Unique and Novel Urinary Metabolomic Features in Malignant versus Benign Adrenal Neoplasms

Dhaval Patel1, Matthew D. Thompson2, Soumen K. Manna3, Kristopher W. Krausz2, Lisa Zhang1, Naris Nilubol1, Frank J. Gonzalez2, and Electron Kebebew1

Abstract

Purpose: Adrenal incidentalomas must be differentiated from adrenocortical cancer (ACC). Currently, size, growth, and imaging characteristics determine the potential for malignancy but are imperfect. The aim was to evaluate whether urinary small molecules (<800 Da) are associated with ACC.

Experimental Design: Preoperative fasting urine specimens from patients with ACC (n = 19) and benign adrenal tumors (n = 46) were analyzed by unbiased ultraperformance liquid chromatography/mass spectrometry. Creatinine-normalized features were analyzed by Progenesis, SIMCA, and unpaired t test adjusted by FDR. Features with an AUC >0.8 were identified through fragmentation patterns and database searches. All lead features were assessed in an independent set from patients with ACC (n = 11) and benign adrenal tumors (n = 46) and in a subset of tissue samples from patients with ACC (n = 15) and benign adrenal tumors (n = 15) in the training set.

Results: Sixty-nine features were discovered and four known metabolites identified. Urinary creatine riboside was elevated 2.1-fold (P = 0.0001) in patients with ACC. L-tryptophan, Nε,Nε-trimethyl-L-lysine, and 3-methylhistidine were lower 0.33-fold (P < 0.0001), 0.56-fold (P < 0.0001), and 0.33-fold (P = 0.0003) in patients with ACC, respectively. Combined multivariate analysis of the four biomarkers showed an AUC of 0.89 [sensitivity 94.7% (confidence interval [CI], 73.9%–99.1%), specificity 82.6% (CI, 68.6%–92.2%), PPV 69.2% (CI, 48.2%–85.6%), and NPV 97.4% (CI, 86.5%–99.6%)] for distinguishing ACC from benign tumors. Of the four, creatine riboside and four unknown features were validated. Creatine riboside, Nε,Nε,Nε-trimethyl-L-lysine, and two unknown features were elevated in ACC tumors.

Conclusions: There are unique urinary metabolic features in patients with ACC with some metabolites present in patient tumor samples. Urinary creatine riboside can differentiate benign adrenal neoplasms from ACC. Clin Cancer Res; 1–9. ©2017 AACR.

Introduction

Adrenal masses detected incidentally by CT scan are highly prevalent with a range from 4% to 10% (1–3). Management is guided by tumor size and growth, patient and imaging characteristics, and biochemical findings (4). Although these characteristics may help differentiate benign adrenal incidentalomas from the rare adrenocortical carcinoma (ACC) with a poor prognosis (5–7), patients frequently undergo adrenalectomy to exclude a cancer diagnosis. Thus, identification of biomarkers that distinguish benign from malignant adrenal neoplasm is needed.

ACCs can be hormonally active; thus, targeted urine metabolomic approaches using gas chromatography mass spectrometry have been utilized to differentiate patients with benign adrenal tumors and patients with ACC. This approach was able to discriminate between patients with benign adrenal tumors and ACC with a sensitivity and specificity of 90% (8). Given these promising results, there may be smaller molecular biomarkers that may improve the sensitivity and specificity in combination, which could be discovered via an untargeted metabolomics approach. Growing evidence also suggests that the metabolome has an important role in cancer initiation and/or progression; thus, an untargeted metabolomic investigation of ACC may provide new information in the pathogenesis of ACC and other cancers.

Metabolomics, the study of small-molecule metabolites, was successfully used to identify biomarkers in urine of patients with ovarian cancer (9), colorectal cancer (10), bladder cancer (11), lung cancer (12), and hepatocellular carcinoma (13). Metabolomic analysis of body fluid samples focuses on identifying metabolites related to tumor biology (14). The two major analytic tools used in metabolomics are nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled to a separation technique. Unlike NMR, MS provides semiquantitative data with high sensitivity and the ability to identify low-abundance metabolites (15). Ultraperformance liquid chromatography (UPLC) MS provides high-throughput analysis and reliable data quality (16). High-throughput UPLC/MS analysis of serum and urine samples is thus a platform that could help discover new biomarkers for ACC.

The objective of this study was to perform an untargeted metabolomic analysis by UPLC/MS to determine whether there is a specific urinary metabolomic signature that can discriminate...
Translational Relevance

Adrenal incidentalomas are a common clinical dilemma, and a diagnosis of adrenocortical carcinoma (ACC) must be excluded in all cases. There are currently no reliable clinical, imaging, or biochemical features to exclude the rare and often fatal diagnosis ACC. Preoperative fasting urine specimens from patients with ACC and benign adrenal tumors were analyzed by unbiased ultraperformance liquid chromatography/mass spectrometry to discover four known metabolites (L-tryptophan, 3-methylhistidine, N,N,N-trimethyl-L-lysine, and creatine riboside) and 65 unknown features that differentiate patients with ACC and patients with benign adrenal neoplasms. Creatine riboside and four unknown features were validated in an independent set of urine samples. These data indicate that patients with ACC can be differentiated from benign adrenal disease, with fasting spot urine specimens leading to improved patient selection for adrenalectomy. During follow-up (median follow-up, 32.8 months; range, 1.0–83.6 months). Three patients had follow-up of less than 1 year, with final pathology showing adrenal hyperplasia. Tissue samples were confirmed to be either malignant or benign by hematoxylin and eosin staining by a pathologist in entirety.

Untargeted global urinary metabolomic analysis and biomarker identification

Urine samples were deproteinized (dilution of 1:5) using a solution of isopropanol/acetonitrile/water (65/30/5) containing 10 μmol/L chlorpropamide or α-aminopinic acid as internal standards for reverse-phase (RP) or hydrophilic interaction liquid chromatography (HILIC), respectively. Supernatants were transferred into 96-well sample plates. All pipetting and dilution were performed using a MICROLAB STARLET automated liquid handler (Hamilton Robotics). For HILIC analysis, 5 μl aliquot of samples was injected in a randomized fashion into a 2.1 × 50 mm Acquity UPLC BEH amide column (1.7 μm) connected to a Waters XEVO G2 ESI-QTOF mass spectrometer (Waters Corporation). Chromatographic separation was achieved by using a mixture of 10 mmol/L ammonium acetate at 90% acetonitrile (A; pH = 9.0) and 10 mmol/L ammonium acetate in 10% acetonitrile (B; pH = 9.0) as mobile phase. The gradient elution was performed over 10 minutes using: 1% to 60% B in 4 minutes, 60% to 80% B at 8 minutes, holding at 80% B to 8.5 minutes, returning to initial conditions for column equilibration. Flow rate was maintained at 0.4 mL/minute, and total run time for each sample was 12.5 minutes. Column temperature was maintained at 40°C. For RP analysis, samples were further diluted with an equal volume of water before a 5 μl aliquot of samples was injected into a 2.1 × 50 mm Acquity UPLC BEH C18 column (1.7 μm). Chromatographic separation was achieved by using a mixture of water containing 1% formic acid (A) and acetonitrile containing 1% formic acid as mobile phase (B). The gradient elution was performed over 6 minutes at a flow rate of 0.4 mL/minute using: 1% to 95% B in 4 minutes, holding at 95% B up to 5.0 minutes, returning to initial conditions for column equilibration (total run time of 10 minutes). Column temperature was maintained at 40°C. The column was reequilibrated with 98% A at the end of each run prior to injection of the next sample. Mass spectrometric analysis (for both RP and HILIC chromatography) was performed in both positive and negative ionization modes. Sulfadimethoxine was used as the lock mass (m/z 311.0814−) for accurate mass calibration in real time. MassLynx software (Waters Corporation) was used to acquire mass chromatograms and mass spectral data in centroid format. Chromatographic separation

Materials and Methods

Urine samples

Fasting urine samples from patients undergoing adrenal surgery were obtained the morning of surgery and stored at −80°C. Urine samples from patients undergoing experimental treatment for ACC were obtained the morning of drug treatment and stored at −80°C. Tissue samples from patients undergoing adrenal surgery were procured at the time of surgery and stored at −80°C. Tissue specimens from patients with ACC and patients with benign adrenal neoplasms were analyzed by unbiased ultraperformance liquid chromatography/mass spectrometry to discover four known metabolites (L-tryptophan, 3-methylhistidine, N,N,N-trimethyl-L-lysine, and creatine riboside) and 65 unknown features that differentiate patients with ACC and patients with benign adrenal neoplasms. Creatine riboside and four unknown features were validated in an independent set of urine samples. These data indicate that patients with ACC can be differentiated from benign adrenal disease, with fasting spot urine specimens leading to improved patient selection for adrenalectomy.

Table 1. Clinical characteristics of study cohorts

<table>
<thead>
<tr>
<th></th>
<th>Training set</th>
<th>Independent set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACC</td>
<td>Benign adrenal tumor</td>
</tr>
<tr>
<td>Number of patients</td>
<td>19</td>
<td>46</td>
</tr>
<tr>
<td>Age (average ± SD)</td>
<td>55.68 ± 10.78</td>
<td>47.85 ± 15.99</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>11/8</td>
<td>26/20</td>
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<tr>
<td>Size (cm)a</td>
<td>15.40 ± 4.74</td>
<td>2.43 ± 1.37</td>
</tr>
<tr>
<td>Syndromeb</td>
<td>Cushing</td>
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<tr>
<td></td>
<td>Subclinical Cushing</td>
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<td></td>
<td>Hyperandrogenism</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nonfunctioning</td>
<td>11</td>
</tr>
</tbody>
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aSize measured in longest dimension for patients with measurable masses undergoing initial surgery for an adrenocortical tumor.
bFunctional status at initial presentation.
Targeted urinary metabolite quantitation

Metabolites in the deproteinated urine samples were quantified in multiple reactions monitoring mode on a Waters XEVO TQ-S triple quadrupole mass spectrometer (Waters Corporation). α-Aminopimelic acid (0.5 μmol/L) was used as an internal standard. The following metabolites were quantified by monitoring characteristic fragmentation reactions (in bracket); α-aminopimelic acid (176→ 112, electrospray ionization [ESI+]), 3-methylhistidine (170→ 96, ESI+), Ne,Ne,N-trimethyllysine (189→ 84, ESI+), L-tryptophan (205→ 118, ESI+), creatine (132→ 90, ESI+), and creatine riboside (264→ 132, ESI+). Creatine was used to standardize creatine riboside due to lack of purified standard.

Chromatographic separation was achieved on a 2.1 × 50 mm Acquity UPLC BEH amide column using the mobile phase as mentioned above. All data were processed using Waters TargetLynx software. Internal standard-normalized area under the peak (response) from serially diluted authentic standard solution was used to build calibration curve for each metabolite. The concentration of metabolite was determined from the calibration curve and divided by creatinine concentration to determine creatinine-normalized excretion of the urine metabolite.

Targeted tissue metabolite quantitation

A subset of tissue samples from patients with ACC (n = 15) and patients with benign adrenal disease (n = 15) from the training set was evaluated for metabolites. Metabolites in the deproteinated and homogenized tissue samples were quantified in multiple reactions monitoring mode on a Waters XEVO TQ-S triple quadrupole mass spectrometer (Waters Corporation). α-Aminopimelic acid (0.5 μmol/L) was used as an internal standard. The following metabolites were quantified by monitoring characteristic fragmentation reactions (in bracket); α-aminopimelic acid (176→ 112, ESI+), 3-methylhistidine (170→ 96, ESI+), Ne,Ne,N-trimethyllysine (189→ 84, ESI+), L-tryptophan (205→ 118, ESI+), creatine (132→ 90, ESI+), and creatine riboside (264→ 132, ESI+). Creatine was used to standardize creatine riboside due to lack of purified standard.

Chromatographic separation was achieved on a 2.1 × 50 mm Acquity UPLC BEH amide column using the mobile phase as mentioned above. All data were processed using Waters TargetLynx software. Internal standard-normalized area under the peak (response) from serially diluted authentic standard solution was used to build calibration curve for each metabolite. The concentration of metabolite was determined from the calibration curve and divided by creatinine concentration to determine creatinine-normalized levels of the tissue metabolites.

Quality control and normalization

The order of sample injection was randomized to avoid order artifact using the rand function in Microsoft Excel. Quality control involved running a standard mix prior to samples to monitor instrument performance over time. Internal standards (α-aminopimelic acid, chlorpropamide) were used for HILIC and RP chromatography, respectively. A set of pooled samples was injected at regular intervals during the analysis. The data were assessed with unsupervised principal component analyses. Creatinine concentration was determined by the Jaffé method (18).

In brief, creatinine was assayed colorimetrically after reaction of creatinine with picrate to generate a chromophore in alkaline solution. The absorbance of the creatinine-picrate chromophore was measured at 500 nm in a 96-well microplate.

Statistical analyses

Samples were classified as ACC or benign adrenal tumor. Data acquired were aligned and deconvoluted using Progenesis software. Urine data were normalized to externally measured creatinine by the Jaffé method (see above). Data were exported and analyzed using a minimal detection rate to eliminate spurious human and instrument variation. Minimal detection rate was defined as a feature that must be present in at least 40% of samples in each group a priori. The data were analyzed in three separate and complementary methods for discovery (Fig. 1). Method 1 used the Progenesis software to compare the groups and a one-way ANOVA with an FDR of q < 0.05 (19). Method 2 consisted of a two-tailed t test and Benjamini–Hochberg test for FDR 5% (19). Method 3 consisted of using soft independent modeling of class analogy (SIMCA) with the construction of principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) plots with a P correlation of 0.5 for inter-group discrimination.

Potential features that were statistically significant by any of the three methods were then analyzed using online MetaboAnalyst 3.0 software (http://www.metaboanalyst.ca/) with an AUC cutoff of >0.8 for each feature (20). The multivariate analysis was completed utilizing the online MetaboAnalyst software by calculating predict class probabilities based on each sample and the biomarker panels tested. The multivariate algorithm utilized was a random forest algorithm that performed repeated random sub-sampling cross-validations. The predicted accuracies were then plotted with increasing number of features. The sensitivities, specificities, positive predictive values, and negative predictive values were calculated using the predicted class probabilities for identifying the biomarker panel ability to correctly diagnose patients as either benign or malignant. Each potential feature was then verified by using TargetLynx software (Waters Corporation). To eliminate features related to mitotane administration, PCA, OPLS-DA, and S-plots were constructed using SIMCA software. Features by S-plots that did not show a correlation to mitotane ingestion were considered as related to cancer and were included in further analysis. To account for unequal variance, the urine and tissue features were compared by the Mann–Whitney U test and correlated by Spearman rank test. A P value of less than 0.05 was considered significant.

Results

Unsupervised PCA plots show discrimination and clustering between patients with ACC and benign adrenal tumors in RP-positive, RP-negative, HILIC-positive, and HILIC-negative modes (Figs. 2A and B, and 3A and B). Supervised OPLS-DA analysis showed separation and features associated with ACC and benign adrenal tumors (Figs. 2C and D, and 3C and D). S-plot analysis showed features associated with ACC and benign adrenal disease.
for each mode, respectively (Figs. 2E and F, and 3E and F). These data show that patients with ACC and benign adrenal tumors have significantly different urinary metabolites.

Preoperatively, patients with recurrent and metastatic ACC were treated with mitotane. Given the long half-life (18–159 days) and the possibility that metabolites discovered may be mitotane metabolites or related to mitotane metabolism, further drug analysis was performed. Unsupervised PCA plots show clustering between patients with and without mitotane therapy (Supplementary Data, Supplementary Figs. S1A, S1B, S2A, and S2B). OPLS-DA plots and S plots showed separation and features associated with mitotane use, which were excluded from further analysis (Supplementary Data, Supplementary Figs. S1C–S1F, and S2C–S2F). To verify that these were metabolites of mitotane, MS/MS fragmentation pattern of a top hit was identified as a mitotane metabolite.

Metabolites not associated with mitotane ingestion were identified and further studied. The significant features as discovered by different methods are summarized (Supplementary Table S3). Sixty-nine features were discovered. Specific m/z masses and retention times that are significantly different between the two groups are summarized in Supplementary Table S4). The significant features of unique parent compounds were combined in a multivariate random forest model to classify benign and malignant adrenal tumors. Of the 69 features, 46 unique features were parent compounds and not related to mitotane ingestion. A combination of the 46 unique features included in the model showed that the AUC was 0.928 with the inclusion of all features (Supplementary Fig. S3A). The predicted accuracy of classification by urinary metabolic analysis utilizing these features was 84% (Supplementary Fig. S3C).

Among the features discovered, four features were identified through MS/MS fragmentation and online metabolomics database searches. The four metabolites identified through fragmentation patterns and database searches were creatine riboside, Ne, Ne,Ne-trimethyllysine, 3-methylhistidinid, and L-tryptophan (Supplementary Fig. S4A–S4D). These four metabolites were quantitated on a Waters Xevo triple quadrupole mass spectrometer (Waters Corporation) in multiple reaction monitoring mode. Urinary creatine riboside was elevated over 2-fold in patients with ACC compared with patients with benign adrenal tumors (Fig. 4A, 2.1-fold change, \( P = 0.0001, \text{AUC} = 0.793 \)). Urinary Ne,Ne,Ne-trimethyllysine (Fig. 4B, 0.56-fold change, \( P < 0.0001, \text{AUC} = 0.820 \)), 3-methylhistidinid (Fig. 4C, 0.33-fold change, \( P = 0.0003, \text{AUC} = 0.782 \)), and L-tryptophan (Fig. 4D, 0.33-fold change, \( P < 0.0001, \text{AUC} = 0.860 \)) were each lower in patients with ACC compared with patients with benign adrenal tumors. Using the four identified metabolites as a panel, the AUC was 0.89 (Fig. 5A). Use of the four features further showed a sensitivity of 94.7% [confidence interval (CI), 73.9%–99.1%] and specificity of 82.6% (CI, 68.6%–92.2%). The positive predictive value was 69.2% (CI, 48.2%–85.6%) and the negative predictive value was 97.4% (CI, 86.5%–99.6%). The model was predicted to be 81.9% accurate in diagnosing malignancy when using a cross-validation model (Fig. 5C).

The four identified metabolites and 65 unknown features were analyzed in an independent set of urine samples consisting of 11 patients with ACC and 46 patients with benign adrenal tumors (Table 1; Supplementary Table S2). Creatine riboside was validated and continued to show a significant difference between the two groups (Fig. 4E). L-Tryptophan, Ne,Ne,Ne-trimethyllysine, and 3-methylhistidinid were found to be not significantly different. Ten of the 65 unknown features were validated and found to be significantly different between the two groups. Of the 10 features, the top four unknown features (Supplementary Table S4, denoted by an asterisk) included m/z 334.186 (Supplementary Fig. S5A, 3.9-fold

Figure 1. Workflow and approach to identifying potentially discriminating metabolites between ACC and benign adrenal tumors.

for each mode, respectively (Figs. 2E and F, and 3E and F). These data show that patients with ACC and benign adrenal tumors have significantly different urinary metabolites.

Preoperatively, patients with recurrent and metastatic ACC were treated with mitotane. Given the long half-life (18–159
change, \( P = 0.0001 \), HILIC positive), \( m/z \) 306.153 (Supplementary Fig. S5B, 5.0-fold change, \( P = 0.0001 \), HILIC positive), \( m/z \) 209.030 (Supplementary Fig. S5C, 2.5-fold change, \( P = 0.0281 \), HILIC negative), and \( m/z \) 427.231 (Supplementary Fig. S5D, 4.8-fold change, \( P = 0.0002 \), RP negative). The top four unknown features and creatine riboside were combined and differentiated between the two groups with an AUC of 0.875 (Supplementary Fig. S6A).

A subset of patients who presented with initial surgery for ACC was compared with patients with benign adrenal tumors for clinical data, including size of the mass on imaging and initial clinical presentation. Clinical presentation was not predictive of malignancy. Therefore, size on imaging and the validated marker creatine riboside were utilized to create a composite model. Quantified creatine riboside data and size improved the AUC to 0.96 (Supplementary Fig. S6A).

To determine whether these biomarkers originated from the tumor, a subset of available tissue samples was selected, and targeted metabolic studies were conducted. Creatine riboside, feature \( m/z \) 334.186, and feature 306.153 were identified and optimized in adrenal tissue samples. Feature \( m/z \) 209.030 and feature \( m/z \) 427.23 were not identified in the tissue. L-Tryptophan and 3-methylhistidine were not found to be different between patients with malignant and benign adrenal tumors. Levels of creatine riboside (\( P < 0.0001 \)), feature \( m/z \) 306.153 (\( P = 0.0063 \)), and feature \( m/z \) 334.186 (\( P < 0.0001 \)), and \( \text{Ne}_2\text{Ne}_2\text{Ne}_2 \)-trimethyllysine (\( P = 0.0357 \)) in ACC tumor tissue samples were compared with benign adrenal tumors (Supplementary Fig. S7A–S7D). The levels of creatine riboside in the tissue were compared with the levels of creatine riboside in paired urine samples, and there was a significant positive association (Supplementary Fig. S4FC, \( r = 0.5681, P = 0.0016 \)).

Discussion

This study is the first untargeted metabolomics examination of the urine of patients with benign adrenal tumors and ACC. An unbiased examination of the data shows that the two groups can be discriminated by a urinary metabolomics approach. Furthermore, mitotane and mitotane-associated metabolites were identified in patients that could be used to direct steroid replacement therapy and measure compliance. Analysis of specific features identified that patients with ACC had higher levels of creatine riboside and lower levels of L-tryptophan, \( \text{Ne}_2\text{Ne}_2\text{Ne}_2 \)-trimethyllysine, and 3-methylhistidine when compared with patients with benign adrenal tumors. Creatine riboside and four unknown features were validated in an independent set of samples. The combination of creatine riboside and size of adrenal tumor had a high degree of discrimination between ACC and benign adrenal tumor. Furthermore, targeted tissue analysis showed elevated levels of creatine riboside, feature \( m/z \) 306.186, feature \( m/z \)
334.153, and Ne,Ne,Ne-trimethyl-L-lysine to be higher in patients with ACC compared with patients with benign adrenal tumors. Levels of creatine riboside in the tissue and urine were positively associated.

Creatine riboside, which was significantly elevated in patients with ACC, was recently described as a noninvasive urinary marker in patients with lung cancer. In addition to elevated levels in the urine, increased creatine riboside was found in the tumors of patients with lung cancer compared with normal adjacent tissue (12). Creatine riboside, therefore, may not be specific to lung cancer or ACC, but may increase the index of suspicion of a potential malignant neoplasm. Although the function of creatine riboside has yet to be elucidated, the fact that both lung and ACC have elevated levels may indicate that this metabolite is involved in tumorigenesis. In the validation set, creatine riboside was the only known metabolite that was validated. Furthermore, creatine riboside was found to be elevated in the tissue of patients with ACC compared with benign adrenal tumors. This may indicate that creatine riboside is produced in the tumor and secreted in the urine. In support of this, creatine riboside levels in the tissue and urine were positively associated.

The three other identified metabolites, L-tryptophan, Ne,Ne,Ne-trimethyllysine, and 3-methylhistidine were not validated. These metabolites may have been false positives or possibly related to lack of fasting in the validation cohort. Each of these three metabolites is associated with consumption of food, and therefore, may still be valid with further testing. Further analysis of the 65 features in the validation cohort revealed four features that were significantly different in both the training and validation sets. These validated features were searched in metabolite databases and were not known metabolites. These features may be novel metabolites that will require future characterization, as they may be specific to ACC. These unknown features may have a role in adrenal cancer and will require further study.

Although urinary Ne,Ne,Ne-trimethyllysine was not validated and may have been affected by diet in the independent set, the difference in tissue levels indicates that it may have a role in adrenal cancer. Ne,Ne,Ne-trimethyllysine is associated with trimethylation of lysine residues on histone complexes. Histone lysine methylation is critical in the regulation of gene expression, cell cycle, genomic stability, and nuclear architecture (21). IHC studies have shown that a decrease in trimethylation of lysine 27 at histone H3 by the methyltransferase EZH2 was associated with poor prognosis in patients with breast cancer regardless of estrogen receptor status. The present data showed that the metabolite Ne,Ne,Ne-trimethyllysine was decreased in urine of patients with ACC. However, in the subset of tissue samples, Ne,Ne,Ne-trimethyllysine showed an elevated level in patients with ACC compared with benign adrenocortical neoplasms. Drelon and colleagues recently reported that EZH2 to be overexpressed in ACC and was possibly the result of inactivation of the P53/RB/E2F pathway.
The increased Ne,Ne,Ne-trimethyllysine would be explained by these findings; however, the paradoxical levels in the urine may indicate that the metabolite may have other sources as well such as muscle (22).

The annual number of adrenalectomies has been increasing in the United States. Murphy and colleagues reported that the
Number of both open and laparoscopic adrenalectomies has increased from 3,241 in 1998 to 5,323 adrenalectomies in 2006. Furthermore, the majority of adrenalectomies were performed for benign disease (83%; ref. 23). Biomarkers able to discriminate between malignant and benign adrenal disease are needed to avoid surgery and reduce the morbidity of adrenalectomy for patients with benign adrenal disease. Given the high negative predictive value of the discovered metabolites and features in combination with adrenal tumor size, clinicians may be able to avoid an adrenalectomy in both large and small adrenocortical tumors or stratify patients for further work-up including functional imaging (24). However, prior to clinical adaptation, the biomarkers will have to be validated in independent cohorts seen at high volume adrenal surgery centers as well as patients undergoing active surveillance for incidentally discovered adrenal tumors.

There are several limitations to this study, including sample size. The limited sample size is due to the rarity of ACC (two cases per million). To overcome this limitation, a training set and validation set were used to reduce a type I error of discovered metabolites. A second limitation to this study is that not all patients in the validation set had fasting urinary samples available for analysis. This limitation may have resulted in a type II error of features that were not validated. However, this finding does emphasize that the validated features/metabolites are not affected by diet and are truly different between the two groups. A third limitation is that many patients were on mitotane as either adjuvant therapy or for disease progression. The authors attempted to control for steroid metabolite alterations by analyzing samples of patients who were treated with mitotane. In addition, steroid supplementation may have affected the urinary metabolome. Although an important limitation, the impact may be limited, given that both benign and malignant cohorts in both the learning and independent set had patients with hypercortisolism or patients receiving steroid supplementation.

In this study, urine samples from patients with benign adrenal tumors and ACC were analyzed using a metabonomics approach in an untargeted unbiased fashion. Unique urinary metabolic features were identified in benign and malignant adrenocortical tumors. Urinary creatine riboside may provide additional diagnostic information on adrenal incidentalomas. Unique unknown features may be novel metabolites that would be able to differentiate patients with malignant adrenocortical tumors and benign adrenal disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D. Patel, F.J. Gonzalez, E. Kebebew
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Patel, M.D. Thompson, S.K. Manna, K.W. Krausz, N. Nilubol, E. Kebebew
Writing, review, and/or revision of the manuscript: D. Patel, M.D. Thompson, S.K. Manna, N. Nilubol, F.J. Gonzalez, E. Kebebew
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Zhang
Study supervision: F.J. Gonzalez, E. Kebebew

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