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Urinary Metabolomics Profile of Genetically Obese Rats Using Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry

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Abstract

As the prevalence of obesity continues to increase further research investigating the body's metabolic response to increased adiposity can help us better understand the disease and identify biomarkers that pave way for the development of new prevention and treatment strategies. The urinary metabolite profile can provide detailed information regarding the metabolic network at any disease stage and may be useful for investigating the effect of obesity on water-soluble metabolite profiles in genetically obese rats using a nontargeted metabolomics approach. Urine from 24 week old male obese *fa/fa* (*n*=8) and lean (*n*=8) Zucker rats were extracted and analyzed using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). Data were analyzed by moderated T-test ($P<0.05$), fold change (≥2) and a prediction model using partial least squares discrimination analysis. The body composition of obese rats was 48.1 ± 0.9% fat compared to 16.9 ± 0.8% fat for lean rats. Untargeted urine metabolomics detected 1046 entities with 43 metabolites significantly different between obese and lean rats. Among these metabolites, N_1-methylnicotinamide, spermine, hexadecyl acetyl glycerol and 3-mercaptolactic acid were significantly elevated in the urine from obese rats. The increase of water-soluble metabolites may suggest an obesity effect on the metabolism of arginine (increased levels of spermine) as well as nicotinamide metabolism (increased levels of N_1-methylnicotinamide) which can lead to oxidative stress. Urinary metabolites observed in obese rats provided new insights into biological mechanisms associated with an obese state.

Keywords: Obesity; Urinary Metabolomics; Spermine; N_1-Methylnicotinamide

Abbreviations

LC-MS: Liquid Chromatography Mass Spectrometry;

QTOF: Quadruple Time-of-Flight;

LC-QTOF-MS: Liquid Chromatography Quadruple Time-of-Flight Mass Spectrometry;

m/z: Mass-to-Charge Ratio;

MHQ: Mass Hunter Qualitative;

MPP: Mass Profiler Professional;

MFE: Molecular Feature Extraction;

TIC: Total Ion Chromatograms;

RT: Retention Time;

PLSD: Partial Least Square Discrimination;

NNMT: Nicotinamide N-Methyltransferase

Introduction

Obesity is a condition characterized by the excessive accumulation and storage of fat in the body [1]. The prevalence of obesity worldwide has more than doubled between 1980 and 2014 with 39% of adults over 18 years of age being classified as overweight (BMI ≥ 25 to ≤ 29.9 kg/m^2) and 13% being classified as obese (BMI ≥ 30 kg/m^2) in 2014 [2]. In Canada, approximately one in four Canadian adults are obese, according to measured height and weight data from 2007-2009 [3]. Obesity has been deemed one of the greatest public health challenges of the 21st century and has reached epidemic proportions worldwide [2] due to its adverse health effects that reduce life expectancy in association with many chronic diseases, including hypertension, cardiovascular disease, diabetes, arthritis and certain types of cancer [4,5]. It has been estimated that 44% of diabetes, 23% of ischemic heart disease and up to 41% of cancer related cases in the world are attributable to being overweight or obese [6].

Metabolomics has emerged as a valuable approach capable of readily detecting subtle changes in the metabolic network and it provides a detailed snapshot of the body's processes at any particular disease stage [7]. Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind [8]. It is a sensitive and unbiased approach [9] that allows for the comprehensive analysis of low molecular weight (typically defined as <1000 daltons or <1500 daltons) metabolites in biological samples. Low molecular weight biomolecules include but are not limited to amino acids, carbohydrates, lipids, peptides, purines, pyrimidines, vitamins, and numerous metabolites involved in biosynthetic and biodegradation pathways [10].

A metabolomics approach can be used for targeted and untargeted analysis of metabolites in biological fluids and tissues. The type of biological samples used for metabolomics is highly dependent on the objective of each study. Blood is often regarded as the most common biological fluid for traditional laboratory tests, however, collection of blood may be considered invasive. Urine on the other hand is considered to be the most commonly used biological fluid for metabolomics; it is a non-invasive diagnostic tool that requires little to no subject preparation and can be collected in large quantities [11,12]. Urine is often ideal for the discovery and screening of potential water-soluble metabolites since cellular metabolism generates numerous by-products, many rich in nitrogen that require elimination from the bloodstream. These by-products are eventually expelled from the body during urination, the primary method for excreting water-soluble compounds from the body.

Liquid chromatography mass spectrometry (LC-MS) based metabolomics is characterized by its capacity to produce large and complex datasets [13] that can generate valuable information. Within the past few years, LC-MS has emerged as a very promising method for global metabolic profiling and rapid scanning when quadrupole time-of-flight (QTOF) analytical instrumentation was added [14]. LC-QTOF-MS is a high speed and sensitive analytical technique with shorter analysis times and greater mass to charge (*m/z)* value accuracy compared to other methodologies.

Physical markers of obesity such as increased adiposity do not provide information regarding the body's metabolic response or cellular environment nor do they assess or identify metabolic phenotypes that help us better understand the disease. Monitoring urinary biomarkers may be a better non-invasive way to understand obesity. Some studies to date have identified potential urinary biomarkers of diet-induced obesity from a high fat diet using gas chromatography mass spectrometry [15] and proton nuclear magnetic resonance analytical techniques [16-18]. However, with metabolism in an obese state continuously changing over time, it is important to profile obesity metabolites independently from any diet intervention. In the current study, we monitored water-soluble urinary metabolites in obese *fa/fa* Zucker rats through the use of a nontargeted LC-QTOF-MS metabolomics approach and compared the metabolic profile to that of lean Zucker rats.

Materials and Methods

Chemicals

High pressure liquid chromatography grade acetonitrile (≥99%) and the internal standard D-norvaline (99%) were purchased from Sigma-Aldrich. Mass spectrometry formic acid (98%) as well as the analytical standard spermine were purchased from Fluka, while N-methylnicotinamide was purchased from Santa Cruz Biotechnology. The electrospray ionization low concentration tuning mix and atmospheric pressure ionization time-of-flight reference mass solutions were obtained from Agilent Technologies. Deionized water was prepared in-house using an Aqua-Summa II Reagent grade Water Polisher system.

Animals

The protocol for the animal study was approved by the Animal Care Committee at the University of Manitoba. Male obese *fa/fa* (*n*=8) and lean (*n*=8) Zucker rats (Charles River Laboratories) were housed individually in polypropylene cages and maintained at a temperature of 22 ± 2°C with a 12 hour light/dark cycle. Sixteen week old rats were fed a semi-purified control diet consisting of cornstarch (363 g), maltodextrin (132 g), sucrose (100 g), egg white (212.5

g), cellulose (50 g), biotin mix (10 g of 200 mg biotin per kg dextrose), choline (2.5 g), AIN-93-VX vitamin mix (10 g), AIN-93-MX mineral mix (35 g) and soybean oil (85 g) for 8 weeks. A semi-purified diet was used to eliminate the variable composition of rodent chow as a confounding factor. All animals were transferred to metabolic cages during week 8 and fasted (provided water but not feed) for 5 hours in order to collect urine. Samples were stored at -80°C until analysis. On a different day, whole body composition was assessed *in vivo* using an EchoMRI-700™ whole body Qualitative Magnetic Resonance instrument (Echo Medical Systems). At 24 weeks of age, the rats were euthanized, weighed, and the fat pads were dissected and weighed. The *fa/fa* Zucker rat was selected for the present study since they are a widely used spontaneous genetic obesity model. They have a single-gene autosomal recessive defect in the leptin receptor that affects the hormone involved in regulating body weight and energy homeostasis [19]; thus they exhibit hyperphagia, hyperinsulinemia and hyperlipidemia in comparison with their lean counterparts [20]. Furthermore, *fa/fa* Zucker rats develop obesity on a standard (low fat) rodent diet whereas variable diet compositions are used for different diet-induced obesity models.

Sample Preparation

Urine samples (250 µL) were mixed with 10 µL of norvaline (0.03 mg/mL in deionized water) in 2 mL microfuge tubes, briefly vortexed and centrifuged for 10 min (10,000 *g* at room temperature). The supernatant was transferred into a clean 2.0 mL microfuge tube and mixed with 500 µL of acetonitrile, vortexed vigorously and placed at -20°C for 30 min (quenching) followed by centrifugation for 20 min (10,000 *g* at room temperature). The supernatant was transferred to clean tube and dried under a gentle stream of nitrogen. Prior to LC-QTOF-MS analysis, extracts were reconstituted with 200 µL of deionized water and acetonitrile (4:1) with 0.1% formic acid and placed into glass inserts in autosampler vials. All extractions were performed in triplicate.

LC-QTOF-MS Analysis

Nontargeted metabolomics analysis was performed using an Agilent 1260 Infinity LC system coupled to a Agilent 6538 UHD Accurate Mass QTOF mass spectrometer (Agilent Technologies) with electrospray ionization (ESI) in positive mode. A 2 µL aliquot of sample was injected and metabolites were separated on a 4.6 mm x 100 mm, 1.8 micron Zorbax High Resolution HT SB-aqueous column (Agilent Technologies) set at 65°C and eluted with a gradient mobile phase of deionized water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was maintained at 0.7 mL/min with a gradient of 2, 98, 98, 2 and 2% of solvent B, respectively at 0, 8, 10, 12 and 12.5 min. Fast polarity referencing solution was used in positive ESI mode with reference ions of *m/z* 121.0509 and *m/z* 922.0098. The mass spectrometer scanned from *m/z* 50 to *m/z* 1700. The MS parameters were as follows: gas temperature, 300°C; drying nitrogen gas flow rate, 11 L/minute; fragmentor voltage, 175 volts; skimmer voltage, 50V, OCT 1RF Vpp voltage, 750 volts and nebulizer pressure, 50 psig.

Metabolites were identified based on their exact masses corresponding to the *m/z* peaks by searching them against the Metlin (>79,000 metabolites) database. Targeted MS/MS in positive mode was used to confirm identities of selected metabolites against commercially pure standards using the same chromatographic parameters described above. Collision energy was applied by setting an appropriate equation having a slope value of 5 and offset value of 2.5. A full range mass scan from *m/z* 50 to *m/z* 1700 with an extended dynamic range of 2 GHz standardized at 3200 was applied. Data acquisition was maintained at a rate of 3 spectra/s at a time frame of 333.3 ms/spectra with a transient/spectrum ratio of 1932.

Data Processing and Statistical Analyses

Agilent MassHunter Qualitative (MHQ, B.05) and Mass Profiler Professional (MPP, 12.6) were utilized for data processing. Raw data files were originally acquired and stored as "*.d" files in order to be processed in MHQ. The Molecular Feature Extraction (MFE), a naïf extraction procedure, was the first algorithm applied to the total ion chromatograms (TIC) files. The MFE parameters were set to allow the extraction of detected features with absolute abundances of >4,000 counts providing information regarding $[M+H]^+$, isotopes and their corresponding Na^+ adducts. The resulting extracted ions were treated as single features for which a potential formula was generated. The collected information summarizing retention time (RT), exact masses and ion abundances were converted into compound exchange format (*.cef) and were exported to MPP for further comparative and statistical analyses. Using alignment and normalization procedures, individual "*.cef" files were binned and combined to generate new "*.cef" files. These new files were reopened in MHQ for a further data mining procedure using a "Find by Ion" algorithm. This targeted feature algorithm helped with minimizing the false positive and negative features. A second series of individual "*.cef" files were created from original individual "*.d" files and exported into MPP for statistical and differential analyses. Frequency filtration was used to only accept features that were detected in at least one condition. Additional MPP filtering procedures such as number of detected ions (set at 2) and charge states (set to all charge states permitted) were also applied. The RT compound alignment parameters were set at 0.15 min with a mass tolerance of 2.0 mDa. The data were normalized using a percentile shift algorithm set to 75 and were adjusted to the baseline values of the median of all samples.

Statistical analysis of the metabolomics data was performed with MPP using a moderated T-test (unpaired modification of unpaired T-test) at a significance level of $P<0.05$ with a fold change set at ≥2. Partial least square discrimination (PLSD) analysis was used as a prediction model for the two types of rats. Lorenz curves were used for both obese and lean rats to visualize the ordering of this measure for each group. Selected rat characteristics were analyzed by an independent T-test using SPSS (version 22) and a significance level set at $P<0.05$.

Results

The body weights of obese rats (748 ± 45 g) were significantly heavier (P<0.001) than the aged-matched lean rats (490 ± 23 g). The whole body fat composition assessed *in vivo* was also significantly (P<0.001) higher in obese (48.1 ± 0.9% fat) compared to lean (16.9 ± 0.8% fat) rats. Visceral fat (sum of perirenal, epididymal and mesenteric fat) was significantly (P<0.001) greater in obese *fa/fa* rats compared to lean Zucker rats 9.4 ± 1.3 and 5.5 ± 1.0 g per 100 g body weight, respectively.

The raw urinary MS data from obese and lean rats analyzed by LC-QTOF-MS were used for the class prediction PLSD algorithm that was applied to >1000 entities. The clusters of metabolites in the PLSD loading showed a separation between obese and lean rat groups (Figure 1*).* The Lorenz Curves generated for each pair of treatments (obese and lean rats) showed it was possible to accurately predict which samples belonged to a particular treatment group (8 rats in each group) as indicated by the break in the slope at the midpoint of the line.

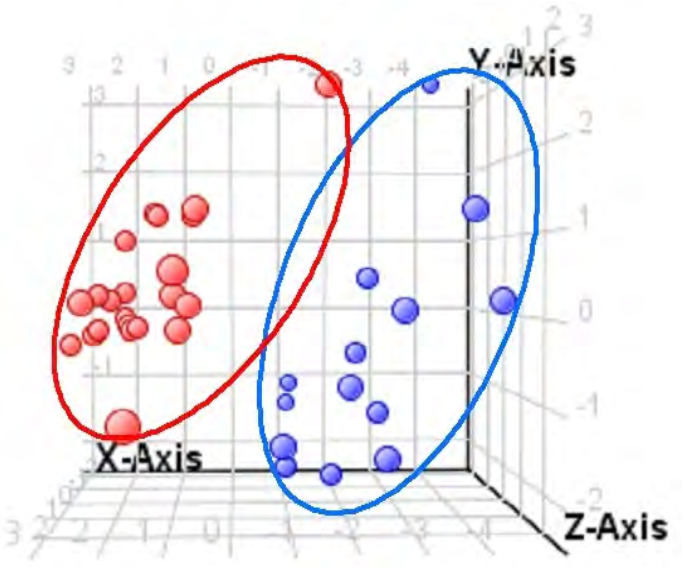

Figure 1. An overview of the t-scores from partial least square discrimination (PLSD) analysis with urinary metabolites from obese *fa/fa* and lean Zucker rats (*n*=8 rats per group). Blue is obese rat urine and red is lean rat urine.

The untargeted metabolomics approach detected 1046 entities with 43 metabolites that were significantly different (P<0.05) between urine extracts from obese and lean rats. Metabolites identified by the Metlin database are shown in Table 1. Metabolite concentrations have been calculated based on the internal standard norvaline and are presented in µg per mL of urine. Since the volume of urine collected over the time period did not significantly vary between groups (obese: 5.2 ± 2.0 mL, lean: 3.6 ± 1.8 mL; P=0.125), the concentrations that were obtained represent actual differences in amounts of metabolites present in the urine of obese and lean rats. Seven metabolites were significantly elevated in obese rat urine, including N_1-methylnicotinamide, spermine, hexadecyl actyl glycerol, 3-mercaptolactic acid and N-(4-hydroxyphenyl)propanamide. Of these metabolites, two metabolites, spermine and N_1-methylnicotinamide were found to be novel and were further investigated.

The metabolite, spermine, was shown to be abundant in the urine of obese rats (0.46 ± 0.14 µg/mL urine) but was not detected in the urine of lean rats (Figure 2A). Originally identified by the Metlin database according to its elemental composition and fragment patterns, spermine was further confirmed using a pure spermine standard (1.02 µg/mL in deionized water). The accurate mass of the ion was identified using targeted MS/MS at a collision energy of 10.1 volts. The fragment ions *m/z* 129.1387 [M+H]+ and *m/z* 112.118 [M+H]+ from the parent ion *m/z* 203.2225 [M+H]+ eluted at a RT of 1.252 min were identified in both the standard and obese rat urine extracts as well as with the Metlin Library, therefore confirming this compound as spermine (Figure 3).

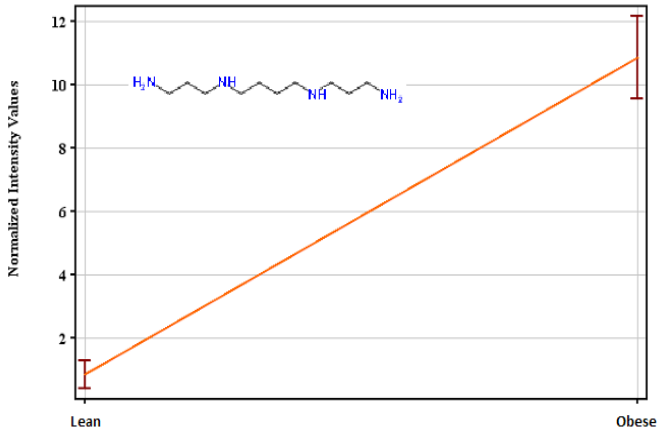

Figure 2A. Intensity increases in spermine of obese and lean rat urine extracts (*n*=16 samples performed in triplicate). (Moderated T-test P<0.05)**.** Error bars represent the standard deviation of the mean.

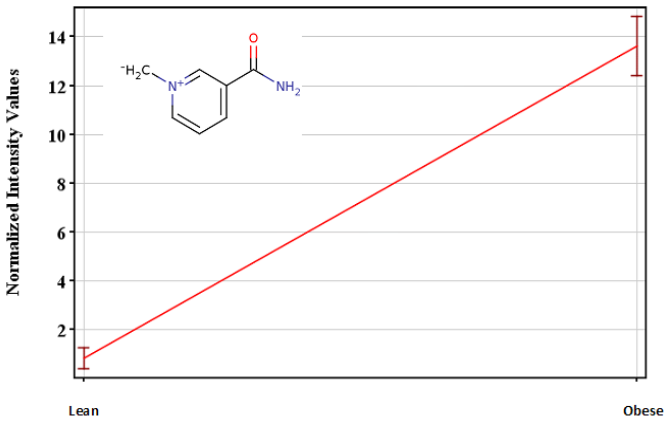

Figure 2B. Intensity increases in N_1-methylnicotinamide of obese and lean rat urine extracts (*n*=16 samples performed in triplicate). (Moderated T-test P<0.05)**.** Error bars represent the standard deviation of the mean.

Table 1. Statistically significant metabolites identified in obese *fa/fa* and lean Zucker rat urine (P<0.05).

Urinary Metabolites	Obese (μg/mL urine)[a]	Lean (μg/mL urine)[a]	Formula	ESI + m/z	Retention Time (min.)	Classification
N_1-methylnicotinamide	0.40 ± 0.12	ND	$C_7H_9N_2O$	137.0708	1.579	Pyridine
Spermine	0.46 ± 0.14	ND	$C_{10}H_{26}N_4$	203.2225	1.252	Polyamine
Hexadecyl acetyl glycerol	1.92 ± 0.70	ND	$C_{21}H_{42}O_4$	359.3130	8.600	Diacyl-glycerol analog
3-Mercaptolactic acid	0.75 ± 0.60	ND	$C_3H_6O_3S$	144.9922	1.202	Alpha hydroxy acid
N-(4-hydroxyphenyl) propanamide	0.38 ± 0.20	ND	$C_9H_{11}NO_2$	166.0855	2.782	Drug
Monoglyceride (0:0/18:0/0:0)	ND	0.93 ± 0.80	$C_{21}H_{42}O_4$	381.2977	8.577	Glycerol lipid
D-α-hydroxyglutaric acid	ND	1.40 ± 1.00	$C_5H_8O_5$	166.0721	2.593	Alpha hydroxy acid
Prolylhydroxyproline	ND	0.42 ± 0.30	$C_{10}H_{16}N_2O_4$	229.1185	1.715	Dipeptide
Lys Phe	ND	0.49 ± 0.40	$C_{15}H_{23}N_3O_3$	294.1816	3.013	Peptide
5,8-Tetradecadienal	ND	1.76 ± 0.40	$C_{14}H_{24}O$	231.1715	2.044	Fatty aldehyde
N^5-(1-Iminoethyl)-L-ornithine	ND	0.35 ± 0.15	$C_7H_{15}N_3O_2$	174.1231	2.600	Nitric oxide synthase inhibitor
7α-Hydroxydehydrocostus lactone	ND	0.49 ± 0.16	$C_{15}H_{18}O_3$	247.1326	5.459	Sesquiterpenoid
Nadolol	ND	0.44 ± 0.11	$C_{17}H_{27}NO_4$	310.2015	4.507	Beta blocker drug
2,4-Dimethyl-2E,4E-hexadienal	0.37 ± 0.10	0.24 ± 0.07	$C_8H_{12}O$	125.0956	4.103	Fatty aldehyde
Oleoyl 3-carbacyclic phosphatidic acid	ND	0.31 ± 0.07	$C_{22}H_{41}O_5P$	439.2553	3.333	Lysophosphatidic analog
Metalaxyl	0.37 ± 0.10	0.24 ± 0.07	$C_{15}H_{21}NO_4$	280.1542	3.573	Fungicide
Selenocysteine	ND	0.15 ± 0.02	$C_3H_7NO_2Se$	163.9778	1.488	Amino acid
3α,12α-Dihydroxy-5β-chol-7-en-24-oic acid	3.50 ± 2.0	4.62 ± 0.60	$C_{24}H_{38}O_4$	391.2844	9.023	Bile acid
Arg Arg Gln	0.50 ± 0.02	1.32 ± 0.10	$C_{17}H_{34}N_{10}O_5$	459.2801	4.212	Tripeptide
2-[3-Carboxy-3-(methylammonio)propyl] L-histidine	ND	0.96 ± 0.125	$C_{11}H_{18}N_4O_4$	271.1413	2.460	Alpha amino acid

[a]ND: not detected

Similarly, N_1-methylnicotinamide, also known as trigonellinamide, was significantly increased in the obese (0.40 ± 0.12 μg/mL urine) compared to the lean (not detected) rat urine extracts in our study (Figure 2B). N_1-methylnicotinamide was originally identified as N-methylnicotinamide, however, with further targeted MS/MS analysis of the pure compound N-methylnicotinamide, the parent ion *m/z* 137.0708 [M+H]+ was broken to the fragment ion *m/z* 108.0436 [M+H]+ which did not correspond with the compound we tentatively identified as N-methylnicotinamide. Further investigation of the compound structure and product breakdown in the obese rat urine extract revealed that the compound we identified as N-methylnicotinamide contained a methyl group on N_1 located on the pyridine ring which gave the fragment ion *m/z* 94.0694 [M+H]+; this was confirmed to be N_1-methylnicotinamide with Metlin: Metabolite and Tandem MS Library where the same fragment ion *m/z* 137.0708 using a collision energy of 10 volts in positive mode was optimized (Figure 4).

In the urine extracts, 13 identified metabolites including monoglyceride (0:0/18:0/0:0), D-α-hydroxyglutaric acid, prolylhydroxyproline, lys phe, 5,8-tetradecadienal, N^5-(1-iminorthyl)L-ornithine, 7α-hydroxydehydrocostus lactone, nadolol, oleoyl 3-carbacyclic phosphatidic acid, selenocysteine, 3α,12α-dihydroxy-5β-chol-7-en-24-oic acid, arg arg gln, 2-[3-carboxy-3-(methylammonio)propyl]L-histidine were significantly increased in lean rat urine. Interestingly, some lipid-soluble metabolites such as hexadecyl acetyl glycerol and monoglyceride (0:0/18:0/0:0) were detectable using the water-soluble extraction method which is likely due to a collection of lipids being dissolved in the acetonitrile fraction.

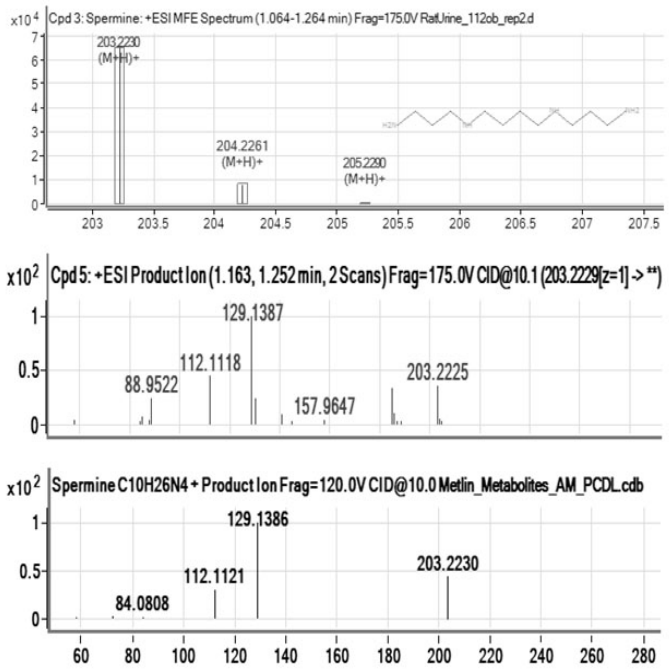

Figure 3. Mass spectras for spermine in ESI + mode; MS spectra of urine sample, targeted MS/MS spectra of urine sample and targeted MS/MS spectra of pure standard of spermine (1.02 µg/mL solution).

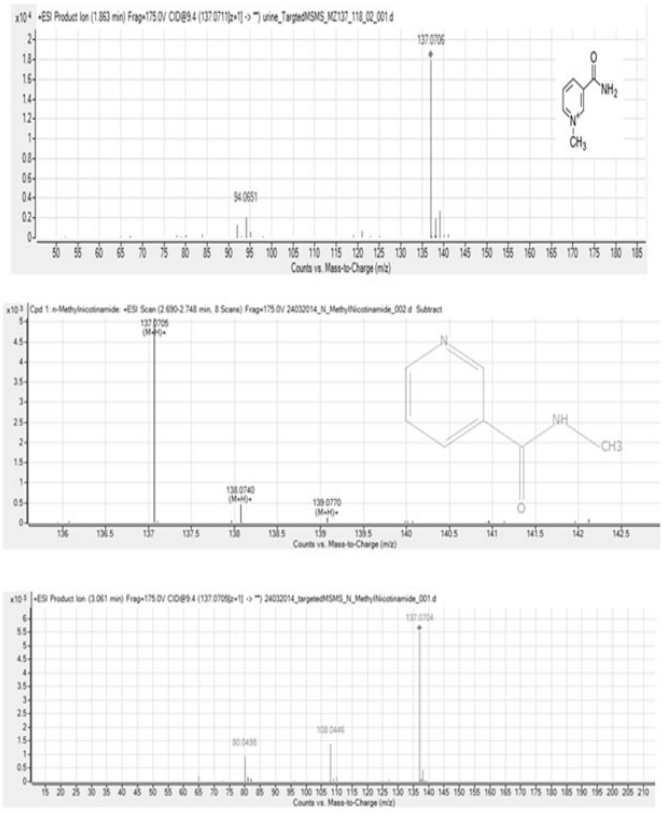

Figure 4. Mass spectras of N_1-methylnicotinamide and N-methylnicotinamide in ESI + mode; targeted MS/MS spectra of urine sample (N_1-methylnicotinamide), MS spectra and targeted MS/MS spectra of pure standard of N-methylnicotinamide (1 µg/mL solution).

Discussion

In the present study, we demonstrated several significant differences between the urinary metabolic profiles of obese and lean Zucker rats using LC-QTOF-MS. Among those differences, two metabolites, spermine and N_1-methylnicotinamide were considered to be novel and their associations with metabolic pathways were further investigated. Polyamines, namely spermine and spermidine which are involved in cellular metabolism of all eukaryotic cells, have been previously reported to stimulate adipose triacylglycerol formation from the sn-glycerol-3-phosphate pathway by activation of several enzymes including *sn-glycerol-3-phosphate acyltransferase*, *Mg(2+)-dependent phosphatidate phosphohydrolase* and *diacylglycerol acyltransferase* [21]. A study by Jamdar et al. (1996) [21] reported an increased activation of adipose triacylglycerol synthetic enzymes such as *Mg(2+)-dependent phosphatidate phosphohydrolase* as well as a 4-fold increase in the concentration of spermine in the adipocytes from adipose tissues of obese Zucker rats compared to lean rats. The higher concentration of spermine in the urine of obese rats in the current study could be explained by *fa/fa* Zucker rats having a greater adipose mass and increased adipocyte spermine concentrations. In addition, spermine has been identified in the urine of aristolochic acid nephrotoxicity rats using ultra performance liquid chromatography quadrupole time-of-flight high definition mass spectrometry which suggests that spermine could also be present due a poisonous effect on the kidneys [22].

Spermine is also an important amine derived from arginine. Arginine is a precursor of nitric oxide which is an important cellular signaling molecule involved in many physiological and pathological processes including oxidative stress [23]. A study by Codoñer-Franch et al. [23] reported that serum polyamine levels were significantly higher in obese children (n=60) compared to non-obese children (n=42) aged 7 to 14 years old. In serum, spermine was the most highly represented polyamine associated with increased obesity. They also determined that polyamines were related to biomarkers of oxidative stress, inflammation and leptin. *Fa/fa* rats display markedly elevated leptin levels [24] and oxidative stress [25] compared with their lean counterparts which can explain the high levels of urinary spermine in our obese model. Additionally, spermine has been well documented for its role as a direct intercellular free radical scavenger capable of protecting DNA from reactive oxygen species damage [26]. Enhanced levels of the reactive oxygen and nitrogen species in an obese state could be attributed to increased utilization of molecules capable of preventing or delaying cell destruction [27]. As a natural free radical scavenger, spermine can remove free radical intermediates from chain reactions in the cell that are responsible for damage or death to the cell, particularly in conditions such as obesity where the oxidative reactions of cells may be accelerated.

N_1-methylnicotinamide is a metabolite involved in nicotinamide metabolism produced primarily in the liver; it has also been linked to oxidative stress. An increase in urinary 1-methylnicotinamide was previously reported in a study

where C57BL/6 mice were fed a high fat diet (HFD, 60% energy from fat)(18). The study found that the HFD affected nicotinamide metabolism by increasing levels of 1-methylnicotineamide and nicotinamide-N-oxide, which can lead to systemic oxidative stress [18]. The greater concentrations of N1-methylnicotinamide in the fa/fa Zucker rat model indicates that the increase is due to obesity and not from the consumption of a high fat diet. The increased urinary concentration of N1-methylnicotinamide in the obese rats may be due to the increased expression of nicotinamide N-methyltransferase (NNMT), an enzyme that catalyzes the conversion of nicotinamide to methylnicotinamide in adipocytes and liver. NNMT is a novel histone methylation modulator that regulates energy metabolism and is an indicator of NNMT activity in serum that is strongly associated with obesity and diabetes in humans [28]. Increased NNMT expression has been previously reported in white adipose tissue and liver of obese and diabetic mice [29]. The study by Kraus et al. (2014) [29] reported that NNMT is an important regulator of the polyamine flux and may be a target for treating obesity. Inhibition of NNMT both upregulates ornithine decarboxylase and spermidine-spermine N1-acetyltransferase activity, which acetylates spermine, and enhances their expression, thus leading to an increase in overall polyamine flux, greater adipocyte secretion of diacetylspermine and elevated urinary excretion [29]. Therefore, NNMT provides a link between N1-methylnicotinamide and spermine in an obese state, and may prove to be a useful target for preventing metabolic changes affecting energy utilization in the obese state.

Although, the study focused on two novel metabolites that were increased in urine of obese rats, it is important to note that several other metabolites not previously linked to obesity or obesity-related disorders were higher or lower in an obese state. For example, 3-mercaptolactic acid, a thiol previously confirmed to be found in urine [30] was increased in obese rat urine and this may suggest an obesity effect on the metabolism of cysteine. Plasma concentrations of cysteine have been reported to be correlated with fat mass and BMI of women and men, and cysteine has been linked to obesity-related disorders such as cardiovascular disease and metabolic syndrome [31]. Cysteine is catabolized to 3-mercaptolactic acid, which may explain the abundance of 3-mercaptolactic acid in the urine of obese rats in our study.

Metabolites detected in lean rat urine, but with lower abundance or not detectable in obese rat urine, can also contribute to understanding obesity. For example, selenocysteine and D-α-hydroxyglutaric acid, a compound previously reported to be found in urine and related to a neurometabolic disorder [32], were present in the lean urine extracts but were not detectable in the obese rat urine. This may suggest that metabolic pathways involving the selenocompound and C5-branched diabasic acid are not as active in an obese state, however, further identity confirmation of these metabolites is required.

In the present study we detected alterations in the metabolite profiles of obese rats that occurred independent of any diet interventions. However, further research is required to determine the applicability of our results in genetically obese rats with disrupted leptin metabolism to models of diet-induced obesity in rodents and humans to further our understanding of metabolic alterations associated with obesity. Urine is an important non-invasive tool that represents whole body metabolism and analysis of specific tissues will be needed to delineate whether the urinary metabolites represent alterations specifically in adipose tissue or various tissues such as the liver which are heavily impacted by obesity.

Conclusion

The investigation of urinary metabolite profiles in obese *fa/fa* and lean Zucker rats using LC-QTOF-MS can be applied to better understand how metabolism is altered in an obese state. Enzymatic changes in cellular metabolism within adipose tissue may be responsible for the increases of spermine and N_1-methylnicotinamide in the urine extracts from obese rats. N_1-methylnicotinamide and spermine have been previously associated with physiological pathways related to oxidative stress in an obese state [18,23]. In addition, this study revealed other metabolites that were either elevated or reduced in an obese state, however, further research is required to investigate their relationship to other biochemical pathways and selected metabolites that may be affected in obese models. This study provides a platform for further research examining the effects of external factors (e.g. dietary interventions) on urinary water-soluble metabolites related to obesity.

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