient cases.

The robust performance of agmatine as a diagnostic marker for *Enterobacterales* infections across four separate cohorts suggests that this metabolite is a good candidate for clinical applications. As an LC-MS assay, agmatine screening could be used to rapidly identify patients with clinically significant bacteriuria. This screening approach is 18 h faster than the traditional urine culture workflow and could accommodate thousands of analyses per day[28]. Alternatively, agmatine screening for UTIs could also be enabled via point-of-care testing tools, such as lateral flow assays, which could be made possible via existing commercial antibodies for agmatine[29]. If such a point-of-care tool were made, it would complement existing dipstick-style UTI screening tests (i.e., nitrate and leukocyte esterase)[30]. In this context, agmatine screening would be both favorable from a performance perspective (Supplementary Table 7) and because the *Enterobacterales*-specific agmatine test could be used to help guide more precise prescribing decisions.

Although we demonstrated the robust performance of our metabolomics-based UTI screening concept, there are some important limitations to this strategy that warrant discussion. Firstly, the biomarkers we identify here report bacteriuria (high levels of bacteria in the

urine), not infection. This distinction is important as asymptomatic bacteriuria is common and does not necessarily warrant antimicrobial therapy[31]. Thus, agmatine/N6-methaladenine screening would need to be paired with symptomatic evaluation by a clinician to enable UTI diagnosis (i.e., following existing practice). Secondly, the gold-standard diagnostic workflow involves both species-level identification of the pathogen and empiric antimicrobial susceptibility testing, neither of which is possible with our screening approach alone. However, most UTIs are treated without supporting lab data. Therefore, we envision agmatine/N6-methaladenine screening as a tool to improve empiric prescribing practices rather than as a replacement for culture-based diagnostics. Thirdly, the biomarkers we report here have clinically significant blind spots, especially in the context of UTIs from hospitalized patients. New biomarkers covering *Candida*, *Enterococcus, Pseudomonas*, and group B streptococcus must be identified if our proposed metabolism-based UTI screening concept is to be applied to hospitalized patients. Despite these limitations, the agmatine/N6-methyladenine screening approach we introduce here is robust, reproducible over multiple independent clinical cohorts, and could add significant value as a point-of-care or patient-near tool to enable more precise empiric prescribing decisions for uncomplicated UTIs.

Methods

Ethics

Discarded residual human urine samples were used in this research, collected under the approved conjoint institutional Research Ethics Board (REB) protocol # REB19-0442 at the University of Calgary, which waived the need for informed consent. Consent was waived because all testing was conducted on residual samples, in no way did test results impact patient care, and all research results were delinked from patient demographic information. Moreover, samples came from a multitude of locations and it would be impossible to contact patients to gain consent. A limited number of healthy urine samples were collected with written, informed consent from adult volunteers, authorized under this same REB protocol.

Experimental design

Untargeted analysis for biomarker discovery was performed directly on patient urine samples (77 culture positive, 33 culture negative) using a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer. Putative biomarkers were identified using MS/MS analysis and matched to standards using fragmentation spectra and retention times. To identify agmatine thresholds that differentiate culture positive ($\geq 10^7$ CFU/L; $n = 240$) vs culture negative urines ($<10^7$ CFU/L; $n = 279$), patient urines were spiked with a known concentration of labeled [U-^{13}C]agmatine, purified using solid phase extraction (SPE), and analyzed on the TSQ Quantum™ Access MAX Triple Quadrupole mass spectrometer. Agmatine concentrations were quantified based on the signal ratio between isotope-labeled and native species. Quantitative analyses of N6-methyladenine were performed directly on patient urine samples (71 culture positive non-*Enterobacterales*, 24 culture negative) using external standards following analysis via the Q Exactive™ HF platform. To demonstrate that agmatine and N6-methyladenine originate from microbial metabolism, *E. coli* ($n = 3$) and *S. aureus* ($n = 2$) were incubated in sterilized urine and the production of biomarkers was monitored. Inhibition of agmatine production by boric acid was also monitored in filter-sterilized urine seeded with *E. coli* during a 24-h incubation period. Two clinical cohorts were used to validate discovered biomarkers. The first was performed using SPE-purified urine samples ($n = 587$) on the TSQ Quantum™. In the second (dilute and shoot approach), methods were developed to quantify agmatine, N6-methyladenine, and creatinine directly in urine using a single injection. For these analyses, urine from residual clinical urine samples ($n = 1042$) was analyzed after a 1:200 dilution (V:V in 50% methanol final) using the TSQ Altis™ triple quadrupole mass spectrometer. The specific LC-MS methods for each experiment are described below.

Urine sample collection

Mid-stream urine samples were collected from Alberta Precision Laboratories' standardized urine analysis workflow, consisting of samples submitted for urine culture from symptomatic ambulatory (74%), hospitalized (18%), and nursing home patients (9%) in the Calgary metropolitan area[7]. These infections have well-known sex-linked differences in prevalence that have been studied in the Calgary metropolitan region. Our study draws samples from the entire breadth of the Calgary metropolitan clinical sample pool and thus appropriately reflects the sex biases inherent to this type of infection. Age demographics are divided as follows: 20/1000 for ages <1; <20/1000 for ages 1–59; 24/1000 for ages 60–69; 32/1000 for ages 70–79; 100/1000 for ages 80–89; 851/1000 for ages >90. Incidence rates in females are 30/1000 and males are 5/1000. Samples were de-identified prior to transfer to the study team. In brief, urine was plated either manually with a loop or using automated equipment onto UriSelect™ Media (Bio-Rad, Canada) and incubated aerobically for 18 h. The growth of bacteria was enumerated as per standard recommendations[12] and colonies present in significant quantities were speciated using the Vitek™ MS system (bioMérieux, Canada). Residual samples from this standard diagnostic lab workflow were fixed 1:1 with 100% methanol within 48 h post-receipt at Alberta Precision Laboratories, and then frozen at −80 °C. All samples were thawed, centrifuged at 14,800 × *g*, and diluted ten-fold further into 50% HPLC-grade methanol prior to LC-MS analysis.

Untargeted mass spectrometry

Untargeted analysis of positive and negative urine samples was performed for UTI biomarker discovery and quantification of N6-methyladenine thresholds. Methods were adapted from previously published studies[32–37]. Briefly, metabolic analyses were performed on a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific) coupled to a Vanquish™ UHPLC System (Thermo Fisher Scientific). Chromatographic separation of metabolites was performed on Syncronis™ HILIC UHPLC column (2.1 mm × 100 mm × 1.7 μm, Thermo Fisher Scientific) at a flow rate of 600 μL/min using a binary solvent system: solvent A, 20 mM ammonium formate pH 3.0 in mass spectrometry grade H_2O and solvent B, mass spectrometry grade acetonitrile with 0.1% formic acid (%*v*/*v*). For initial biomarker discovery experiments, the following 15-min gradient was used: 0–2 min, 100% B; 2–7 min, 100–80% B; 7–10 min, 80–5% B; 10–12 min, 5% B; 12–13 min, 5–100% B; 13–15 min, 100% B. The mass spectrometer was run in positive and negative full scan mode at a resolution of 240,000 scanning from 50 *m*/*z* to 750 *m*/*z*. Metabolite data were analyzed by El-MAVEN (v. 12.0) software package[38,39]. Metabolites were identified by matching observed *m*/*z* signals (±10 ppm) and chromatographic retention times to those observed from a commercial metabolite library of standards (MSMLS; Sigma-Aldrich). Assignments were verified on the Thermo Fisher Scientific Q Exactive™ HF platform via MS/MS fragmentation analysis and co-elution of standards with the identified signals from urine.

Targeted mass spectrometry

Targeted mass spectrometry analysis for validation of agmatine as a UTI biomarker and *Clinical cohort 1* were performed on a TSQ Quantum™ Access MAX mass spectrometer (Thermo Fisher Scientific). For quantitative analyses the following transitions were monitored using a fixed collision energy of 15 eV: ^{12}C agmatine, 131.2 → 72.4 *m*/*z*; [U-^{13}C] agmatine, 136.2 → 76.4 *m*/*z* in positive ionization mode. The same HPLC column and buffers were used as described above, with the following modified 6-min gradient: 0–0.5 min, 100% B; 0.5–2 min, 100–5% B; 2–3.5 min, 5–0% B; 3.5–4.5 min, 0% B; 4.5–5 min, 0–100% B;

5–6 min, 100% B. For targeted experiments using the TSQ Quantum™, electrospray ionization source conditions were as follows: spray voltage of 3000 V, sheath gas of 25 (arbitrary units), auxiliary gas of 10 (arbitrary units), sweep gas of 0 (arbitrary units), capillary temperature of 275 °C, auxiliary gas temperature of 325 °C.

Dilute and shoot

For *Clinical cohort 2* ("dilute and shoot" approach), LC-MS/MS analysis was performed on a Vanquish™ UHPLC system, with an integrated Vanquish™ charger module (Thermo Fisher Scientific), coupled to a TSQ Altis™ triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization (HESI-II) probe. The UHPLC-MS platform was controlled by an Xcalibur™ data system (Thermo Fisher Scientific). Chromatographic separation was achieved on a Syncronis™ HILIC column (50 × 2.1 mm, 1.7 μm, Thermo Fisher Scientific) using a binary solvent system composed of LC-MS grade H_2O with 20 mM ammonium formate, pH3 (solvent A) and LC-MS grade acetonitrile with 0.1% (*%v/v*) formic acid (solvent B). The following 3.2 min gradient was used: 0–0.5 min, 95% B; 0.5–1.75 min, 95–30% B; 1.75–1.8 min, 30–5% B; 1.8–2.3 min, 5% B; 2.3–2.4 min, 5–95% B; 2.4–3.2 min, 95% B. The flow rate was 1 mL min^{-1} and the sample injection volume was 2 μL. The auto sampler and charger were kept at 6 °C and the column at 30 °C. For quantitative analyses, the following transitions were monitored using the following polarities, collision energies, and transitions: agmatine, positive, 16 eV, 131.0 → 97.1 *m/z*; N6-methyladenine, positive, 23 eV, 150.0 → 122.9 *m/z*; creatinine, positive, 13 eV, 114.1 → 86.1 *m/z*. Electrospray ionization source conditions were as follows: spray voltage of 3000 V (2500 V, negative mode), vaporizer temperature of 250 °C, sheath gas of 35 (arbitrary units), auxiliary gas flow of 15(arbitrary units) and sweep gas flow of 2 (arbitrary units), capillary temperature of 275 °C. The analytical characteristics of this method were tuned according to Food and Drug Administration guidelines for chromatographic assays[40] (Supplementary Figs. 3 and 4 and Supplementary Tables 8–13).

Determination of agmatine threshold values using [U-^{13}C] agmatine

Targeted quantitative analysis was conducted to establish agmatine thresholds between culture-positive and culture-negative urine samples (*Validation of agmatine as a UTI marker*) and to assess performance thresholds (*Clinical cohort 1*). Quantitative analyses of agmatine in 519 urine samples were undertaken using a known concentration of a [U-^{13}C]agmatine isotope as an internal standard in the urine sample (see [U-^{13}C]agmatine standard biosynthesis and purification below). Prior to MS analysis, samples were purified using a SPE 96-well HyperSep™ Silica plate (Thermo Fisher Scientific). Columns were equilibrated with methanol followed by water, and then loaded with the sample of interest. Columns were then washed with methanol, water, methanol with 0.1% formic acid, and water with 0.1% formic acid. The target analyte was eluted with water containing 2% formic acid. LC-MS analysis was performed using a TSQ Quantum™ Access MAX Triple Quadrupole mass spectrometer (Thermo Fisher Scientific). For the clinically-adapted blinded evaluation, clinical urine specimens were centrifuged and the supernatant was spiked with the [U-^{13}C]agmatine isotope. These samples were analyzed directly on a TSQ Quantum™ Access MAX instrument (Thermo Fisher Scientific).

[U-^{13}C]agmatine standard biosynthesis and purification

E. coli (strain MG1665) was seeded into M9 minimal media containing 22.2 mM [U-^{13}C]glucose, grown overnight, and then seeded into fresh [U-^{13}C]glucose-containing media. This culture was then incubated under agitation in a 37 °C, 5% CO_2 incubator. Glucose consumption was monitored using a blood glucose monitoring system (Bayer Contour Next). When glucose levels in the media reached 5 mM, the culture was centrifuged, the supernatant was retrieved, adjusted to pH 7 with ammonium bicarbonate, and steri-filtered. [U-^{13}C]agmatine was isolated and purified using a SPE column (HyperSep™ Silica Cartridges, # 60108-712, Thermo Fisher Scientific), as described above, and scaled accordingly to the column volume. The fraction was concentrated to 1/10th of its initial volume in a vacuum concentrator at 4 °C and labeled [U-^{13}C]agmatine in the final sample was quantified by stable isotope dilution via LC-MS/MS using an unlabeled internal standard curve.

Validation of microbial metabolic capacity

E. coli and *S. aureus* were inoculated in sterilized urine to verify that agmatine and N6-methyladenine originate from microbial metabolism. In vitro microbial samples were cultured from reference strains of bacteria (*E. coli*: MG1665, ATCC 25922, ESBL ATCC BAA-196; *S. aureus*: ATCC 25923, ATCC 43300 (MRSA)). Briefly, cryogenic stocks were grown overnight in Mueller-Hinton medium, then inoculated at 10^5 CFU/mL in sterilized urine and grown at 37 °C. *E. coli* cultures were spiked with [guanido-$^{15}N_2$]arginine (herein referred to as "[$^{15}N_2$]arginine") such that the urine contained an approximate 1:1 ^{12}C arginine to [$^{15}N_2$]arginine ratio. The disappearance of [$^{15}N_2$]-arginine and appearance of [guanido-$^{15}N_2$]agmatine (herein referred to as "[$^{15}N_2$]agmatine") was monitored over 5 h using LC-MS/MS. Similarly, the appearance of the N6-methyladenine signal was assessed in *S. aureus* cultures at 0 h, 4 h, 8 h, 10 h, and 12 h with LC-MS.

. Inhibition of agmatine production by boric acid preservative, at concentrations used in BD Vacutainer Plus Tubes (2.63 mg/mL boric acid, 3.95 mg/mL sodium borate, 1.65 mg/mL sodium formate) was monitored in filter sterilized urine seeded with *E. coli* (three reference strains—MG1665, ATCC 25922, ATCC BAA-196—and four clinical isolates) over a 24-h period and analyzed on the Q Exactive™ HF platform (Thermo Fisher Scientific).

Blinded performance evaluation of agmatine vs traditional urine analysis pipeline

Mid-stream urine samples for the blinded head-to-head performance evaluation were collected from Alberta Precision Laboratories' standardized urine analysis workflow (described above in *Urine sample collection*). These samples were stripped of diagnostic information and assigned a unique identifier prior to their transfer to the analytical team. Following analysis using a clinically adapted metabolomics platform, diagnostic data were un-masked and the results of the quantitative agmatine-based MS approach were scored against the clinical diagnostic laboratory calls, using 174 nM as the threshold for UTI-positive samples.

Targeted quantitative assays were conducted in two independently blinded trials using slightly differing methods. In trial 1, targeted mass spectrometry was performed to quantify the levels of agmatine in a blinded cohort of 587 urine specimens. This was done following the targeted mass spectrometry workflow described above using spiked in [U-^{13}C]agmatine and SPE sample cleanup.

In trial 2, 1042 urine specimens were analyzed using a simplified dilute and shoot targeted workflow that accurately captures the two target analytes as well as creatinine in a single injection. This approach negates the use of SPE, and instead, a 1/20 dilution of the sample was directly injected onto the MS following our chromatographic methods described above using a 3.2-min gradient and external agmatine standard curves were used for quantification.

Data analysis and statistics

Raw mass spectrometry files were converted into.mzXML files via MSConvertGUI (ProteoWizard Tools)[41]. All full scan MS analyses were conducted in El-MAVEN (v. 12.0). MS/MS data were analyzed using Xcalibur 4.0.27.19 software (Thermo Fisher Scientific). Untargeted mass spectrometry data were grouped according to co-variance, co-retention, and similarity to common adducts/fragments using previously published software developed in R Statistics[42]. Violin plots,

ROC curves, and scatter plots were all generated in R Statistics using existing packages, (FUGU-MS, pROC, and vioPlot). A two-tail unequal variance Welch *T*-test was used to test differences in agmatine and N6-methyladenine levels in culture-positive urine samples compared to negative controls. Alpha thresholds for significance were corrected for multiple hypothesis testing via Bonferroni correction.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information. Source data are provided with this paper.

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Author contributions

S.D.W., C.C.Y.C., D.B.G., D.G.B., T.R., and I.A.L. designed and performed the experiments. S.D.W., D.B.G., D.G.B., C.C.Y.C., R.A.G., R.A., and T.R. collected and interpreted mass spectrometry data. K.P., N.V.B., and I.A.L. interpreted the results and wrote the manuscript in consultation with all authors.

Competing interests

Drs. Lewis, Gregson, and Mr. Groves are authors of a patent relating to the use of LC-MS for detecting urinary tract infections. These authors have a relationship with Rapid Infection Diagnostics Inc., a company that is developing commercial microbiology diagnostic tools. No other competing interests were declared.

Additional information

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