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Comprehensive urinary metabolomic characterization of a genetically induced mouse model of prostatic inflammation

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Abstract

Dysfunction of the lower urinary tract commonly afflicts the middle-aged and aging male population. The etiology of lower urinary tract symptoms (LUTS) is multifactorial. Benign prostate hyperplasia, fibrosis, smooth muscle contractility, and inflammation likely contribute. Here we aim to characterize the urinary metabolomic profile associated with prostatic inflammation, which could inform future personalized diagnosis or treatment, as well as mechanistic research. Quantitative urinary metabolomics was conducted to examine molecular changes following induction of inflammation via conditional Interleukin-1 β expression in prostate epithelia using a novel transgenic mouse strain. To advance method development for urinary metabolomics, we also compared different urine normalization methods and found that normalizing urine samples based on osmolality prior to LC-MS most completely separated urinary metabolite profiles of mice with and without prostate inflammation via principal component analysis. Global metabolomics was combined with advanced machine learning feature selection and classification for data analysis. Key dysregulated metabolites and pathways were identified and were relevant to prostatic inflammation, some of which overlapped with our previous study of human LUTS patients. A binary classification model was established via the support vector machine algorithm to accurately differentiate control and inflammation groups, with an area-under-the-curve value of the receiver operating characteristic of 0.81, sensitivity of 0.974 and specificity of 0.995, respectively. This study generated molecular profiles of non-

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Conflict of interest

The authors declare no competing interests.

bacterial prostatic inflammation, which could assist future efforts to stratify LUTS patients and develop new therapies.

Keywords

Metabolomics; LC-MS; Normalization; Inflammation; Osmolality; Support vector machine

1. Introduction

Lower urinary tract symptoms (LUTS) commonly affect the middle-aged and aging male population and arise due to a number of possible contributors: benign prostatic hyperplasia, fibrosis, and inflammation [1–4]. The annual costs of LUTS diagnosis and treatment in the USA is estimated at approximately \$4 billion per year [5], but the condition's root causes and mechanistic basis are still unclear. Mice are often used to model some of the key risk factors for LUTS, including bladder and prostate inflammation [3,6–9]. Animal models are useful for studying mechanisms underlying symptoms, but how relevant some animal models are to human patients is unknown. A major challenge is that LUTS are diagnosed by patient-reported symptoms and cannot be assessed the same way in animals. While a variety of urodynamic and biochemical endpoints can be measured in humans and animals, it is unclear which most closely associate with human symptoms [10].

With the advancements of molecular biology, genetically engineered mouse models have been developed to recapitulate many aspects of the corresponding human diseases with both histological and genetic accuracy [11,12]. The tetracycline-dependent regulatory system is an effective approach for manipulating strength and latency of gene expression to model human diseases [13]. The doxycycline (Dox)-inducible reverse tetracycline transactivator (rtTA) enables conditional (Dox-dependent) gene expression in both tissue and temporal specific manner. Rao et al. [14] developed a rtTA regulatory system that employs the cis-regulatory elements of the prostate and clones restricted *Hoxb13* gene to mediate transgene expression in the prostate of the transgenic mice. The same rtTA regulatory system was used to develop a novel prostatic inflammation mouse model by driving proinflammatory cytokine Interleukin-1 beta (IL-1 β) expression in the prostate epithelium [14–16,2,17–19].

Urine is the most accessible non-invasive biofluid for studying lower urinary tract inflammation. Profiling the urinary metabolome of mice with defined pathologies can enable us to identify molecular fingerprints associated with each pathology, as a means to deconstruct the complex etiologies of LUTS and perhaps inform future personalized diagnostics and treatments. These molecular fingerprints also inform hypothesis testing about disease mechanisms and pathologies that trigger urinary inflammation. Multiple analytical platforms have been used to study metabolomics, including nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and capillary electrophoresis-mass spectrometry (CE-MS), each with its own advantages and drawbacks [20–30]. LC-MS-based approaches has led the field of urinary metabolomics, with various application to the studies of kidney cancer, prostate cancer, and many other diseases [24,26,31–33].

In our previous study, we developed a comprehensive approach combining LC–MS-based metabolomics and machine learning bioinformatics to discover putative LUTS biomarkers in urine of human patients [34]. Both our metabolomic and proteomic studies of human LUTS revealed a subset of biomarkers related to fibrosis and inflammation [34–36]. This is important because fibrosis is associated with LUTS severity [37] and prostate inflammation is associated with risk of clinical progression of LUTS [38]. Here, we examine the urinary metabolite profile of mice with genetically induced prostatic inflammation, which could correlate mouse models with human patients and inform future efforts to develop objective biomarkers for more precise treatment decisions in patients with LUTS.

2. Materials and methods

2.1. Transgenic mouse model and urine collection

Mouse handling and sample collection procedures were approved by the University of Maryland Baltimore County Animal Care and Use Committee. The mouse model of genetically induced prostatic inflammation was developed by a tightly regulated induction of the pro-inflammatory cytokine Interleukin-1 beta, using a *Hoxb13*-driven reverse tetracycline transactivator system, described previously [14,19]. Doxycycline was administered in drinking water to the transgenic mice for 6 weeks to induce IL-1 β , resulting in localized prostatic inflammation. Details of the construction of the inducible IL-1 β system and the inflammatory phenotype will be published elsewhere. Mouse urine samples were collected from six transgenic mice at baseline and again from the same mice after 6 weeks of doxycycline administration. Mice were restrained by hand and massaged on the lower abdomen to induce voiding. Urine was collected in a sterile weigh boat and transferred to a microfuge tube that was immediately placed on dry ice and stored in –80 degree celsius for further analysis.

2.2. Mouse urine sample preparation

Mouse urine aliquots were thawed on ice and centrifuged at $10,000 \times g$ for 10 min to remove cell debris and particulates. Urinary metabolites were obtained by collecting flow-through fractions from 3 kDa molecular weight cut-off filters (Millipore Amicon Ultra, Billerica, MA). Osmolality of each metabolite fraction was measured by a freezing point depression osmometer (Osmometer Model 3250, Advanced Instruments, MA). For normalization to osmolality, each sample was diluted to achieve the same osmolality, 250 osmoles/kg H₂O. Another set of samples were prepared without normalization by diluting each metabolite fraction 4-fold. A quality control (QC) sample was prepared by mixing equal volumes of all urinary metabolite fractions. Aliquoted QC samples and all mouse urine samples were stored at –80 °C.

2.3. LC–MS analysis

LC–MS analyses of mouse urine metabolites were carried out on a Dionex UltiMate 3000 LC system coupled with a high-resolution accurate-mass Q-Exactive™ Orbitrap mass spectrometer (Thermo, San Jose, CA). Chromatographic separation was conducted on a Phenomenex HILIC column (2.1 $\times$ 100 mm, 1.7 μ m, 100 Å) at 30° column temperature and 0.3 mL/min flow rate. Mobile phase A was 13 mM ammonium acetate in LC–MS grade

water and mobile phase B was LC–MS grade acetonitrile. The LC gradient for ESI(+) was set as follows: 0–4 min, 90–80% solvent B; 4–11 min, 80–45% B; 11–14 min, 45–5% B; 14–16 min, 5% B; 16–20 min, 90% B. The LC gradient for ESI(–) was: 0–8 min, 90–50% solvent B; 8–9 min, 50–5% B; 9–10 min, 5% B, 10–14 min, 90% B. The full MS scan range was m/z 70–1000. Resolution was 70 K. Automatic gain control (AGC) target was 1×10^6 . Maximum injection time (IT) was 100 ms. For targeted LC–MS/MS analyses, MS parameters were set as follows: resolution of 35 K; AGC target of 5E5, maximum IT of 100 ms, isolation window of 2 m/z , and normalized collision energy (NCE) of 30 with higher-energy collisional dissociation (HCD) fragmentation. The injection volume was 5 μ L with two technical replicates.

2.4. Data analysis

Raw data files were acquired by Thermo Scientific Xcalibur software and subjected to SIEVE™ 2.2 software for peak alignment and framing. A QC data file was used as the reference file for peak alignment with a maximum retention time shift of 0.2 min. The ICIS algorithm was used for peak detection with an intensity threshold for component extraction of 1E6, and a signal-to-noise ratio of 3. The frame time width was 2 min and m/z width was 5 ppm. Chromatographic peak area values were log-transformed and a two-tailed Student's *t*-test was used to compare log-transformed peak area values between groups with and without inflammation. *P*-values were corrected for multiple hypothesis testing by the Benjamini-Hochberg procedure, as implemented in R, and the threshold of statistical significance was set as 0.05 [39]. Principal component analysis (PCA) was performed via MetaboAnalyst [40]. As described in our previous human urine metabolomics study [34], machine learning feature selection with the support vector machine (SVM)-based attribute evaluation and information gain (IG)-based attribute filtering was performed to rank all detected features based on their contribution in the separation of disease and control groups in the WEKA software [41]. The top 100 features in SVM and IG evaluation were overlapped with statistically significant features to generate the final list of features for subsequent metabolite identification. This method combines the traditional *p*-value based statistical tests and advanced machine learning feature selection, which has been proven to enable effective selection of candidate biomarkers in human urine samples [34]. For metabolite identification, accurate mass matching (mass error ≤ 3 ppm), MS/MS matching, and standard confirmation were conducted based on the designed flowchart described previously [34]. To identify altered metabolic pathways in the mouse model, significantly dysregulated metabolite identities were mapped into the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using MBRole (Metabolites Biological Role) platform [42].

3. Results and discussion

3.1. Comparing different normalization methods for mouse urine

Urine volume and solute concentration are affected by water consumption, diet, behavior, and physiology, distinguishing urine from many biofluids used in metabolomic studies. To minimize the impact of these factors on downstream analyses, researchers often normalize urine samples to urinary creatinine content, osmolality, specific gravity, or perform normalization procedures after metabolic profiles are acquired (post-acquisition

normalization) [43–48]. Normalization to endogenous creatinine, a common practice in clinical sciences, is unsuitable for metabolomics analysis because urinary creatinine content is affected by physical activity and external stressors such as kidney impairment [45,46]. Single point normalization to creatinine can also be problematic for thousands of detected metabolites in a metabolomic experiment. Pre-acquisition normalization methods are generally reported to perform better than post-acquisition normalization techniques [44,47]. Warrack et al. showed that normalization to osmolality prior to instrumental analysis performed better than other methods based on the premise that osmolite concentration can represent the total urinary metabolite content [43].

Here, we investigated several normalization methods, including pre-acquisition normalization to osmolality, pre-acquisition normalization to volume, post-acquisition normalization to osmolality, post-acquisition normalization to creatinine, post-acquisition normalization to the median, and post-acquisition normalization to the sum. These methods were evaluated based on peak alignment and ability to differentiate between mice with and without inflammation. We first compared chromatographic peak alignment scores across normalization methods. Pre-acquisition normalization to osmolality (0.912 ± 0.044 for ESI(+)) and 0.910 ± 0.037 for ESI(–)) performed the best with peak alignment scores closest to 1. Pre-acquisition normalization to volume yielded alignment scores of 0.851 ± 0.054 for ESI(+)) and 0.886 ± 0.058 for ESI(–)). We next used PCA to determine which method separates control mice, prostate inflammation mice, and QC samples into three distinct clusters. Pre-acquisition normalization to osmolality also outperformed other methods, obtaining the complete separation of noninflammation control, inflammation, and QC groups (Fig. 1). Only conducting post-acquisition normalization to median, sum or creatinine did not provide complete separations of different groups (figures not shown). Pre-acquisition normalization methods performed better than the post-acquisition normalization methods which was consistent with previously reported studies [47]. Sample normalization in early steps of sample preparation performs the best to reduce sample variation before instrumental acquisition (e.g., LC–MS) and can be combined with post-acquisition normalization method for data analysis.

Based on the results above, urinary metabolite fractions were normalized to osmolality before instrumental analysis. The denominator for normalization method should represent the differences among different samples but not be significantly changed between comparison groups. The average osmolality of mouse urine before and after induced inflammation was 733 ± 285 osmoles/kg H₂O and 809 ± 96 osmoles/kg H₂O, respectively. Urinary metabolite fraction osmolalities did not significantly differ before and after Dox administration (p -value = 0.56), suggesting that total urinary metabolite output is not influenced by prostate inflammation.

3.2. Technical reproducibility of global metabolomics platform

The complexity of both biological samples and LC–MS instrumentation introduces systematic variations that confound quantitative metabolomics. We prepared a pooled QC urine sample to evaluate the run-to-run reproducibility of the LC–MS platform in this study. The QC samples were analyzed before the real samples to equilibrate the instrument and

then every 10 injections to monitor instrument stability in both ESI(+) and ESI(−) modes. A total of 812 compounds were detected in LC–MS ESI(+) and 490 compounds were detected in LC–MS ESI(−) of the QC sample. Technical replicates of QC samples are highly correlated ($r^2 > 0.99$) (Fig. 2). The consistent mass accuracy ($\text{ppm} < 3$), retention time (peak alignment score > 0.9) and peak area (median relative standard deviation = 6.1%) ensured the reliability of the metabolomics platform for subsequent comparison of urinary metabolic profiles of mice before and after induced inflammation.

3.3. Comparative urinary metabolomics of mice before and after induced inflammation

Global urinary metabolomics analysis generated a total of 1296 compounds in mouse urine. Base peak chromatograms were highly reproducible and well-aligned by retention time (Fig. 3). The most abundant peak in mouse urine samples is creatinine, detected in ESI(+) mode. Three hundred and sixty compounds changed significantly after induction of prostatic inflammation, with corrected p -values < 0.05 and fold-change greater than 2 or less than 0.5 (Fig. 4). Combining statistical analysis and machine learning feature selection, a total of 159 compounds were selected and over 100 of these were identified with the previously designed flowchart [34]. Machine learning classification was carried out to stratify urinary metabolite profiles from control and prostate inflammation mice using the support vector machine algorithm. A receiver operating characteristic (ROC) curve was then created by plotting the true positive rate (sensitivity) as the y-axis and the false positive rate (1-specificity) as the x-axis, which represents the classification performance. Predictive accuracy of a classification model can be denoted by the area-under-the-curve (AUC) of the ROC curve. A perfect AUC ROC statistic is 1.0, and AUC ROC of 0.5 is no better than random chance. A classification model using the top 15 ranked metabolites (Table 1) achieved greatly improved performance and higher predictive accuracy compared to the classification model using the entire metabolomics dataset, with AUC ROC of 0.81 and 0.67, respectively (Fig. 5).

The urinary metabolites significantly differing in abundance between control and prostate inflammation mice were used for metabolite enrichment and pathway analysis with MBRole tool [42]. The p -values of the enrichment terms were calculated by weighing the number of compounds in the uploaded dataset against the background dataset in MBROLE. Ten KEGG pathways and eleven HMDB taxonomies were enriched with corrected p -value < 0.05 (Fig. 6). Metabolites differing most between control and prostate inflamed mouse urine were fatty acyls, amino acids and derivatives, organic acids, and ketones. Many inflammatory diseases are associated with changes in ketones and fatty acid abundance because ketone bodies are generated by inflammatory leukocytes [49–51]. Inflammation has also been associated with other differentially expressed metabolites in this study including uracil, glycine, histidine, serotonin, sugar acids, and nucleic acids [52–55].

The top three dysregulated metabolic pathways are: the histidine metabolism pathway, the tryptophan metabolism pathway, and the arginine and proline metabolism pathway. Histidine is considered as an anti-inflammatory and antioxidant factor and is less abundant in urine from prostate-inflamed mice compared to controls. This observation comports with a previous study showing that plasma histidine content is reduced by chronic kidney inflammation [56]. Histidine decarboxylation produces histamine which can affect blood

flow and urinary metabolite profiles [57]. We showed previously that arginine metabolism is dysregulated in human LUTS patient urine [34]. Arginase activity can also influence collagen content, which has been implicated in LUTS [37,58,59]. The prostate synthesizes high levels of polyamines, which derive from the arginine metabolism pathway and can regulate Ca^{2+} influx and K^{+} channel and affect lower urinary tract smooth muscle activity [60–62]. Of note here, α_1 -blockers, drugs that relax smooth muscle by antagonizing the α_1 -adrenoceptor, remain a mainstay treatment for LUTS [63].

Prostatic inflammation can contribute to the development of LUTS by a combination of several mechanisms: prostatic enlargement from glandular and stromal hyperplasia [64,65], increased sensitivity to bladder filling and detrusor overactivity from neurotrophic and inflammatory signaling effects on afferent nerves and detrusor muscle, and increased urethral resistance from periurethral fibrosis and stromal cell hyperplasia [3,7,8,37,64–66]. Human patients with LUTS have a more complicated disease mechanism compared with tightly controlled mouse models and may in fact harbor multiple diseases deriving from multiple etiologies. Human and mouse have both shared and distinct metabolic pathways which increases the difficulty to correlate mouse model with human patients. Disease mechanistic studies on prostatic inflammation are needed to validate the targets generated in this study and further elucidate the correlation between mouse model and human patients.

4. Conclusions

We used a comprehensive metabolomics approach to characterize the urinary profile associated with non-bacterial prostatic inflammation in mice. We evaluated several methods of urine normalization and determined that pre-acquisition normalization to osmolality outperformed other methods. We also identified urinary metabolites and associated pathways that differ between mice with prostate inflammation and controls that overlap with our previous metabolomic study in human LUTS patients [34]. Although mouse models cannot provide information about urinary symptoms like those that are used to define LUTS in human patients, mice are tractable, the contribution of individual risk factors can be evaluated in isolation, and important clues can be gleaned about molecular mechanisms underlying LUTS. Our study provided important molecular targets to study the underlying mechanisms of urinary inflammation and the functional correlations between mouse models and human LUTS. This metabolite profile of non-bacterial prostate inflammation could also inform future efforts to develop diagnostic biomarkers for personalized LUTS diagnosis and treatment.

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Fig. 1. Principal component analysis of mouse urine samples using different pre-acquisition and post-acquisition normalization methods. Urine from uninflamed control mice (blue), urine from prostate inflammation disease mice (induced inflammation, red), QC urine (green).

Fig. 2.
Technical reproducibility of LC–MS platform using a pooled QC urine sample.

Fig. 3. Base peak chromatograms of control (blue) and induced prostate inflammation (red) groups in ESI(+) (left), and ESI(-) (right) modes. Mouse urine samples were collected from six transgenic mice at baseline and after 6 weeks of doxycycline administration to induce localized prostate inflammation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Fig. 4.

Volcano plot of all quantified urinary compounds in the comparative metabolomics dataset between disease (induced prostate inflammation) and control groups. Statistically up-regulated compounds were shown in red (corrected p -value < 0.05, ratio > 2), and statistically down-regulated compounds were shown in blue (corrected p -value < 0.05, ratio < 0.5) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Fig. 5. ROC analysis using the top 15 dysregulated urinary metabolites to differentiate control and induced prostate inflammation group, comparing to the ROC curve using all detected compounds in the metabolomics dataset. Support vector machine algorithm was used to construct binary classification models.

Fig. 6. Metabolic enrichment analysis revealed dysregulated KEGG metabolic pathways and HMDB taxonomies associated with non-bacterial prostate inflammation in mice. Corrected p -values are calculated by weighing the number of compounds in the upload dataset against the background dataset in MBROLE software.

Table 1

Top fifteen dysregulated urinary metabolites in induced prostate inflammation mice vs. control.

Name	<i>m/z</i>	ppm ^c	ESI	Time	Ratio	Corrected <i>p</i> -value	HMDB ID	KEGG ID	Pathway
Hydroxyproline ^a	132.0660	0.5	+	6.1	0.4	1.5E-02	0000725	C01157	Arginine and proline metabolism
Creatine ^a	132.0773	0.01	+	6.5	1.5	2.3E-02	0000064	C00300	Arginine and proline metabolism
Imidazole-acetaldehyde ^b	111.0558	0.3	+	2.5	0.5	1.1E-04	0003905	C05130	Histidine metabolism
Serotonin ^a	177.1026	1.1	+	4.3	0.3	6.1E-09	0000259	C00780	Tryptophan metabolism
Indole (+NH4) ^b	135.0921	0.9	+	3.9	>100	1.8E-04	0000738	C00463	Tryptophan metabolism
Acetyl-hydroxylysine ^b	205.1187	0.6	+	9.3	7.2	7.0E-07	0033891	NA	Lysine degradation
Pyridoxal ^b	168.0660	0.4	+	1.6	0.5	5.9E-05	0001545	C00250	Vitamin B6 metabolism
Acetyl-vanilalanine ^b	254.1026	0.9	+	0.9	0.1	5.2E-07	0011716	NA	Tyrosine derivatives
Aspartyl-Proline ^a	231.0978	1.3	+	3.9	5.5	7.7E-06	30765	NA	Di-peptide
Succinic acid ^a	117.0187	0.7	-	0.8	0.2	2.9E-04	0000254	C00042	Citrate cycle
Aconitic acid ^a	173.0085	0.6	-	1.4	0.1	4.4E-09	0000072	C00425	Citrate cycle
Oxoglutaric acid ^a	145.0135	1.4	-	3.2	0.2	1.0E-07	00208	C00026	Citrate cycle/Histidine metabolism
Oxoisovalerate ^a	115.0395	0.2	-	2.4	1.8	4.2E-06	00019	C00141	Valine, leucine and isoleucine degradation
Glyceric acid ^a	105.0187	0.8	-	2.7	33.1	3.7E-06	06372	C00258	Pentose phosphate pathway
Propionyl-carnitine ^b	216.1237	0.5	-	2.0	2.3	1.7E-08	0000824	C03017	Acylcarnitine

^aMetabolite ID was confirmed with standard compound.

^bMetabolite ID was confirmed with MS/MS fragmentation.

^c ppm mass error = $1 \times 10^6 | \text{detected } m/z - \text{theoretical } m/z | / \text{theoretical } m/z$.