Volatile metabonomic profiling in urine to detect novel biomarkers for B-cell non-Hodgkin's lymphoma

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Received August 11, 2017; Accepted February 8, 2018

DOI: 10.3892/ol.2018.8352

Abstract. To date, there have been a limited number of useful biomarkers for the screening and monitoring of B-cell non-Hodgkin's lymphoma (B-NHL), which leads to the impetus to discover novel biomarkers for the disease. In the present study, gas chromatography-mass spectrometry (GC-MS) combined with head-space solid-phase micro-extraction (HS-SPME) was employed to analyze the volatile metabolites in the urine samples of 131 subjects. The subjects were divided into 4 main groups: Aggressive B-NHL, indolent B-NHL, benign lymphatic diseases patients and healthy volunteers. The differences of the concentrations of the potential biomarkers among the groups were assessed by non-parametric Wilcoxon's test. The ability of the potential biomarkers to discriminate between the four aforementioned groups was evaluated by receiver operating characteristic curves (ROC). The present study indicated that 4-heptanone, 2-methylpyrazine, 2-methylbutanal, 2,6-dimethyl-7-octen-2-ol and decanoic acid may serve as potential biomarkers for B-NHL. The area under the curve (AUC) values of single potential biomarker ranged from 0.634 to 0.901. The diagnostic models established with combined biomarkers exhibited higher diagnostic values (AUC, 0.824-0.968) compared with the models established with single biomarkers. The present study indicated that urinary volatile metabolites might be potential biomarkers for screening and monitoring of B-NHL.

Introduction

Non-Hodgkin's lymphoma (NHL) is the most common hematological cancer with a rapidly increasing incidence among adults. In adults, ~85% of cases of NHL were of B cell origin (1). A lack of specific clinical symptoms and clear risk factors hinders the early diagnosis of B-cell non-Hodgkin's lymphomas (B-NHL), which results in poor prognoses (2). Population-based screening undoubtedly contributes to earlier diagnosis and improved prognosis. However, this is unfeasible for B-NHL due to a lack of useful screening methods. During the course of the treatment for B-NHL, the therapeutic effects need to be monitored by clinicians regularly. Following treatment, the disease needs to be monitored in long-term follow-up in case of recurrence or progression. At present, apart from computed tomography (CT), magnetic resonance imaging (MRI) and other imaging methods, tumor biomarkers are often employed to screen malignant diseases, monitor treatment responses and assess the status of the disease, including α-fetoprotein for liver cancer and prostate-specific antigen for prostate cancer (3,4). Compared with imaging methods, tumor biomarkers are easier and more economical. Unfortunately, the sensitivities and specificities of the commonly used biomarkers for lymphoma (e.g., lactate dehydrogenase and β 2-microglobulin) are limited (5,6).

B-NHL consists of aggressive and indolent subtypes according to its clinical features. Indolent lymphoma accounts for nearly one-third of NHL cases, and it is considered incurable (7). Indolent lymphoma includes low-grade (grade 1-2) follicular lymphoma (FL), chronic lymphocyte leukemia/small lymphocytic lymphoma, mucosa-associated lymphoid tumors (MALT), minority of mantle cell lymphoma (MCL) and lymphoplasmacytic lymphoma (8). Aggressive B-NHL comprises diffuse large B-cell lymphoma (DLBCL), most cases of MCL, grade 3 FL and Burkitt's lymphoma (9). Occasionally, several indolent subtypes may transform into aggressive subtypes. For example, low grade FL often changes into aggressive B-NHL, which for most of the cases are DLBCL, spontaneously. This transformation is referred to as transformed lymphoma (TL) and has a reported incidence of 10-70% (10). Since the outcomes of the two subtypes are very different, it is of clinical significance to monitor the transformation during long-term follow-up. However, pathological examination is not suitable for the long-term follow-up of TL due to its invasiveness. Therefore, a new non-invasive method should be developed to monitor the transformation.

Metabonomic analysis, a brand-new approach, employs comprehensive metabolic profiling methods to provide

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Key words: B-cell non-Hodgkin lymphoma, urine, volatile metabolites, screening, monitoring

systemic views of the disordered biological processes of diseases. Urine is considered ideal for metabonomic analysis as sampling of urine is non-invasive, and it is relatively easy to obtain sufficient volume for analysis (11). Since cancer often induces severe metabolic disorders, the comprehensive analysis of metabolites in urine may help to discover useful biomarkers for cancer (12,13). Silva *et al* (14) observed lower levels of hexanoic acid in the urine of patients with cancer compared with healthy controls using solid phase micro-extraction (SPME) in combination with gas chromatography-mass spectrometry (GC-MS). Guadagni *et al* (15) also detected higher concentrations of hexanal in the urine samples of patients with lung cancer compared with healthy controls using head-space SPME-GC-MS. Here, we present a pilot study using head-space SPME-GC-MS to assess the possibility of volatile metabolites in urine to satisfy the aims stated above.

Materials and methods

Recruitment of subjects. Between March 2014 and June 2014, urine samples were collected from 101 previously untreated patients [71 male and 30 female, average age 56.6 (37-78) years old] with a diagnosis of lymphatic disease and 30 healthy volunteers [21 male and 9 female, average age 57.1 (38-76) years old] at the First Affiliated Hospital of Anhui Medical University, (Hefei, China). The diagnoses of lymphatic diseases (B-NHL and benign lymphatic diseases) were confirmed by pathology. The clinical stage of the B-NHL was determined according to the Ann Arbor staging system (16). The prognosis of B-NHL was assessed using International Prognostic Index (IPI) score. The 30 healthy volunteers employed in the present study were family members of the patients. The exclusion criteria for the present study included: i) History of other types of cancer; ii) pregnant or lactating female; iii) presence of metabolic diseases, including diabetes; iv) liver or urinary diseases; v) smoker or drinker; vi) use of specific drugs, including antibiotics, hormones and non-steroid anti-inflammatory drugs. A questionnaire was designed to investigate the dietary habits of the subjects, and no significant difference was observed among the groups. All subjects have signed an informed consent to participate in the present study. The present study was approved by the Ethics Committee of Anhui Medical University, and the use of human urine samples was in accordance with the Guidelines of the Declaration of Helsinki.

Materials and equipment. Methanol standards (purity ≥99.0%) and 30 quantitatively volatile metabolites (purity ≥95.0-99.5%) were purchased from 7 different chemical reagent companies (AladdinCo.,Ltd,Shanghai,China(http://www.aladdin-e.com); Energy-Chemical Co., Ltd., Shanghai, China (https://www.energy-chemical.com); Alfachina Co., Ltd., Shanghai, China (http://www.alfachina.cn); Brain Biotecnology Co., Ltd., Shanghai, China (http://www.brain-biot.com); Sinopharm Chemical Reagent Co., Ltd., Shanghai, China; Xibao Biotecnology Co., Ltd., Nanjing, China (http://seebio.biomart.cn); Zhongwei Chemical Co., Ltd, Beijing, China (http://www.wechem.cn). The pH values of samples were tested using a portable pH test pen (Hengaodeyiqiyibiao Co., Ltd., Beijing, China). The 30 ml sterile glass vials were purchased from Sanyuanhuaboyiqi Co., Ltd., (Hefei, China). The 20 ml GC vials that were capped with Teflon (PTFE) septa were obtained from (Shimadzu Corporation, Kyoto, Japan). SPME manual holder and the carboxen-polydimethylsiloxane (CAR-PDMS) fiber were produced from Sigma-Aldrich, (Merck KGaA, Darmstadt, Germany). GC-MS QP 2010 Plus equipped with HP-5MS column and NIST 05 library was obtained from Shimadzu Corporation. The HP-5MS column (size x I.D., 30 m x 0.25 mm; thickness, 0.25 μ.) was obtained from Agilent Technologies, Inc., (Santa Clara, CA, USA).

Sample collection and preparation. Each subject (either patient or healthy control) fasted and did not drink water overnight. The next day, the subject was requested to pass mid-stream morning urine into a 30 ml sterile glass vial, which was sealed thereafter. The samples were analyzed on the same day of sample collection (transported at 4˚C to the laboratory and analyzed within 1 h of collection). In the laboratory, 10 ml urine samples were transported into GC vials that were previously added with 3 g sodium chloride. Either 0.1 ml 5 mol/l HCl, 5 mol/l NaOH or ultra-pure water was added into the GC vials depending on the required final pH as described in a previous study (17). In this way, acids and sulfur compounds are better extracted at an acidic pH, whereas the extraction of alcohols and heterocyclic compounds in an alkaline environment is favored.

SPME procedure. The volatile metabolites in the headspace of urine were extracted using CAR-PDMS fiber as described in a previous study (18). Briefly, SPME fibers were pre-conditioned by inserting them into the GC injector port as recommended by the manufacturer (Sigma-Aldrich; Merck KGaA). The GC vials containing urine samples were sealed and placed in an oven (Shimadzu Corporation) at 40˚C for 30 min with vibration. Next, the vials were equilibrated in the same oven without vibration for 30 min. Following equilibration, static extraction in the headspace of urine was performed for 30 min using the CAR-PDMS fiber. At the end of the extraction time, the fiber was inserted into the GC injector port for the thermal desorption of volatile metabolites.

GC-MS analyses. The GC injector port temperature was 250˚C. The carrier gas used for GC was helium with a flow rate of 1 ml/min, and the splitless mode was used. The temperature profile of the column oven was set as follows: i) 35˚C for 2 min; ii) increase in temperature for 6˚C/min until 150˚C, iii) increase in temperature for 12˚C/min until 250˚C, and the temperature is maintained for 3 min. The ion source temperature of the mass spectrometer was 200˚C, and the detector operated in a mass range of 40-350 m/z. The chromatographic data sets were analyzed as described in previous studies by the present authors (19,20). Using the GC-MS Post Run software (GCMS Solution, Shimadzu Corporation), peak detection and volatile metabolite identification were carried out. The identification of each volatile metabolite was achieved by comparing the fragmentation patterns (i.e., presence and intensity of the signals) with those in the NIST 2005 library, and this was verified by evaluating the retention time using standard compounds, when available. The fragment ion m/z values of the identified volatile metabolites that have the highest abundance and

the matching percentages in the NIST library were assessed. Additionally, the occurrence rates of the volatile metabolites in urine samples were assessed.

Samples for quantitation of volatile metabolite and method validation. Calibration samples were prepared in 20 ml GC vials containing a set of six volumes of standard metabolites (i.e., 30 volatile metabolites that are present in 100% of the urine samples) prepared in methanol, 10 ml urine (from a healthy volunteer) and 3 g sodium chloride. These samples were used to establish the calibration curves in order to quantify the volatile metabolites in the urine samples. The calibration samples were not prepared by adding the metabolite standards into water due to the complexity of the components of human urine as the complex components of urine may have a notable effect on the quantification of target volatile metabolites. Either 0.1 ml 5 mol/l HCl, 5 mol/l NaOH or ultra-pure water was added into the GC vials containing calibration samples depending on the required final pH. The control samples were prepared with 10 ml urine and 3 g sodium chloride (NaCl). Similar to the preparation of the calibration samples, either 0.1 ml 5mol/l HCl, 5 mol/l NaOH or ultra-pure water was also added into control samples depending on the required final pH. Blank samples were prepared by adding 10 ml ultra-pure water into 20 ml GC vials (previously added with 3 g sodium chloride and either 0.1 ml l 5 mol/l HCl, 5 mol/l NaOH or ultra-pure water. Calibration samples were analyzed together with the control samples, for the quantification of volatile metabolites. The blank samples were adopted for the evaluation of contamination in the environment, accuracy, precision, detection and quantification limits.

Quantification of volatile metabolites and method validation. Calibration curves were established as described in a previous study (15). The chromatographic peak areas of target volatile metabolites in the calibration samples were subtracted with areas from the control samples. The subtraction is required due to the endogeneity of the volatile metabolites. Here, limit of detection (LOD) and limit of quantification (LOQ) of the detection method are defined as the concentration of the volatile metabolite that results in a noise ratio of 3 and 10, respectively. Due to the endogenous nature of the volatile metabolites, blank samples were used instead of control samples. The signal to noise ratios of the chromatographic peaks of the blank samples that were added with decreasing concentrations of volatile metabolites were measured to evaluate the LODs and LOQs of this detection method. The accuracy of the detection method was evaluated by the percentage of deviation between the calculated concentration value (from the calibration curve) and the actual value of standard added in the blank samples. The precision of the detection method was calculated as relative standard deviation (RSD) of the peak area values by detecting blank samples that were added with a standard of constant concentration at three different days (1st, 7 and 30th day; 3 times per day).

Statistical analyses. To avoid bias and over-fitting of the data, urine samples were coded prior to data acquisition and randomized using MATLAB (R2008a; MathWorks, MA, USA). The volatile metabolites that exhibited significant differences (P-value=0.05) among the groups were determined from the head-space SPME-GC-MS results using the SPSS 23.0 package for Windows (SPSS, Inc., Chicago, IL, USA) by the non-parametric Wilcoxon's test. Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic values of the potential biomarkers. Binary logistic regression analyses were performed to construct ROC curves of the combination of potential biomarkers. The concentrations of volatile biomarkers that exhibited statistically significant differences between the groups were included as variables for the ROC curves, and the groups were used as the dependent variables.

Results

Clinical characteristics of enrolled subjects. A total of 131 subjects were recruited in the present study. The subjects were divided into four groups. Subjects with aggressive B-NHL were included in the first group. A total of 35 patients with DLBCL were recruited in this group. The second group consisted of 33 subjects with indolent B-NHL (23 cases with low grade FL and 10 cases with MALT). The third group included 33 reactive hyperplastic lymphadenitis patients. The fourth group included 30 healthy controls. There was no difference in the distribution of age and sex among the groups (P>0.05). The details of the subjects are stated in Table I.

Qualitative and quantitative analyses of volatile metabolites. A total of 227 volatile metabolites were identified under acidic, basified and unmodified pH. The identified volatile metabolites included various chemical families: Aldehydes, ketones, acids, alcohols, benzene derivatives, phenols, esters, furan and sulfur-containing compounds. A total of 35 of the 227 volatile metabolites were present in 100% of the urine samples. A total of 125 volatile metabolites were detected in the acidic samples, and 22 of the metabolites were ubiquitous. A total of 102 metabolites were detected in the basified urine samples and 19 of the metabolites were present in 100% of the urine samples. A total of 85 volatile metabolites were detected in the unmodified pH urine samples, and 22 of the metabolites were ubiquitous. A total of 7 volatile metabolites were present independent of pH values. These 7 metabolites were acetone, 2-butanone, 4-heptanone, dimethyl disulfide, furan, 2-methylbutanal and 2-methylpyrazine.

The relative heights of the mass spectra of the ubiquitous volatile metabolites in the samples and NIST library were similar indicating the absence of disruptors. Out of the 35 ubiquitous volatile metabolites, 1 was excluded because of chromatographic column bleed. A total of 2 metabolites were excluded due to the unavailability of a high-purity calibration standard, and the other 2 metabolites were considered as environmental contamination as the abundances of these metabolites were similar in urine and blank samples. Therefore, 30 volatile metabolites were further analyzed (Table II).

Potential biomarkers and their diagnostic values for B-NHL. Various cross-comparisons between the groups

Table I. Clinical characteristics of enrolled subjects.

A, B-NHL

Characteristics	AB, (n=35)	IB, (n=33)
Age, years, mean, (range)	54.3 (37-71)	56.7 (40-78)
Sex		
Male	24	24
Female	11	9
CD20		
Positive (+)	20	12
Negative (-)	15	21
Stage		
Early (I-II)	27	22
Advanced (III-IV)	8	11
IPI score		
0-2	19	25
3-5	16	8

B, Non-lymphoma

Characteristics	BLD, (n=33)	HC, (n=30)
Age, years, mean, (range)	58.9 (40-75)	57.1 (38-76)
Sex		
Male	23	21
Female	10	9
CD20		
Positive (+)	-	-
Negative (-)	-	-
Stage		
Early (I-II)	-	-
Advanced (III-IV)	-	-
IPI score		
0-2	-	-
3-5	-	-

AB, aggressive B-NHL; B-NHL, B-cell non-Hodgkin's lymphoma; IB, indolent B-NHL; BLD, benign lymphatic diseases; HC, healthy control; IPI, International Prognostic Index.

were carried out as follows: i) B-NHL (aggressive B-NHL and indolent B-NHL; n=68) vs. non-lymphoma (benign lymphatic disease and healthy control; n=63); ii) B-NHL (n=68) vs. BLD (benign lymphatic disease; n=33); iii) B-NHL (n=68) vs. healthy control (n=30); iv) aggressive B-NHL (n=35) vs. indolent B-NHL (n=33); v) $CD20^+$ B-NHL (n=32) versus $CD20^-$ B-NHL (n=36); vi) early-stage B-NHL (n=49) vs. advanced B-NHL (n=19); (7) low IPI score (0-2) B-NHL (n=44) vs. high IPI score (3-5) B-NHL (n=24). The comparisons are indicated in Fig. 1.

A total of 3 volatile metabolites (4-heptanone, 2-methylpyrazine and 2-methylbutanal) were detected to significantly discriminate B-NHL from non-lymphoma. The mean concentrations of these metabolites ranged from 24.76 to 494.27 ng/ml in the four groups (Fig. 2 and Table III). The concentrations of the 3 volatile metabolites also were detected to be significantly different between patients with B-NHL and healthy controls (Table III). Notably, the concentration of 4-heptanone was significantly higher in patients with B-NHL compared with those with BLD (Table III). In addition, the concentrations of the 3 volatile metabolites (4-heptanone, 2-methylbutanal and decanoic acid) were significantly different between aggressive B-NHL and indolent B-NHL (Table III). The mean concentrations of the 3 volatile metabolites ranged from 45.50 to 494.27 ng/ml in the aggressive B-NHL and indolent B-NHL groups (Table III). Two volatile metabolites (4-heptanone and 2,6-dimethyl-7-octen-2-ol) were detected to able to significantly discriminate early-stage lymphoma from advanced lymphoma. A total of 2 volatile metabolites (2-methylbutanal and decanoic acid) were able to distinguish the patients with $CD20^-$ B-NHL and $CD20^+$ B-NHL with significantly higher concentration levels in patients with $CD20^-$ B-NHL (Table III). Additionally, 2-methylpyrazine was detected at higher concentration in patients with high IPI score B-NHL compared with those with low IPI score B-NHL (Table III).

The applicability of the potential biomarkers identified in the Wilcoxon's test was assessed with ROC curve (Fig. 3 and Table IV). The sensitivities of the potential biomarkers ranged from 62.9 to 97.1% and the specificities ranged from 55.6 to 91.0%. Their AUC values ranged from 0.634 to 0.901. In order to construct ROC curves of a combination of the different biomarkers, binary logistical regression analyses were performed. The AUC values ranged from 0.824 to 0.968. Therefore, diagnostic models that use a combination of different biomarkers may be preferable.

Linearity, LOD, LOQ precision and accuracy. The LOD, LOQ, linear coefficient (R^2) and linear range for each potential volatile biomarker are presented in Table V. For the identified volatile biomarkers, the LODs ranged from 0.8 to 4.8 ng/ml, and the LOQs ranged from 2.5 to 9.5 ng/ml, which demonstrated that the detection method was sensitive. The RSD values for intra-and inter-day precision were <5%, which demonstrated that the detection method was reliable. The accuracy of detection method was also evaluated, and this ranged from 97 to 108% (Table V).

Discussion

The majority of patients with B-NHL were diagnosed at an advanced stage resulting in poor prognoses because of being asymptomatic at early stages of the disease (21). For example, 70-75% of patients with DLBCL were diagnosed at an advanced stage (22), and only 25% of patients with FL were diagnosed at early stage (23). The patients with aggressive B-NHL that were diagnosed at early stages of the disease not only had improved prognosis compared with patients diagnosed at an advanced stage (5-year overall survival, 90 vs. <70%) but also had advantages of reduced dosages, toxicities of therapy and less economic burden (24-26). In the present study, the concentration levels of 3 volatile metabolites (4-heptanone, 2-methylpyrazine and 2-methylbutanal) in urine samples from

Table II. Ubiquitous volatile metabolites identified under acidic/basified/unmodified pH in the urine samples.

Name	CAS no.	Chemical group	m/z	Ubiquitous or not		
				Acidic	Basified	Unmodified
2-Butanone	78-93-3	Ketone	43	Y	Y	Y
4-Heptanone	123-19-3	Ketone	43	Y	Y	Y
Furan	110-00-9	Furan	68	Y	Y	Y
Acetone	67-64-1	Ketone	43	Y	Y	Y
2-Methylbutanal	96-17-3	Aldehyde	41	Y	Y	Y
2-Methylpyrazine	109-08-0	Pyrazine	94	Y	Y	Y
Dimethyl disulfide	624-92-0	Sulfide	94	Y	Y	Y
2,6-Dimethyl-7-octen-2-ol	17,042-16-9	Ketone	59	N	Y	Y
Decanoic acid	334-48-5	Acid	60	Y	N	Y
6-Methyl-3-heptanone	624-42-0	Ketone	57	N	Y	Y
Methylpropyldisulfide	2,179-60-4	Thioether	80	Y	N	Y
Phenol	108-95-2	Phenol	94	Y	N	N
Methylphenol	620-17-7	Phenol	107	Y	N	N
Benzaldehyde	100-52-7	Aldehyde	77	Y	N	Y
α-Calacorene	21,391-99-1	Alkene	157	Y	N	N
1,2-Dihydro-1,1,6-trimethylnaphthalene	30,364-38-6	Naphthaline	157	Y	N	N
2,5-Dimethylfuran	625-86-5	Furan	96	Y	N	N
Nonanal	124-19-6	Aldehyde	57	Y	N	Y
3-Nonen-2-one	14,309-57-0	Ketone	55	N	Y	Y
Furfural	98-01-1	Aldehyde	96	Y	N	Y
Hexanal	66-25-1	Aldehyde	44	Y	N	Y
Indole	120-72-9	Indole	117	N	Y	Y
Methanethiol	74-93-1	Alcohol	47	N	Y	Y
2-Methyl-3-phenyl-2-propenal	101-39-3	Aldehyde	145	Y	N	N
3-Methyl-1-butanol	123-51-3	Alcohol	55	N	Y	Y
2-Ethyl-5-methylfuran	1,703-52-2	Furan	95	N	Y	Y
6-Methyl-5-hepten-2-one	110-93-0	Ketone	43	N	Y	Y
2-Pentanone	107-87-9	Ketone	43	N	Y	N
3-Methyl-3-buten-2-one	814-78-8	Ketone	43	N	Y	N
1,2,4-Trimethylbenzene	95-63-6	Benzene	105	N	Y	N

CAS no., chemical abstracts service identifier.

patients with B-NHL were significantly higher compared with non-lymphoma subjects, and the concentration of 1 of the metabolites (4-heptanone) was significantly different between early and advanced stages of lymphoma, indicating that 4-heptanone may be a potentially useful biomarker for screening of B-NHL.

The prognosis of TL is quite poor. A retrospective study indicated a median overall survival (OS) of 1.2 years from the time of transformation (27). The misdiagnosis of TL delays opportunities for treatment. In the present study, 4-heptanone, 2-methylbutanal and decanoic acid were identified as novel volatile biomarkers with satisfactory accuracy to differentiate aggressive B-NHL from indolent B-NHL. Therefore, it is possible to monitor the transformation of indolent lymphoma using volatile biomarkers in urine.

In the present study, volatile metabolites in urine were identified to be capable of discerning the critical features of B-NHL, including $CD20^+/CD20^-$, early/advanced stage and low/high IPI scores. The sensitivities of the metabolites ranged from 68.4 to 78.0%, and the specificity values ranged from 69.4 to 91.0%. Currently, the determination of clinical stage of B-NHL is primarily based on expensive imaging methods, including CT, MRI, positron emission tomography-CT and invasive pathologic analysis of bone marrow samples. However, the detection of volatile metabolites in urine is cheap and noninvasive, which means a better method for determination of clinical stage of B-NHL. The present classification of CD20 status mainly relies on immunohistochemistry, which is subjective and requires a suitable quality of lymphoma tissue and qualified pathology physicians (28). Due to the lack of expertise in pathology, the false judgment of CD20 is encountered in hospitals (21). By contrast, volatile metabolites in urine, as an objective diagnostic method, may be a valuable method for improving the diagnostic accuracy

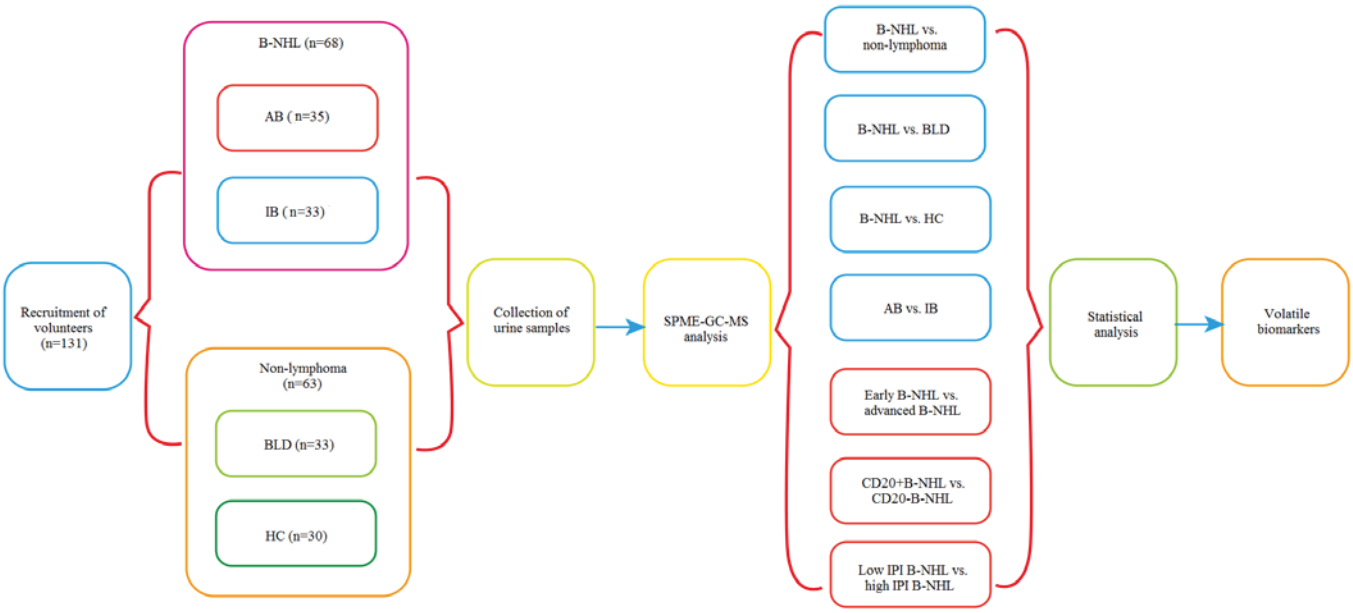

Figure 1. Flowchart of the study. B-NHL, B-cell non-Hodgkin's lymphoma; AB, aggressive B-NHL; IB, indolent B-NH; BLD, benign lymphatic diseases; HC, healthy control; IPI, International Prognostic Index.

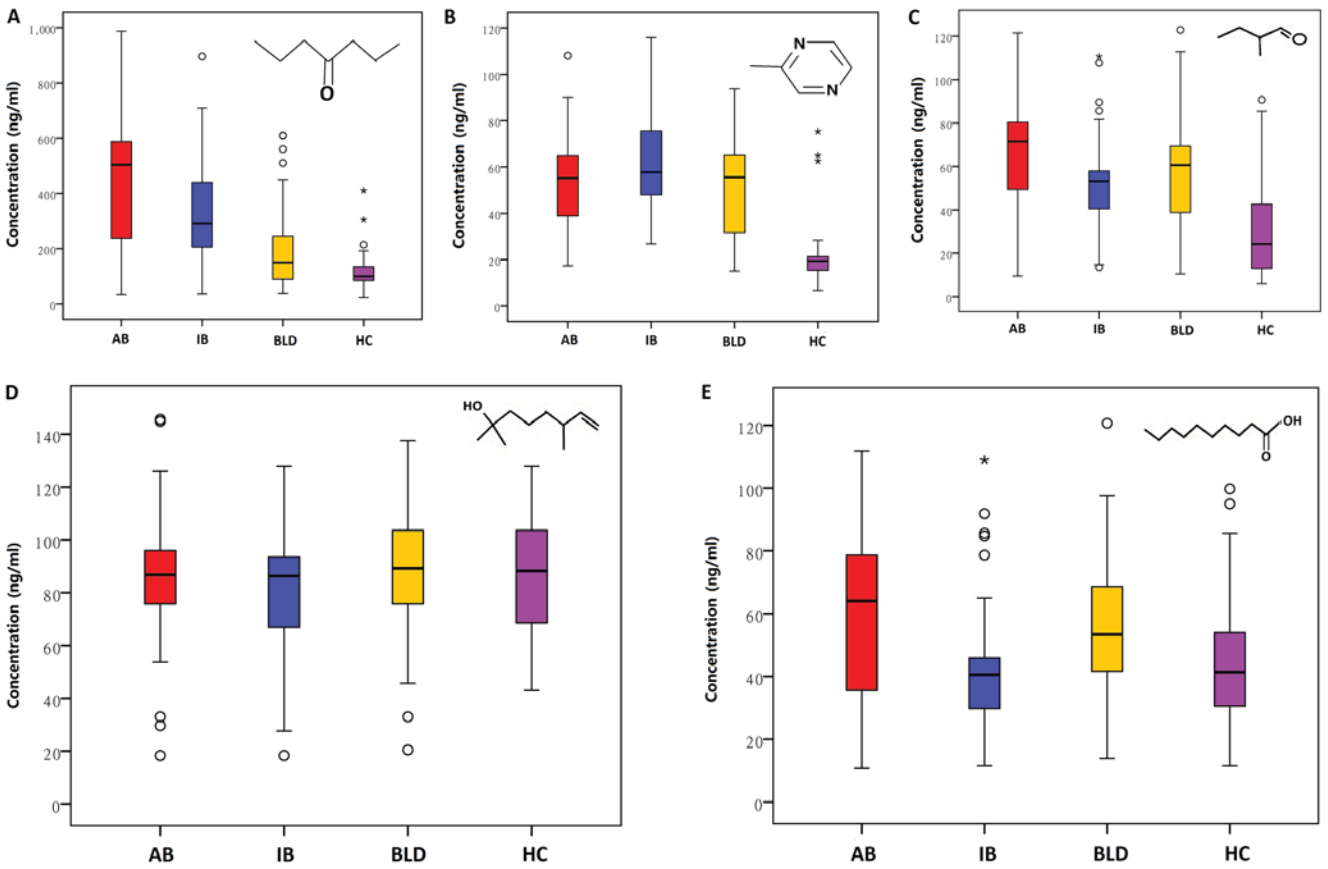

Figure 2. Levels of volatile biomarkers in urine among the groups. Box-whisker plots of the concentrations of (A) 4-heptanone, (B) 2-methylpyrazine, (C) 2-methylbutanal, (D) 2,6-dimethyl-7-octen-2-ol and (E) decanoic acid. The data are presented as the median value (black line), interquartile range (box), and 5th and 95th percentiles (whiskers). AB, aggressive B-NHL; IB, indolent B-NHL; BLD, benign lymphatic diseases; HC, healthy control. The circles represent values >1.5 times the amount of the interquartile value and ≤3 times the amount of the interquartile value. *Represent values >3 times the amount of the interquartile value.

of CD20 status. Moreover, different volatile metabolites were detected in in urine samples from B-NHL patients with low and high IPI scores, which indicate that volatile biomarkers in urine have the potential to predict the prognosis of B-NHL.

Table III. Identified VOCs with significant statistical differences between the study groups in urine samples from healthy volunteers, patients with B-NHL and patients with BLD.

	Concentration, M ± SD[a] (ng/ml)				P-value (Wilcoxon's non-parametric test)					
	B-NHL		Non-lymphoma							
Volatile metabolite	AB	IB	BLD	HC	B-NHL vs. non-lymphoma	B-NHL vs. BLD	B-NHL vs. HC	AB vs. IB	$CD20^+$ vs. CD20	Early-stage	Low IPI vs. high IPI
4-Heptanone	494.27±239.65	356.09±208.59	206.31±165.40	119.86±76.96	<0.001	0.002	<0.001	0.036	0.858	<0.001	0.864
2-Methylpyrazine	55.78±21.83	61.26±19.92	51.99±20.43	24.76±20.24	<0.001	0.238	<0.001	0.177	0.659	0.098	0.005
2-Methylbutanal	66.26±30.15	51.25±23.60	58.42±25.37	31.43±23.03	0.005	0.131	<0.001	0.029	<0.001	0.095	0.165
2,6-Dimethyl-7-octen-2-ol	94.56±40.18	82.02±30.63	92.92±35.64	87.91±22.48	0.428	0.680	0.978	0.334	0.798	0.003	0.218
Decanoic acid	60.34±26.31	45.50±22.21	58.22±24.82	45.98±21.40	0.127	0.447	0.06	0.017	<0.001	0.156	0.258

[a]The highest level of concentration was selected when volatile metabolite was detected in the same urine sample with different pHs. B-NHL, B-cell non-Hodgkin's lymphoma; IPI, International Prognostic Index; AB, aggressive B-NHL; IB, indolent B-NH; BLD, benign lymphatic diseases; HC, healthy control; M, mean; SD, standard deviation.

The latent volatile biomarkers may origin from a variety of endogenous biochemical pathways and exogenous sources (environmental pollutions, food, tobacco and alcohol) (29). For example, increased reactive oxygen species can attack the polyunsaturated fatty acids in the cell membranes and generate volatile organic compounds (VOCs), which result in a process known as oxidative stress (30). In the present study, the concentrations of 3 VOCs (4-heptanone, 2-methylpyrazine and 2-methylbutanal) were significantly different between the B-NHL group and healthy control group. However, only 1 VOC, 4-heptanone, was able to distinguish B-NHL from benign lymphatic disease. This result can be explained as benign lymphatic diseases have the level of oxidative stress similar to B-NHL. In terms of endogenous sources, it has been proposed that new VOCs can be produced or the levels of VOCs can change in pathological processes (31). The VOCs could be biomarkers for early diagnoses of malignancies based on the hypothesis that tumors have marked metabolic abnormalities even in the early stages (32). To reduce the confounders from exogenous contamination, smokers and drinkers were excluded in the present study. Furthermore, no significant differences in dietary habits of the volunteers were observed among the groups.

It has been reported that 4-heptanone is a β-oxidation product of 2-ethylhexanoic acid from plasticizers in a study on the *in vivo* metabolism of humans (33). Since plasticizers are considered as carcinogen (34), 4-heptanone can be an exogenous biomarker for B-NHL. Hanai *et al* (35) detected increased concentrations of 2-methylpyrazine in the urine of human lung tumor-bearing mice. Nevertheless, 2-methylpyrazine was not considered as a suitable candidate for lung cancer biomarker, since it was not likely to be released from lung cancer cell. A study by Calejo *et al* (36) revealed that 2-methylbutanal had a higher level of concentration in the urine samples of smokers compared with non-smokers. The authors suggested that 2-methylbutanal as a potentially useful biomarker to identify smoking habits. However in the present study, smokers were excluded, which indicates that there are different sources of 2-methylbutanal. Previous studies indicated that abnormal expression of 10-formyltetrahydrofolate dehydrogenase and alcohol dehydrogenase in cancer may increase aldehyde levels (37,38). However, the association between the enzymes and 2-methylbutanal has not been demonstrated. Another potential biomarker, 2,6-dimethyl-7-octen-2-ol, had been identified in urine samples from patients with prostate cancer, and the diagnostic value of 2,6-dimethyl-7-octen-2-ol was similar to PSA (39). Decanoic acid was detected in several types of fats and may be responsible for the mitochondrial proliferation associated with the ketogenic diet (40,41). To date, decanoic acid has not been reported to be a biomarker of cancer. In the present study, significant differences in the concentration of decanoic acid were detected between the different subtypes of B-NHL.

There are several limitations in the present study. Firstly, all the volunteers in the present study were non-smokers and non-drinkers from the same place and of the same ethnic origin. Therefore, diverse populations should be tested to evaluate the effects of these confounding factors in further studies. Secondly, the sample size of the present study is limited. In many other disease-screening studies (e.g., lung cancer) that asses the levels of volatile metabolites, subtypes of lung cancer are often pooled in one group due to the limitation of sample size (42-44). For

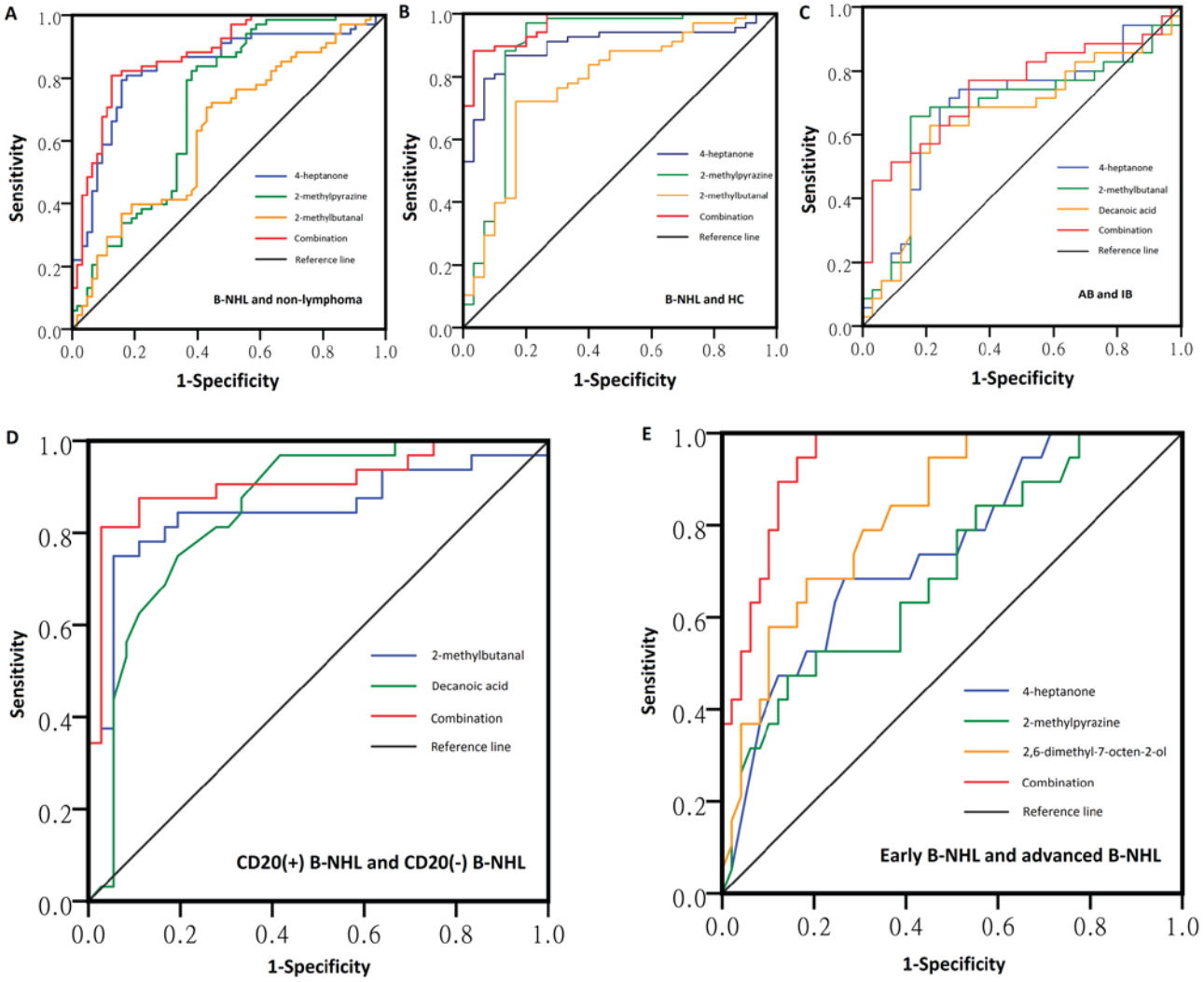

Figure 3. ROC curves of volatile biomarkers for the diagnosis of (A) B-NHL from non-lymphoma, (B) B-NHL from healthy control, (C) aggressive B-NHL from indolent B-NHL, (D) $CD20^+$ B-NHL from $CD20^-$ B-NHL, (E) early-stage B-NHL from advanced B-NHL using different combinations of 4-heptanone, 2-methylpyrazine, 2-methylbutanal, 2,6-dimethyl-7-octen-2-ol and decanoic acid. The AUC values are 0.878, 0.968, 0.824, 0.908 and 0.941, respectively. AUC, area under curve; B-NHL, B-cell non-Hodgkin's lymphoma; ROC, receiver operating characteristic.

the same reason, different subtypes of B-NHL were pooled to differentiate B-NHL from healthy control and to distinguish aggressive B-NHL from indolent B-NHL. Even biomarkers with high diagnostic values were found in our study, caution is needed to explain the results. Taken together, the present study indicated that volatile metabolites in urine might be potentially used as biomarkers for screening and monitoring of B-NHL. The possibility of volatile biomarkers in urine samples for the screening of different subtypes of B-NHL will be assessed in future studies with larger sample sizes.

Acknowledgements

The authors would like to thank the staff of Anhui Institute for Food and Drug Control for their technical assistance.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81472750), the Nature Science Research Project of Anhui province (grant no. 1508085MH171) and the Scientific Research Project for Young and Middle-aged Staff by Wannan Medical College (grant no. KY85880317).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QLH, LW and CL made substantial contributions to acquisition and analyses of data. They also drafted the manuscript and revised it critically for important intellectual content. LLH and YZZ were major contributors in method design and GC-MS operation. HL made substantial contributions to study conception and design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Anhui Medical University (no. 20140141), and the use of

Table IV. ROC curves of potential volatile biomarkers.

Volatile metabolite	Cut-off value	Sensitivity	Specificity	AUC	P-value	AUC 95% CI
4-Heptanone						
B-NHL vs. non-lymphoma	224.27	0.794	0.841	0.835	<0.001	0.763-0.907
B-NHL vs. BLD	198.02	0.809	0.758	0.775	<0.001	0.677-0.874
B-NHL vs. HC	144.87	0.868	0.867	0.901	<0.001	0.838-0.964
AB vs. IB	440.38	0.686	0.758	0.687	0.008	0.555-0.818
Early-stage vs. advanced	412.45	0.684	0.735	0.742	0.002	0.613-0.870
2-Methylpyrazine						
B-NHL vs. non-lymphoma	38.79	0.824	0.619	0.714	<0.001	0.623-0.805
B-NHL vs. HC	21.78	0.971	0.800	0.885	<0.001	0.792-0.978
Low IPI vs. high IPI	66.05	0.696	0.818	0.765	<0.001	0.634-0.897
2-Methylbutanal						
B-NHL vs. non-lymphoma	42.86	0.721	0.556	0.634	0.008	0.539-0.730
B-NHL vs. HC	42.86	0.721	0.833	0.777	<0.001	0.673-0.881
AB vs. IB	69.94	0.743	0.909	0.798	<0.001	0.678-0.919
$CD20^+$ vs. $CD20^-$	69.59	0.750	0.840	0.848	<0.001	0.746-0.950
2,6-Dimethyl-7-octen-2-ol						
Early-stage vs. advanced	333.77	0.737	0.694	0.734	0.003	0.608-0.860
Decanoic acid						
AB vs. IB	48.04	0.629	0.788	0.657	0.026	0.523-0.792
$CD20^+$ vs. $CD20^-$	40.70	0.780	0.910	0.851	<0.001	0.874-0.986
Combined ROC models						
B-NHL vs. non-lymphoma	-	-	-	0.878	<0.001	0.819-0.937
B-NHL vs. HC	-	-	-	0.968	<0.001	0.938-0.997
AB vs. IB	-	-	-	0.824	<0.001	0.806-0.912
$CD20^+$ vs. $CD20^-$	-	-	-	0.908	<0.001	0.830-0.986
Early-stage vs. advanced	-	-	-	0.941	<0.001	0.890-0.992

AUC, area under the curve; B-NHL, B-cell non-Hodgkin's lymphoma; AB, aggressive B-NHL; IB, indolent B-NH; BLD, benign lymphatic diseases; CI, confidence interval; HC, healthy control; IPI, International Prognostic Index; ROC, receiver operating characteristic.

Table V. Method validation of each potential biomarker (ng/ml).

Volatile metabolite	LOD	LOQ	Linear range	Coefficient (R^2)	Precision (RSD), %	Accuracy (%)
4-Heptanone	2.6	7.5	10-600	0.992	3.8	105
2-Methylpyrazine	0.9	2.5	5-100	0.995	2.5	108
2-Methylbutanal	1.5	2.8	5-150	0.999	1.8	99
2,6-Dimethyl-7-octen-2-ol	4.8	9.5	10-250	0.997	4.8	97
Decanoic acid	0.8	2.5	5-100	0.995	2.3	102

LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

human urine samples was in accordance with the Guidelines of the Declaration of Helsinki. All subjects provided informed consent to participate in the present study.

Consent for publication

No identifying information of the patients or volunteers were included in our manuscript.

Competing interests

The authors declare that they have no competing interests.

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