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Identification of biomarkers for melamine-induced nephrolithiasis in young children based on ultra high performance liquid chromatography coupled to time-of-flight mass spectrometry (U-HPLC–Q-TOF/MS)

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ABSTRACT

Milk products contaminated with melamine caused renal disease in young children in mainland China in 2008. The present study was designed to identify potential markers and assess the underlying metabolomic mechanisms of melamine-induced nephrolithiasis in young children. Urine samples were collected from healthy children ($n = 74$) and from children diagnosed with nephrolithiasis ($n = 73$) with either a positive ($n = 40$) or a negative ($n = 33$) history of melamine exposure. Ultra high-performance liquid chromatography coupled to time of flight mass spectrometry (U-HPLC–MS/MS) was applied to profile the abundances of metabolites. Partial least squares-discriminant analysis (PLS-DA) was used to discriminate between the samples. Seven compounds were found to highly discriminate between healthy controls and nephrolithiasis patients with a history of melamine exposure. The critical markers such as proline and 5C-aglycone were the predominant markers in the control group and detected only rarely in nephrolithiasis patients with a history of melamine exposure. In contrast, hypoxanthine at was the most significant compound that distinguished nephrolithiasis patients with a history of melamine exposure. It was increased to 116.12 ± 23.34 μg/L (mean ± S.D.) in the melamine-induced nephrolithiasis group, whereas the non-melamine group was at the level of 67.47 ± 9.33 μg/L ($p < 0.001$). The biomarkers for melamine-induced nephrolithiasis identified by this study may have clinical application in determining the aetiology of renal disease in young children.

1. Introduction

Melamine is commonly used in the chemical production of inks, plastics and fertilisers. However, this compound was illicitly blended with dairy products as food additive to interference the protein content, which caused the deaths of a large number of domestic cats and dogs in the United States in 2007 and inflicted melamine-induced nephrolithiasis on more than 290,000 young children (0–3 years of age) in China in 2008–2009 [1]. After this large-scale exposure, studies on kidney failure and asymptomatic kidney stones in young children (0–3 years) exposed to melamine have become more urgent [2–5].

Several studies have been conducted over the last several years to aid in the understanding of the formation mechanism of kidney stones in young children and pets. In an epidemiological investigation for screening melamine exposure, Guan et al. [2] found that dietary and lifestyle factors played key roles in stone formation. They also found a causal relationship between kidney disease and prolonged melamine exposure. Using matrix-assisted laser desorption/ionisation–mass spectrometry (MALDI–MS), Tang et al. [3] found the presence of melamine and cyanuric acid in kidney stones from patients who had been exposed to melamine. In a recent study, Xie et al. focused on Wistar rats exposed to melamine and detected the amino acid metabolism with high-dose melamine exposure in the acute renal toxicity in rats. Melamine in the acute renal toxicity at 100, 300 and 600 mg/kg was given vial oral gavage for 15 days [4]. According to their findings, the amino acid metabolisms of rats were disrupted after high exposure to melamine, including tryptophan, polyamine and tyrosine metabolism, and altered TCA and gut microflora structure. By all accounts, the metabolisms and pathogeny on kidney stone disease [5,6] on the melamine-exposed rats had been studied; however, none of these studies have been able to find the

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potential markers differentiating between melamine-induced and non-melamine-induced disease states and propose a reasonable formation metabolism in human cases. Because kidney stones in young children with no history of melamine consumption have been found, and because neither animal nor human experiments have produced evidence to support the direct toxicity of melamine on the kidney, it is necessary to develop methods of identifying pathogenesis related to melamine exposure [7]. In recent years, metabonomics have often been used as promising techniques in disease diagnosis. Biomarkers in urine, serum, saliva or plasma samples have been used to understand the metabolic changes in affected biological systems by external stimuli over time [8–10] and for a variety of diseases. For example, Ma et al. [11] found that the levels of two low-molecular-weight compounds in patients with preoperative colorectal cancer were significantly increased in comparison to healthy volunteers. Using gas chromatography–mass spectrometry (GC–MS) on samples from renal transplantation subjects, Mao et al. [12] found 17 compounds, which included phenylalanine, serine and glycine that were present at significantly higher levels in the transplant rejection group compared with the stable group.

In this study, we attempt to find some biomarkers related with melamine-induced nephrolithiasis in children's urine samples, compared with those of healthy children. Urines samples taken from disease and control groups were detected by ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (U-HPLC–Q-TOF/MS) and the data were analysed by a series of pattern recognition methods. In view of the numerous previous studies that have discussed the biomarkers of kidney stones without melamine exposure (e.g., the Tamm–Horsfall Protein discovered in 1950), samples from nephrolithiasis patients with no history of melamine exposure were not used for the biomarkers of kidney stone disease to avoid repeating previous research, which was instead used to validate the markers and determine whether they were significantly correlated with melamine exposure. Therefore, in our study, samples from nephrolithiasis patients with no history of melamine exposure were only used to validate the potential markers related to melamine exposure. The secondary step was to check the ability and reliability of the predictive model that the training set data to predict the test set data, both randomly divided from raw data.

2. Materials and methods

2.1. Chemicals and reagents

OPTIMA-grade acetonitrile and pure water were purchased from Fisher Scientific (Pittsburgh, PA) for use as the mobile phase. Formic acid (99%) was purchased from Acros Organics (Morris Plains, NJ). Leucine enkephalin and the chemical reference standards were purchased from Sigma–Aldrich (MA, USA).

2.2. Patients and sample preparation

The sick children included in this study were diagnosed with nephrolithiasis using renal ultrasonography. Urine samples (n = 147, provided by Peking University First Hospital) were collected from sick children diagnosed with nephrolithiasis (n = 73) and from a control group of healthy children (n = 74). Table A1 (in the Electronic support material) includes (i) Group 1: samples from healthy controls (n = 74), (ii) Group 2: samples from nephrolithiasis patients with a history of melamine exposure (n = 40) and (iii) Group 3: samples from various nephrolithiasis patients with no history of melamine exposure (n = 33). The samples were collected in the morning and stored without additives at −20 °C until analysis [13]. Urine samples were collected midstream. The median age of the subjects, 60% of whom were boys, was 18 months. We previously found that age, sex and use of formula milk alone or in combination with breast milk are not significantly associated with the presence or absence of melamine-induced nephrolithiasis [2]. Thus, more detailed information on the children was not recorded. This study was approved by the local ethics committee (Beijing) and conducted in accordance with the terms of the Declaration of Helsinki (Declaration of Helsinki – current in 2008 version). Informed assent was obtained from the parents of all study participants.

Fresh urine samples were collected from subjects. A 400-μL volume of urine was diluted twofold with ultrapure water (Milli-Q, Millipore, MA), transferred to a 1.5-mL Eppendorf tube (Eppendorf, New York, USA), vortexed and centrifuged at 13,400 rpm for 30 min. The supernatant was then removed for use in the U-HPLC–Q-TOF/MS analysis. Samples that were not immediately assayed were stored at −20 °C until analysis. The quality control (QC) samples were consisted of the urine spiked with melamine standard at 10 ng/mL. The mixture was shaked well, then stored with other urine samples before analysis. The repeatability of the QC samples was determined by U-HPLC–Q-TOF/MS, and the instrumental conditions were same to those urinary samples. The peak heights, areas and retention times of these samples were extracted and analysed.

2.3. Instrumental analysis

Chromatographic separation was performed using an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size) with an ACQUITY U-HPLC system (Waters, Milford, MA, USA). A Waters Micromass ion mobility Q-TOF mass spectrometer was used for data acquisition. The chromatographic and mass-spectrometric parameters are summarised in Table A2. All analyses were required using the lock spray to ensure accuracy and reproducibility. Leucine-enkephaline at 2 ng/μL was employed as the reference lock mass for all mass-calibration analyses. Its $[M+H]^+$ ion was 556.2771 Da in ESI^+ mode. The lock spray frequency was set as 10 s..

2.4. Analysis of urine residues/stones

Nearly 0.5 mL of urinary residues/stones in 500 μL ultrapure water vortexed for 1 min and then soaked for about 2 h. The mixture was centrifuged at 13,400 rpm for 30 min at 4 °C. The liquid supernatant was collected and transferred into a 1.5 mL Eppendorf tube for analysis. Matrix-assisted laser desorption connecting to TOF/TOF (MALDI–TOF/TOF, Applied Biosystems 4800 Proteomics Analyzers) was applied directly to biological residues/stones analysis, and the MALDI matrices (CHCA) were used to ionise melamine from melamine cyanurate in the positive mode [3]. Twenty microliters of urinary residues/stones was combined with an equal volume of mixed solution (10 mg/mL CHCA in 80% ACN, 0.1% TFA) and spotted onto a MALDI target for analysis. The analysis conditions were as follows: solid state laser wavelength, 355 nm; data acquisition rate, 200 Hz; MS reflector mass range, 50–1000; lasers shots, 1000; grid 1 V, 15.1 kV; mirror 1 V, 14.205 kV; mirror 2 V, 20.5 kV; delay time, 250 ns; MS/MS conditions, 2 kV, CID ON; laser shots, 1500; grid 1 V, 7.564 kV; source 2 V, 15 kV; mirror 1 V, 10.885 kV; mirror 2 V, 18.746 kV; delay time, 300 ns.

2.5. Statistical analysis

A Micromass MarkerLynx™ applications manager (an add-on to Masslynx Version 4.1, Waters, MA, USA) was used to (i) process the total-ion chromatograms (TICs) and detect peaks, (ii) identify retention times (t_R) and (iii) obtain mass spectra. The parameters in MarkerLynx™ were set as follows: peak width, 5%; height, 12 s;

peak-to-peak baseline, 80; intensity threshold, 80 counts; mass window, 0.05 Da; mass tolerance, 0.05 Da; retention time window, 0.2 min; and noise elimination level, 5. The period of retention time was processed from the beginning of the chromatogram at 0.1–15 min at the end. Two or three common peaks were checked manually in the aligned peak list. This procedure can assist in setting the main parameters to be set as a properly defined window, in which common peaks are found in every chromatogram using retention time and mass pair in the spectra. Following U-HPLC–Q-TOF/MS analysis, the raw peak detected by the software to ascertain differences between groups were transformed to a peak table and then exported into MATLAB (Version 7.0, Mathworks, Inc., Natick, MA) for further calibration [14]. Each row of the table corresponds to the urine samples of an individual child, whereas the columns refer to the identified variables (e.g., peak heights, peak areas). The results were exported into MATLAB for further analysis. All MATLAB scripts were written in-house; the steps of data transformation and model construction are described in the Appendices. Some functions in the toolbox of MATLAB were performed (e.g., Pearson correlation coefficient and receiver operator characteristic (ROC) curves). The major background peaks generated from the instrument and solvent were confirmed by spectrograms of blank samples and removed manually from the peak table prior to processing.

Partial least-squares discriminant analysis (PLS-DA), rather than principal component analysis (PCA), was used for the pattern-recognition analysis in this study to enhance the discrimination while processing the observation groups from the complicated dataset [15,16]. ROCs were used to evaluate the performance of the predictive model [17]. Due to the objective of finding markers rather than the calibrated models, the evaluation methods of the predictive models were briefly described in the Electronic support material.

3. Results and discussion

3.1. Chemical signature analysis of urine samples

Urinary metabolic profiling was performed on a U-HPLC–Q-TOF/MS. The analysis of the 20 QC samples showed that the relative standard deviation (RSD) of retention times was 2%. The RSDs of peak areas and heights were both below 20%. The results were acceptable for the good repeatability in the metabolic analysis. All the urine samples were analysed by U-HPLC–Q-TOF/MS using full scan (at m/z 50–1000 Da) in both negative mode and positive mode. In the preliminary analysis, the positive-ion electrospray ionisation (ESI) mode detected more information for peaks than the negative-ion ESI mode and no interesting characteristic response in negative-ion ESI-MS. For this reason, only positive-ion-mode data were provided in this study to construct an initial peak-intensity matrix using 3785 variables from the 114 samples.

The resulting dataset was exported to MATLAB, and an independent t-statistics was used to determine the significant differences between the two groups. T-statistics, in which individual single variables were ranked in order of significance, chose those variables that appeared to be important. Non-significant variables were eliminated from the dataset. The resulting integrative peak intensity matrix included 114 samples × 567 variables. Peak numbers (t_R–m/z pairs) and mass spectra were used to identify each peak, and the peak heights were normalised and standardised. The prepared peak table was optimised by bootstrap to generate the significant components in the model. The details of calibrations had been given in Data transformation of the Electronic support material.

The PLS-DA method was used to group separation. The two-component PLS model ($R^2X = 0.869$, $R^2Y = 0.919$, $Q^2 = 0.942$, respectively) was generated between the extracted matrix (114 samples × 567 variables). The score plot (Fig. 1) indicated that differences existed between the two groups, whereas the construction of three-dimensional graphs clearly demonstrated the direction of the metabolomic changes. Pearson correlation coefficients (Fig. 2) within the groups confirmed the two classifications and supported the similar distribution that was observed with PLS-DA. The two separate clusters in Fig. 2 with higher correlation coefficients indicate that the samples were much more similar within groups on the optimised model. The corresponding peaks for the separated classes in the loading plot are shown in Fig. 3, and the major loadings responsible for the separations observed in the score plot are labelled. Of the top 10 biomarkers, one unknown chemical was not identified, but possible formulas are given in Table 1. The identification standards for the remaining metabolites are reported according to the literature [18]. The most representative and discriminative markers in Groups 1 and 2 were identified by reference standards or by searching online databases, including HDMB [19], NIST [20], Metabolome [21], METLIN [22] and Chemspider [23].

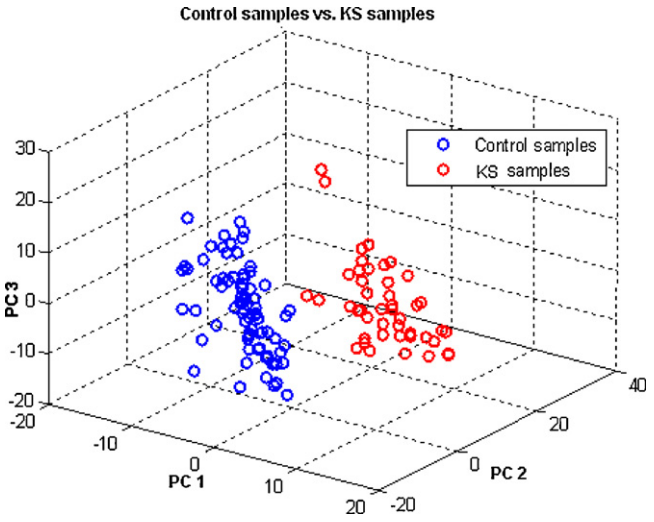

Fig. 1. 3D score plot of the PLS-DA method applied to Group 1 (O) and Group 2 (O) with exposure to melamine. The corresponding loading plot is shown in Fig. 3.

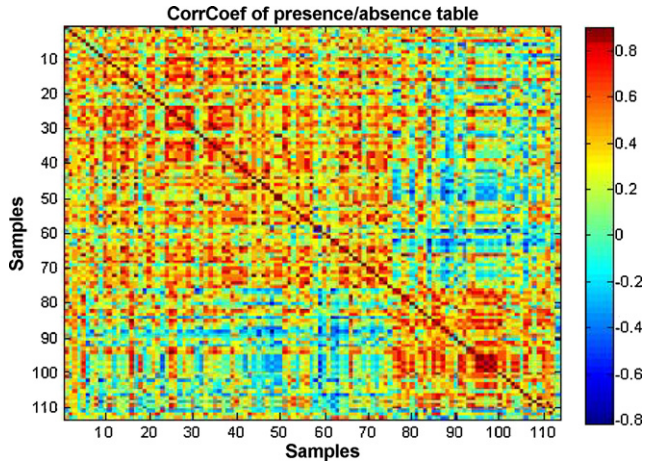

Fig. 2. Correlation–coefficient graph demonstrating the results of the peak table. The red areas in the upper left and bottom right show the two classifications. The legend means that the low correlation coefficient (blue colour) between groups increases toward the highest coefficient (dark red colour) within groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Potential discriminative markers of melamine-induced nephrolithiasis samples ($p < 0.01$) compared with control samples and results of ESI$^+$ mode U-HPLC–Q-TOF/MS.

Identified chemicals	Formula	t_R (min)	$[M+H]^+$	Trend[a]	Reference
L-beta-aspartyl-*L*-glycine	C6H10N2O5[b]	1.64	191.0356	↑	Putatively characterized compound
5C-aglycone	C16H16O4[b]	4.47	273.0805	↓	Putatively annotated compound
Proline	C5H9NO2[b]	2.31	116.0738	↓	Putatively annotated compound
3-Hydroxysebacic acid	C10H18O5[b]	3.23	219.0076	↓	Putatively annotated compound
Urine creatinine	C4H7N3O	0.75	114.0612	↑	Identified compound
Uric acid	C5H4N4O3	0.50	169.0463	↑	Identified compound
7-Methylguanine	C6H7N5O	1.05	166.0755	↑	Putatively characterized compound
L-homocysteine sulfonic acid	C3H9NO3S2[b]	1.59	172.0389	↑	Putatively annotated compound
Hypoxanthine	C5H4N4O[b]	1.25	136.9902	↑	Identified compound
Vanylglycol	C9H12O4[b]	1.16	185.0426	↑	Putatively annotated compound

[a] The trend indicates good separation between nephrolithiasis and control samples: (i) ↑ indicates an increasing trend in nephrolithiasis samples and (ii) ↓ indicates a decreasing trend in nephrolithiasis samples.
[b] During validation of the potential markers by comparison with the non-melamine exposed nephrolithiasis group, the marker was found to be significantly associated with the nephrolithiasis group with melamine-exposure. The unknown chemicals just gave the possible formulas.

To visually indicate whether the differences in the 10 detected markers in two groups were statistically significant, error bars were used to evaluate the results. Mean and standard error (S.E.) (Fig. 4a) were calculated to calibrate the differences of the scaled intensities between Groups 1 and 2. The error lines illustrate the ranges of variability for the marker compounds in the urine samples. Little variation was observed between patients in the range of the given S.E., which indicated that these markers maintained a steady state. The bars (Fig. 4b) corresponding to 95% confidence interval (C.I., mean $\pm$ 2 S.D./$\sqrt{n}$) presented as two separate means [24]: (i) for Group 1 and (ii) for Group 2 (t-test with $p < 0.01$). A small overlap or a large gap between the bars was considered positive evidence of a difference between the groups. The results for the identified markers were confirmed using the comparative PLS-DA method.

The Group 3 samples collected from children with various kidney stone diseases were helpful in validating the ten biomarkers that were common in kidney stone disease or significant markers related to melamine. Four compounds were commonly detected in the nephrolithiasis samples from Group 2 and Group 3 (i.e., children with and without a history of melamine exposure). No significant difference in the levels of these four compounds was observed between the two groups (t-test with $p > 0.1$, Table 1). This result suggests that, although these compounds were associated with renal disease, four chemicals were not specific markers of renal disease induced by melamine. The remaining seven compounds, which were marked by asterisks in Table 1, were potential markers of melamine-induced nephrolithiasis. In the results, the concentration of one compound at m/z 136.9906 was dramatically increased in urine samples from nephrolithiasis patients with a history of exposure to melamine. Two compounds showed decreasing trends, whereas three compounds exhibited increasing trends. The compound at m/z 116.0738 was obviously detected in urine samples from the control group, but it exhibited a much lower occurrence in the disease group. Fig. 5 shows chromatograms of the two extracted ions from the three study groups, which were the significant markers at m/z 136.9906 and m/z 116.0738. The relevance of these compounds to the mechanism underlying the development of melamine-exposure nephrolithiasis is suspect.

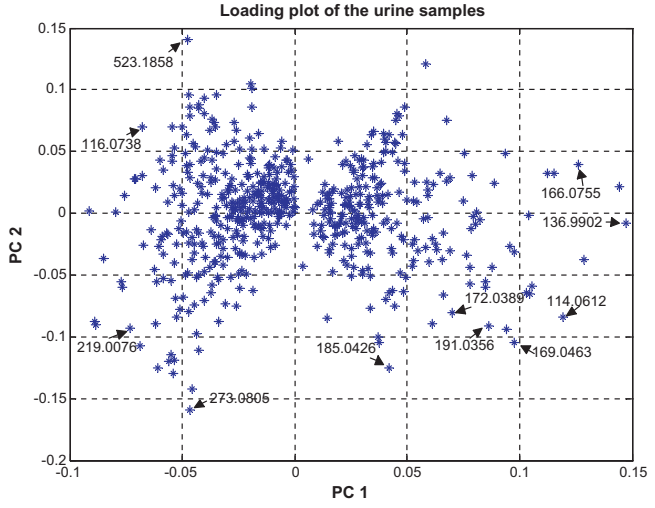

Fig. 3. Loading plot of the urine samples, including labels for the 10 most significant variables. This corresponds to the score plot shown in Fig. 2.

Table 2
The percentages of true and false positives were demonstrated for disease and control groups with autoprediction, bootstrap and test sets for a threshold equal to 0.

Types	Classes	Threshold = 0	
		Disease	Control
Autoprediction	Disease	96.20	4.10
	Control	3.80	95.90
Bootstrap	Disease	94.05	2.36
	Control	4.64	97.64
Test set	Disease	94.36	8.13
	Control	5.64	91.87

To determine the accuracy of the model for the markers, ROC curves were applied to the predictive model. The default value was set as the threshold (threshold = 0) that distinguished between the disease and control groups. In Table 2, the ROC curve for the average percentage in the predictive model in the bootstrap set was highly consistent with the ROC curve for the autoprediction set, which illustrates the applicability of the model for reliable selection of biological markers (Fig. A2, in the Electronic appendices material). The area under the curve (AUC) usually illustrates the result. Its value is closer to 1, which indicates that the separation of the classifications is much better. The AUC values are 0.99, 0.96 and 0.94 for autoprediction, bootstrap and test sets (Fig. A2), respectively. The AUC was slightly lower in the test set than in the autoprediction and bootstrap sets. In Table A3, the average percentage of correct classification (C.C.%) for the model is over 95% and only less 4% misclassified in the crossed groups, which indicates that the selected biological markers achieved a good predictive classification. Therefore, the model is quite generalised without bias for various data sets with good predictive ability.

3.2. Potential markers of the melamine-exposure nephrolithiasis

The multivariate statistical analysis suggested that the discriminative difference between two groups. The seven compounds

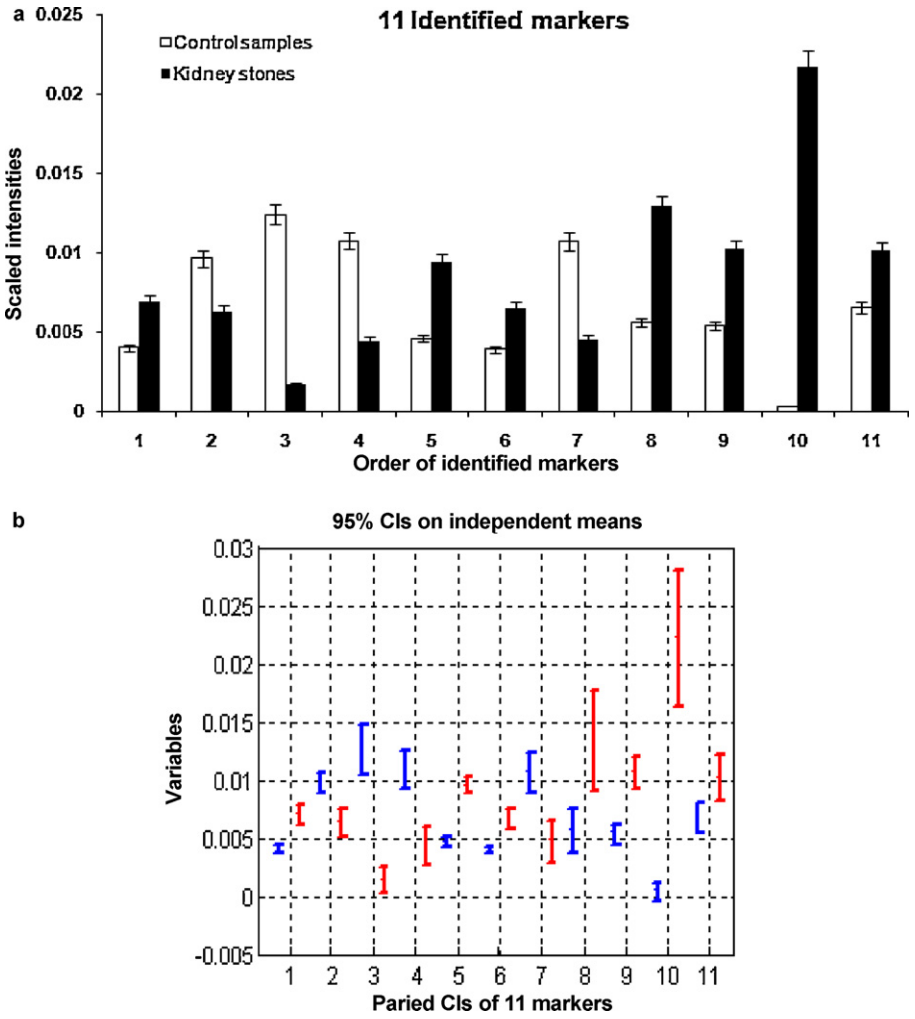

Fig. 4. The *x*-axis indicates the order of identified markers from Table 1, which includes 10 markers in the same order. (a) The levels (mean and S.E.) of 10 biomarkers identified from Group 1 and Group 2 (p value <0.01). The *y*-axis indicates the scaled intensities of the 10 identified markers. (b) Results expressed as 95% confidence interval (C.I.). The variance of these biomarkers between individuals is illustrated by confidence intervals and standard errors; the blue colour represents Group 1 ($n = 74$) and the red colour represents Group 2 ($n = 40$). The *y*-axis shows the scaled peak intensity measures of the identified markers from the peak table.

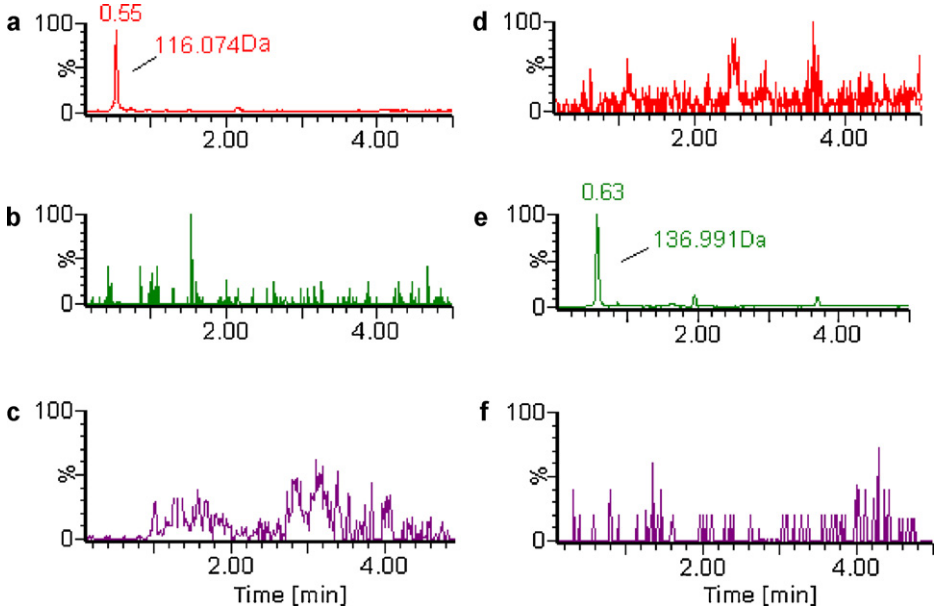

Fig. 5. The ions at m/z 116.074 from (a)–(c) and at m/z 136.991 from (d)–(f), which correspond to the bar plot in Supplementary Fig. 2. These total-ion chromatograms were extracted from the control group (red: a and d), the nephrolithiasis group with exposure to melamine (green: b and e), and the nephrolithiasis group with no exposure to melamine (purple: c and f). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were potential markers that were highly associated with melamine exposure in kidney stone disease (Table 1). These identified markers were effectively screened, yet they did not independently represent a single functional *in vivo* pathway. In our previous clinical studies by Guan et al. [2], high melamine content in the formula was significantly associated with the presence of stones in the renal tract. Consequently, the potential markers of melamine-exposure nephrolithiasis would be related to stone formation.

Hypoxanthine was identified among the compounds that showed a more sharply increasing trend in Group 2 than in healthy young children (*t*-test with $p < 0.001$). Because hypoxanthine was oxidised to uric acid via xanthine oxidase [25], it was not surprising that uric acid levels were also elevated in the disease group, as detailed in Table 1. To validate the levels of hypoxanthine in the urine of the two groups, the remaining urine samples belonging to the non-melamine group (8 samples from Group 1 plus 5 samples from Group 3) and the melamine group (11 samples from Group 2) were completely thawed for analysis by U-HPLC/MS–MS (Waters, Milford, MA, USA). The instrumental conditions are described in Table A4, and the mass conditions are described in Table A5. However, because the urine samples had been thawed and refrozen several times for previous injections, hypoxanthine levels lower than those found in the fresh samples were expected [13]. An independent *t*-test was used to analyse hypoxanthine levels in the healthy and disease groups. Apparent hypoxanthine of urine samples increased to 116.12 ± 23.34 μg/L (mean ± S.D.) in the melamine-induced nephrolithiasis group, whereas the non-melamine group was at the level of 67.47 ± 9.33 μg/L ($p < 0.001$). The result of higher detected intensities in the melamine-exposed group can be easily visualised in the TIC figure; two random single-ion chromatograms using precursor ions plus product ions are given as an example (Fig. A3).

The significant increases in hypoxanthine in nephrolithiasis patients exposed to melamine led to an accumulation of uric acid in the renal tubules. The subsequent crystallisation of uric acid and urate in the urinary tract and the formation of stones could lead to obstruction and kidney failure. For further validation of this reasoning, ten residues/stones of the remaining samples were collected from Group 2. Ammonia urate present in the fifteen urine residuals of this group was found by MALDI–TOF/TOF, as shown in Fig. A4. Ammonia urate was found only in the urine residuals of Group 2, which was exposed to melamine. The spectra figures show randomly selected examples.

The next marker at *m*/*z* 116.0738 that distinguished the control group was identified as proline (*t*-test with $p < 0.001$). The urine samples from Group 2 contained a much lower level of proline than did those from the healthy control group. Proline is a nonessential amino acid that is biosynthesised from the amino acid L-glutamate [26]. This result indicates that, in Group 2, a sharp increase of uric acid *in vivo* would have suppressed the synthesis of glutamate and increased ammonia genesis. The urine creatinine and uric acid levels were both common markers, as illustrated in Table 1 [27], which shows the higher value in the sick patients. The concentration of creatinine depended on the metabolism of nitrogen and the excretory capability of the kidney. Because sick children were fed high-nitrogen food contaminated by melamine, the purine metabolism was disrupted when the concentration of creatinine and uric acid exceed the normal limits. Thus, the capability of kidney reasonably was challenged under the situation. The reduction in the concentration of proline would cause a corresponding increase in the production of ammonia in the renal tubules. The accumulation of uric acid can lead to the formation of ammonium acid urate crystals and subsequent obstruction of the urinary tract, which may ultimately lead to kidney failure. Ammonia urate detected (Fig. A4) in the residuals/stones can be used to validate these results.

Other than hypoxanthine and proline, five compounds (*t*-test with $p < 0.05$) were differentiated between Groups 1 and 2 that showed either a decreasing trend (two compounds) or an increasing trend (three compounds) in nephrolithiasis patients exposed to melamine.

The compounds 5C-aglycone and 3-hydroxysebacic acid were decreased in melamine-exposed nephrolithiasis patients. The compound 5C-aglycone is a metabolite of vitamin K and is detectable in the urine of healthy individuals [28]. Vitamin K is necessary for the production of a urinary protein involved in kidney function, which inhibits the formation of kidney stones. People with diets high in vitamin K often have a low incidence of kidney stones. However, the decreased level of 5C-aglycone in Group 2 is an indicator of abnormal vitamin K absorption, which likely would have resulted in the increased formation of kidney stones *in vivo*. In the study by Muth et al. [29], 3-hydroxydicarboxylic acid was found to be a marker related to glycogen storage disease. In the study by Smit [30], decreased levels of metabolites (3-hydroxydicarboxylic acid and 3-hydroxysebacic acid) revealed that glycogen was not digestive and was stored at increased levels. The common glycogen storage disease tended to cause uric acid (a waste chemical) to accumulate, finally resulting in gout. Therefore, the abnormal urinary excretion of 3-hydroxydicarboxylic acid indicated an abnormal glycogen level that would lead to an accumulation of uric acid in the renal tube and the development of nephrolithiasis [31].

In contrast, the concentrations of L-beta-aspartyl-L-glycine, L-homocysteine sulfonic acid and vanylglycol increased in nephrolithiasis patients exposed to melamine (*t*-test with $p < 0.05$). L-Beta-aspartyl-L-glycine is a proteomic breakdown dipeptide and a normal component of human urine. Due to damage to the renal tube, the intermediate compound L-beta-aspartyl-L-glycine increased along with unbalanced amino acids *in vivo*. L-Homocysteine sulfonic acid is a metabolite of the amino acid homocysteine and is excreted by the kidneys in healthy individuals [32]. In the present study, nephrolithiasis patients exposed to melamine showed an abnormal increase in urinary L-homocysteine sulfonic acid. This increase is an indicator of renal disease *in vivo*.

4. Conclusions

Urine samples were analysed from healthy children and children with nephrolithiasis that had either been exposed or not exposed to melamine. The results by the PLS-DA method showed a promising classification. For the optimised model obtained by the bootstrap method, the AUC curves demonstrated the model's performance. A correct classification rate of approximately 95% indicates that the optimised model exhibits good predictive ability. The model should therefore provide reliable results. The variations in the identified markers of the loading plot were caused by renal disease secondary to melamine poisoning; unbalanced amino acid levels *in vivo*, containing the levels of detected markers highly increased or decreased such as proline, L-beta-aspartyl-L-glycine and L-homocysteine sulfonic acid, are the result of the pathological mechanism by which melamine affects stone formation in melamine-exposed children. The study demonstrates the usefulness of hypoxanthine as a biomarker of melamine-induced nephrolithiasis. Elevated levels of hypoxanthine are responsible for purine metabolism disorders; these effects combine with those of uric acid, a major compound in body fluids, to cause stone formation in the renal tracts. Maintaining normal levels of hypoxanthine and uric acid in the body can decrease disease risk. MALDI–TOF/TOF mass spectrometry detected ammonia urate in urinary residuals/stones from melamine-exposed children, which verified that the crystallisation of uric acid in renal tubules is responsible for the occurrence of kidney stones. A reliable marker could help with

medical management and decrease the incidence rate. Most of the markers came from a large category of amino acid, which is similar to the results by Xie [4]. Their study focused on the toxicity of melamine exposure based on an animal model. Based on their and our results, we conclude that the melamine exposure influences the level of amino acids *in vivo*. However, because the metabolic mechanism in human beings is much more complicated than that in rats [33], the differences in the detected metabolic compounds are not surprising. Prospective studies are warranted to validate these novel biomarkers using urine samples from nephrolithiasis patients with a history of exposure to other chemical contaminants and to establish standardised methods for the evaluation of the metabolomics process of the disease. The biomarkers identified in the present study may represent disease signatures of the specific underlying pathology of the study groups.

Competing interests

The authors have no competing financial interests to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.09.039.

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