

GC-MS-based metabolomics approach to diagnose depression in hepatitis B virus-infected patients with middle or old age

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ABSTRACT

Depression is concomitantly presented in hepatitis B virus (HBV)-infected patients (HB). However, there is still no objective method to diagnose HBV-infected patients with depression (dHB). Therefore, in this study, a gas chromatography-mass spectrometry (GC-MS)-based metabolomic approach was employed to profile urine samples from 59 dHB and 52 HB (the training set) in order to identify urinary metabolite biomarkers for dHB. Then, 41 dHB and 35 HB (the testing set) were used to independently validate the diagnostic generalizability of these biomarkers. In total, 13 differential metabolites responsible for the discrimination between dHB and HB were identified. These differential urinary metabolites belonged mainly to Lipid metabolism and Amino acid metabolism. A panel consisting of six urinary metabolite biomarkers (ethanolamine, azelaic acid, histidine, threitol, 2,4-dihydropyrimidine and levulinic acid) was identified. This panel was capable of distinguishing dHB from HB with an area under the receiver operating characteristic curve (AUC) of 0.986 in the training set. Moreover, this panel could classify blinded samples from the testing set with an AUC of 0.933. These findings indicated that the GC-MS-based metabolomics approach could be a useful tool in the clinical diagnosis of dHB, and the identified biomarkers were helpful for future developing an objective diagnostic method for dHB.

INTRODUCTION

Although preventable with safe and effective vaccines, hepatitis B virus (HBV) infection is still a serious global health concern [1]. It is estimated that there are 400 million people with chronic hepatitis B worldwide, and thousands of patients succumb annually to end-stage liver cirrhosis and hepatocellular carcinoma [2]. Up to now, the available treatment methods could not completely eliminate the HBV from the body. The incurable nature of this disease often causes many negative emotions, such as depression and anxiety disorder. Moreover, the misunderstanding of the infectivity of HBV could increase the psychological pressure on HBV-infected patients, thus promoting the

development of mental disorders, such as depression [3].

Depression is a seriously debilitating mental disorder. The previous study reported that it affected up to 200 million people (approximately 3% of the world's population) in 2015 [4]. It could affect a person's behavior, thoughts and feelings [5, 6]. Gallegos-Orozco et al. reported that depression was prevalent in chronic HBV-infected patients [7]. Depression could decrease the quality of life and social activities in these patients, and it is also linked to a worse outcome in multiple medical disorders including viral illnesses [8]. What's more, depression could impinge on self-management ability and then reduce the patients compliance with

Table 1. Demographic and clinical characteristics of the included patients.

	Training set		P-value	Testing set		
	HB	dHB		HB	dHB	
Sample Size	52	59	-	35	41	
Age (years) ^a	45.7±14.5	45.0±17.1	0.82	45.6±15.2	50.6±16.1	0.17
Sex(F/M) ^b	28/24	28/31	0.50	16/19	23/18	0.37
HDRS ^a	1.7±1.0	23.9±3.9	<0.00001	1.4±1.2	23.4±3.7	<0.00001
BMI ^a	22.8±3.8	22.5±3.7	0.67	21.7±3.9	21.8±3.4	0.93

^aMean ±standard deviation, p-value was from student's t-test. ^b p-value was from Chi-square test. HB: hepatitis B virus infected patients without depression; dHB: hepatitis B virus infected patients with depression; F: female; M: male; BMI: Body Mass Index; HDRS: Hamilton depression rating scale.

prolonged therapeutic regimens [9]. However, the prevention and treatment of depression is often overlooked in HBV-infected patients.

Currently, the pathogenesis of depression is still unclear [10-12]. The psychiatrists still rely on the subjective identification of symptomatic clusters to diagnose depression. But, due to the highly heterogeneous clinical presentation of depression, this method results

in a considerable error rate [13]. Recently, metabolomics has been increasingly used to identify potential biomarkers for neuropsychiatric disorders [14, 15]. In our previous studies, using the nuclear magnetic resonance (NMR) spectroscopy-based metabolomic approach, we firstly observed the divergent urinary metabolic phenotypes between HBV-infected patients with depression (dHB) and HBV-infected patients without depression (HB), and successfully identified several potential biomarkers for dHB [16, 17]. However, considering that no single analytical technology could provide adequate coverage of the entire human metabolome in any biological samples [18, 19], it is important to use the complementary metabolomic platforms to identify novel biomarkers for dHB. Some studies have proved the especially valuable of this complementation for psychiatric disorders, such as BD and schizophrenia [14, 20]. Therefore, in this global metabolite profiling study, a gas chromatography-mass spectrometry (GC-MS)-based metabolomic platform was used to further study the divergent urinary metabolic phenotypes between dHB and HB, and identify some novel urinary metabolite biomarkers for future development of a urine-based diagnostic test for depression in HBV-infected patients.

RESULTS

Baseline data

In total, 87 HB and 100 demographically matched dHB were included in this study. There were 11 patients in the HB group and 16 patients in the dHB group receiving medicines for treating HBV, such as telbivudine, lamivudine, interferon and adefovir dipivoxil. According to the results of our previous metabolomic studies [16, 17], the statistical power could be up to 0.83 when the dHB group had 100 patients and HB group had 87 patients. Using the independent samples to validate the diagnostic performance of the identified panel was essential before it could be used clinically. Thus, the included patients

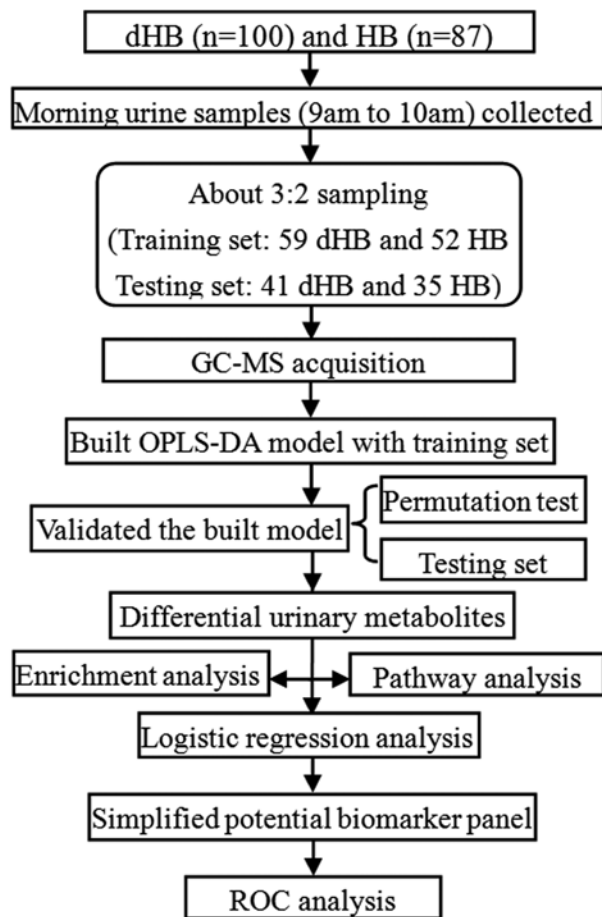


Figure 1. Flow chart of the study strategy in this work.

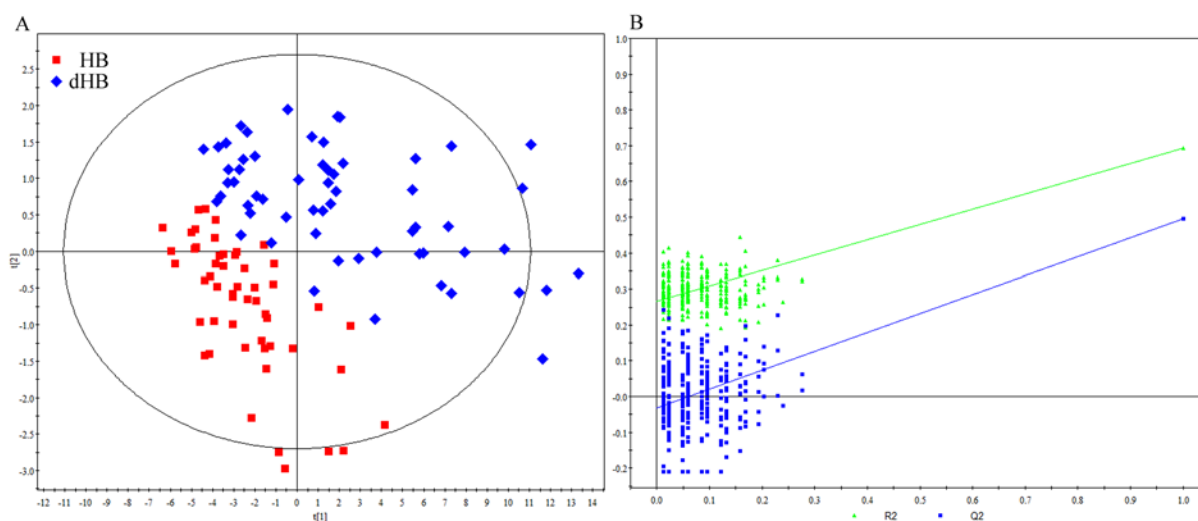


Figure 2. Metabonomic analysis of urine samples from HB and dHB. (A) OPLS-DA score plots showing an obvious separation between dHB (blue diamond) and HB (red square) in the training set; (B) 300-iteration permutation test showing the corresponding permuted values (bottom left) as significantly lower than original R^2 and Q^2 values (top right), demonstrating the OPLS-DA model's robustness.

were divided into training set and testing set. Finally, there were 52 HB (28 female and 24 male) and 59 dHB (28 female and 31 male) in the training set, and 35 HB (16 female and 19 male) and 41 dHB (23 female and 18 male) in the testing set. The training set was used to identify the biomarker panel, and the testing set was used to independently validate the diagnostic performance of the obtained panel. There were no significant differences in age, gender, or body mass index (BMI) between the two groups in both sets. The detailed information was described in Table 1. The flow chart of the study strategy was described in Figure 1.

OPLS-DA model

In total, 61 metabolites were measured. To explore the metabolic differences between HB and dHB, we used the training set to build the OPLS-DA model. As shown in Figure 2A, the score plots of the OPLS-DA model showed that the dHB were obviously separated from HB with little overlap ($R^2X=0.38$, $R^2Y=0.69$, $Q^2=0.50$), implying robust metabolic differences between HB and dHB. Moreover, as shown in Figure 2B, the results of permutation test suggested that the built OPLS-DA model was valid and not over-fitted. Meanwhile, the testing set was used to independently validate the reliability of the built model. As shown in Figure 3, the T-predicted scatter plot from the built OPLS-DA model showed that 31 of the 35 HB and 36 of the 41 dHB were correctly predicted, yielding a predictive accuracy of 88.2%. These results demonstrated that this OPLS-DA model built with urinary metabolites could be a potential method for objectively diagnosing dHB.

Moreover, we found that samples from non-medicated patients showed a similar metabolic phenotype to samples from medicated patients in both groups (see Supplementary file 1).

Differential urinary metabolites

By analyzing the OPLS-DA loadings plot, we found 13 differential urinary metabolites ($VIP>1.0$) responsible

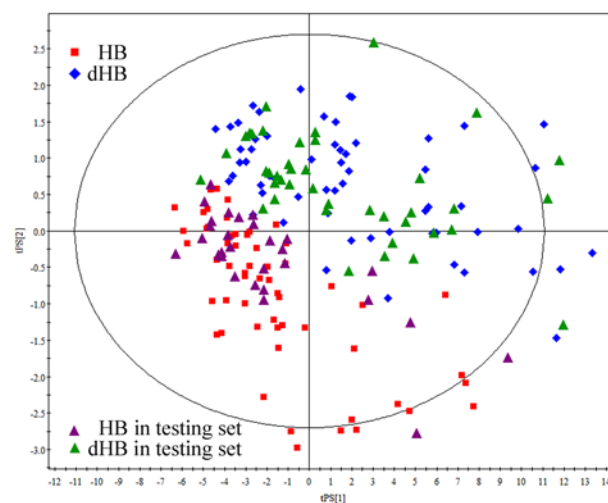


Figure 3. T-Predicted scatter plot from the OPLS-DA model built with HB (red square) and dHB (blue diamond) in the training set. The 31 of the 35 HB and 36 of the 41 dHB were successfully predicted by the OPLS-DA model with an accuracy of 88.2%.

Table 2. Differential urinary metabolites attributed to discriminating HB and dHB patients.

Metabolite	VIP	P-value ^a	P-adjusted ^c	Fold change ^c	Category
ethanolamine	2.67	0.24	0.45	-0.34	Lipid metabolism
azelaic acid	1.32	1.81E-07	1.17E-06	0.98	Oxidative stress
glyceric acid	1.09	0.06	0.14	0.50	Lipid metabolism
histidine	1.51	8.04E-07	2.6E-06	0.96	Amino acid metabolism
methylmalonic acid	1.72	0.57	0.62	-0.11	Amino acid metabolism
hippuric acid	1.20	0.27	0.45	0.32	Amino acid metabolism
2,4-dihydropyrimidine	1.69	0.51	0.66	-0.09	Amino acid metabolism
pyruvic acid	1.06	0.0002	0.0006	0.34	Energy Metabolism
acetic acid	1.40	0.31	0.45	0.20	Lipid metabolism
sucrose	1.24	0.86	0.86	0.11	Energy Metabolism
threitol	1.72	3.51E-07	1.5E-06	1.03	Lipid metabolism
aminomalonic acid	1.28	0.55	0.65	0.32	Amino acid metabolism
levulinic acid	1.94	1.05E-07	1.35E-06	1.09	Amino acid metabolism

^aP-values were derived from non-parametric Mann-Whitney U test. ^bP-values were corrected using Benjamini and Hochberg False Discovery Rate method. ^cPositive and negative values indicate higher and lower levels, respectively, in HB patients with depression.

for discriminating dHB from HB (Table 2). As compared to the HB, the dHB were characterized by higher levels of azelaic acid, glyceric acid, histidine, hippuric acid, pyruvic acid, acetic acid, sucrose, threitol, aminomalonic acid and levulinic acid, along with lower levels of ethanolamine, methylmalonic acid and 2,4-dihydropyrimidine. These differential urinary metabolites belonged to Lipid metabolism, Amino acid metabolism, Oxidative stress and Energy Metabolism. Meanwhile, to assess the correlations between the identified differential metabolites, the Pearson correlation coefficient was used. As shown in Figure 4, there were relatively moderate correlations between pyruvic acid and other metabolites.

Potential biomarker panel

It is not economical and convenient to simultaneously measure 13 metabolites to diagnose dHB. Therefore, these differential metabolites were used as variables to further conduct step-wise logistic regression analysis. We used the BIC rule to determine the minimum number of metabolites in the potential biomarker panel. The results showed that six metabolites (ethanolamine, azelaic acid, histidine, threitol, 2,4-dihydropyrimidine and levulinic acid) could describe the most significant deviations between dHB from HB. The relative concentration of these six metabolites was displayed in Figure 5. The non-parametric Mann-Whitney U test was used to explore the group differences on these metabolites. The panel consisting of these six metabolites could yield acceptably high accuracies of 94.6% and 86.8% in the training and testing set, respectively. The panel consisting of these six metabolites had the similar accuracy to the panel consisting of 13 differential metabolites. The discriminative model was: $P(Y=1) = 1/(1+e^{-y})$; $y = -40.213 * \text{ethanolamine} + 1518.415 * \text{azelaic acid} + 303.788 * \text{histidine} - 15.894 * \text{2,4-dihydropyrimidine} + 28.621 * \text{threitol} + 543.669 * \text{levulinic acid} - 8.966$. This model could be used to calculate the probability of depression in each HBV-infected patient.

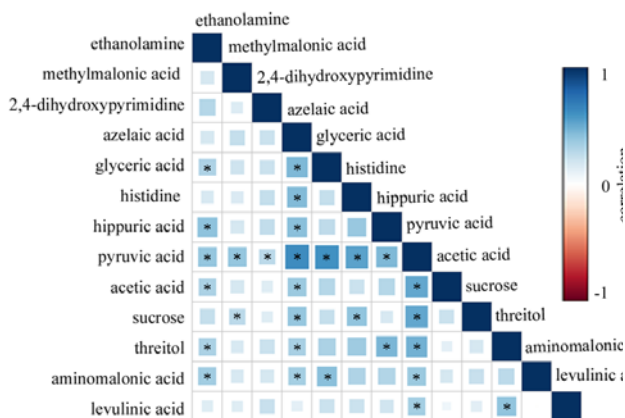


Figure 4. Pearson correlation analysis of differential urinary metabolites using R software.

Diagnostic performance

The ROC curve analysis was used to assess the diagnostic performance of this simplified panel. The areas under the ROC curve (AUC) was calculated to

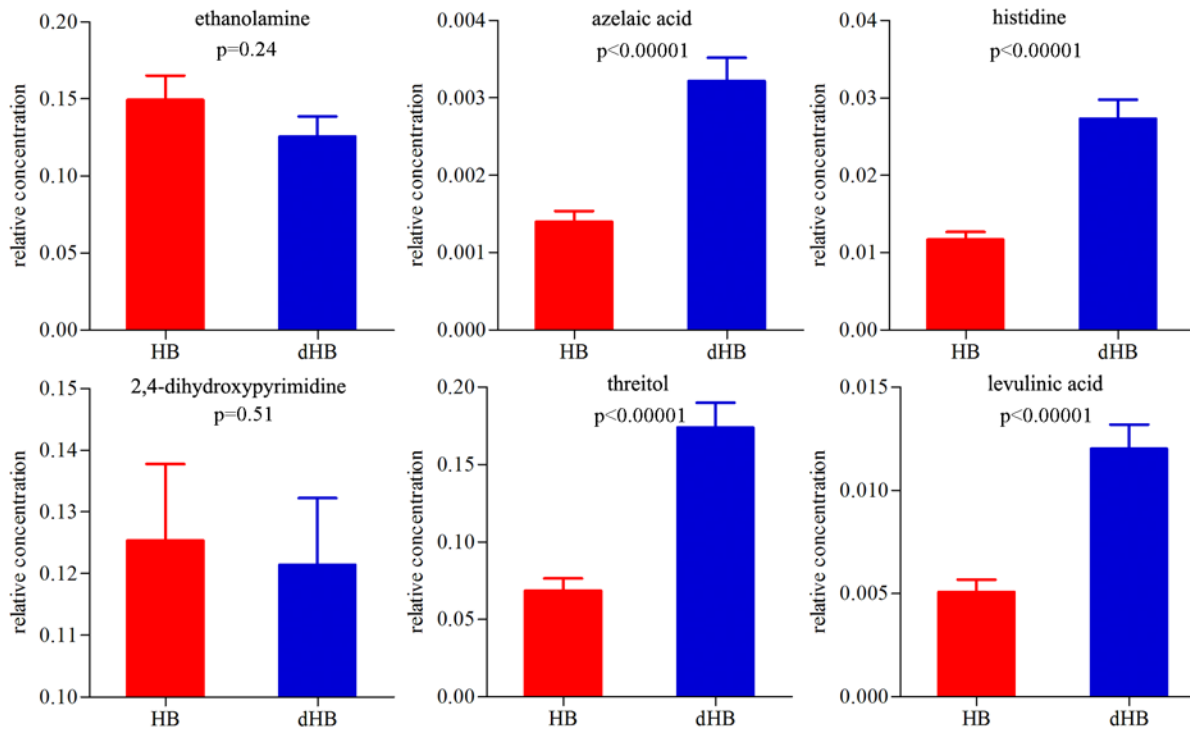


Figure 5. Relative concentrations of these six urinary metabolite biomarkers for dHB.

estimate how well dHB would be discriminated from HB (discriminating power). An AUC of 1.0 represented a perfect discrimination, and 0.5 referred to a case with no discrimination at all. The results showed that this simplified panel could effectively discriminate dHB from HB with an AUC of 0.986 (95% confidence interval (CI) = 0.971-1.00) in the training set (Figure 6). Furthermore, this simplified panel could classify blinded samples from the testing set with an AUC of 0.933 (95%CI=0.870-0.996) (Figure 6). Given the biological reproducibility observed in both sets, we used

all samples to conduct ROC analysis to increase the statistical power. The AUC was 0.970 (95%CI=0.948-0.992) in the whole set (Figure 6). These results showed that the diagnostic performance was similar between this simplified panel and the OPLS-DA model built with all the differential metabolites, suggesting the efficacy of this simplified panel in dHB detection. Meanwhile, we found that these six identified biomarkers had no sex specificity (see Supplementary file 1).

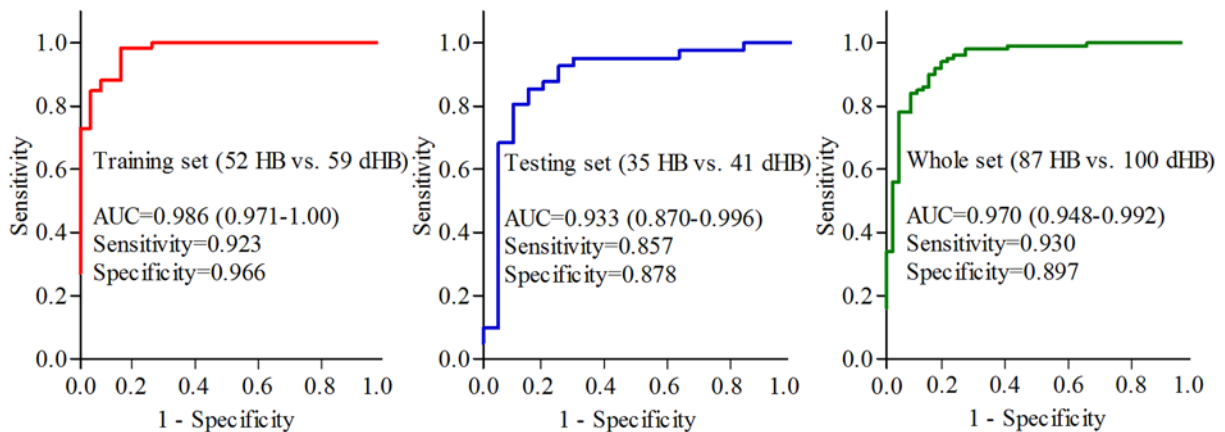


Figure 6. Diagnostic performance of the identified biomarker panel in training, testing and whole sets.

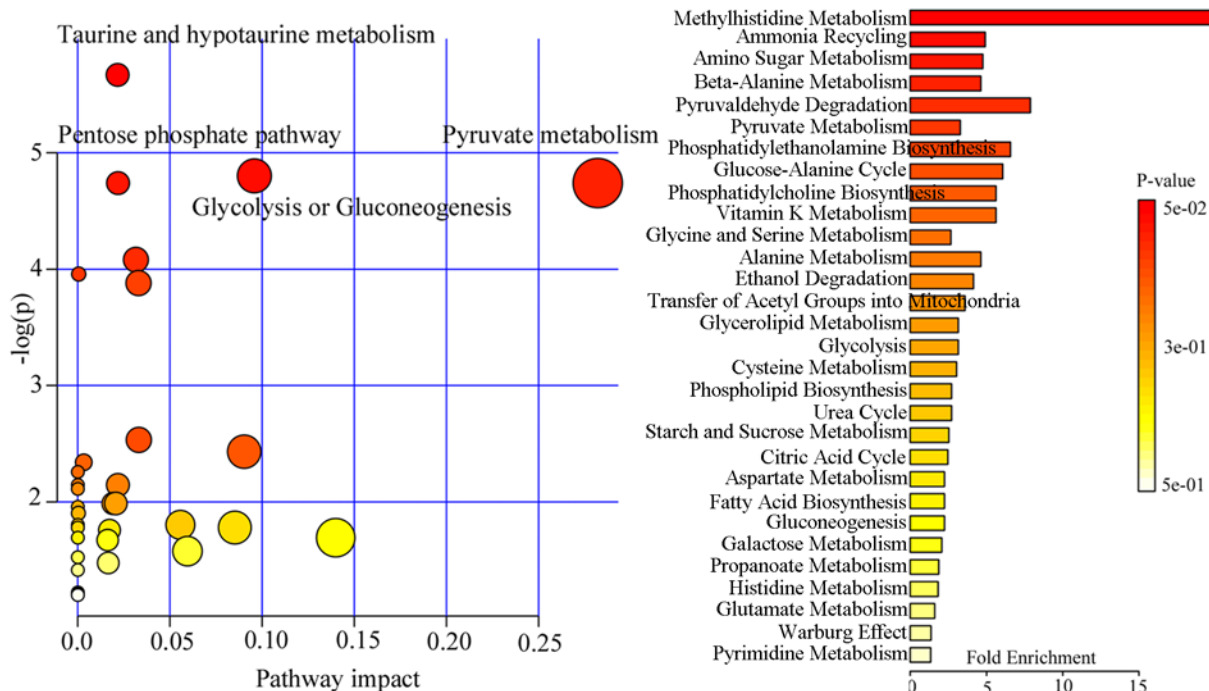


Figure 7. Affected metabolic pathways and functional enrichment analysis using 13 differential metabolites.

Subsequent analysis

The 13 differential metabolites were imported into MetaboAnalyst 3.0 to conduct pathway analysis and functional enrichment analysis. The pathway analysis showed that these metabolites were mainly involved in four metabolic pathways: (p -value <0.01 , impact >0): Taurine and hypotaurine metabolism ($p=0.004$, impact=0.021); Glycolysis or Gluconeogenesis ($p=0.009$, impact=0.096); Pentose phosphate pathway ($p=0.010$, impact=0.022); Pyruvate metabolism ($p=0.010$, impact=0.282) (Figure 7A). The functional enrichment analysis showed that the top 3 infected functions were methylhistidine metabolism, ammonia recycling and amino sugar metabolism (Figure 7B).

DISCUSSION

In this work, we used GC-MS-based metabolomics approach to further study the metabolic changes between dHB and HB. The OPLS-DA model showed that the dHB group could be obviously separated from the HB group. In total, we identified 13 differential urinary metabolites (VIP >1.0) responsible for the discrimination. These differential urinary metabolites belonged mainly to Lipid metabolism and Amino acid metabolism. Further analysis showed that six of the 13 differential metabolites (ethanolamine, azelaic acid, histidine, threitol, 2,4-dihydropyrimidine and levulinic acid) were defined as candidate diagnostic

biomarkers for dHB. The panel consisting of these six metabolites could yield an AUC of 0.986 in the training set and 0.933 in the testing set. Our findings further indicated that the urinary metabolite biomarkers could aid in the future development of objective diagnostic methods for dHB.

The levels of several differential metabolites were not found to be significantly changed (p -value <0.05) by the non-parametric Mann-Whitney U test (univariate analysis). However, the OPLS-DA model (multivariate analysis) still viewed these metabolites as differential metabolites responsible for the discrimination between the two groups. Previous metabolic studies also reported the similar results [27, 28]. This was because the multivariate analysis found the highest discrimination power after adding these metabolites into the discrimination model. These results demonstrated that the multivariate analysis had some advantages over univariate analysis in detecting the potential significance of subtle metabolic changes between the different groups [29].

Metabolomics has been extensively applied to capture the metabolic changes of diseases [30]. The metabolic phenotypes might be different in different disease states. Using GC-MS-based metabolomics approach to compare the urinary metabolic phenotypes of depressed patients and healthy controls, a previous study identified six potential urinary biomarkers (sorbitol, uric acid,

azelaic acid, quinolinic acid, hippuric acid, and tyrosine) for diagnosing depression [31]. In this study, the azelaic acid was also identified as biomarker for diagnosing dHB, and the hippuric acid was identified as differential metabolites. The other four metabolites were not identified as differential metabolites responsible for the discrimination between dHB and HB. Meanwhile, we used these six biomarkers to diagnose dHB in this study, and found that the diagnostic accuracy of these six biomarkers were inferior to the diagnostic accuracy of our panel (72.15% vs. 94.6%). These results suggested that the metabolic phenotypes were different between patients with only depression and dHB. Therefore, it might be inappropriate to use the previous panel to diagnosing depression in HBV-infected patients [31].

Previous study found that the decreased central energy production in depressed patients might be mirrored by the peripheral metabolic perturbations [27]. The significantly decreased level of plasma pyruvic acid (energy metabolism) was found in depressed patients [32]. Here, we found that there were relatively moderate correlations between pyruvic acid and other differential metabolites, and the Pyruvate metabolism was affected in dHB. HBV was frequently accompanied by many complications, such as hepatocellular carcinoma. Moreover, HBV was responsible for over half of hepatocellular carcinoma cases worldwide [33]. Another study reported that the pyruvic acid could be used for lipid synthesis, which was important for tumor cell proliferation and survival [34]. In addition, researchers found the correlation between lipid synthesis and glycolysis [35]. In this study, we found that the glycolysis was affected in dHB. Meanwhile, the Taurine and hypotaurine metabolism was found to be affected in dHB. And, the functional enrichment analysis found that some related functions of amino acid metabolism, such as methylhistidine metabolism, ammonia recycling and amino sugar metabolism, were affected in dHB. Taken together, these results indicated that the lipid metabolism and amino acid metabolism might be disturbed in depressed HBV-infected patients.

Several limitations existed in this study: 1) the number of recruited patients in both training set and testing set was relatively small; future large-scale studies were needed to validate our conclusions; 2) all patients were from the same place; then there might be ethno- and site-specific biases; Future studies with heterogeneous populations were required to conduct across different sites; 3) the diagnostic performance of this panel was confirmed solely by discriminating dHB from HB; Future work should study whether or not these biomarkers could be used to differentiate dHB from HBV-infected patients with other psychiatric disorders;

4) previous metabolic study reported that the medications might have a non-significant effect on the urinary metabolites [36]. Here, we also found the similar results. However, further studies should collect larger samples to determinate the potential influences of drugs on the urinary metabolites; 5) only urinary metabolites were studied here, future studies should collect other biological samples from patients, such as plasma and cerebrospinal fluid, to check whether these urinary biomarkers are physiologically relevant to disease pathogenesis and related to brain functionality; 6) we did not address whether the metabolic changes in urine was a consequence or a cause of depression in HBV-infected patients, which was needed future studies to find out; 7) except for well-matched demographic data, such as gender, age and BMI in the included patients, future studies should also consider the other possible confounding factors, such as smoking status, lifestyle and alcohol consumption; 8) there was lack of imaging and known assays for depression; 9) the number of differential metabolites might be not enough to obtain the robust results of pathway analysis and enrichment analysis, thus future studies were still needed to validate and support these results.

In conclusion, using GC-MS-based metabolomics approach to study the metabolic phenotypes of dHB, 13 differential metabolites responsible for separating dHB from HB were identified, which mainly belonged to Lipid metabolism and Amino acid metabolism. Moreover, a panel consisting of six differential metabolites (ethanolamine, azelaic acid, histidine, threitol, 2,4-dihydropyrimidine and levulinic acid) was identified, which displayed a good diagnostic performance (yielding an AUC of 0.986 in the training set and 0.933 in the testing set).

MATERIALS AND METHODS

Patients recruitment

This study was reviewed and approved by the Ethical Committee of Xinxiang Medical University, and the methods were strictly performed according to the approved guidelines and regulations. The dHB were included in this study if they met the following criteria: i) depression was diagnosed in a Structured Psychiatric Interview using DSM-IV-TR criteria; ii) the 17-item Hamilton Depression Rating Scale (HDRS) score, which was used to assess the symptom severity, was more than 17; iii) patients did not receive any antidepressant medications; iv) cirrhosis patients co-infected with human immunodeficiency virus or hepatitis C virus were not included; v) patients with other psychiatric disorders or any pre-existing bodily disorders were not included; vi) patients with alcohol or

illicit drug problems were not included; vii) patients older than 40 years of age (middle or old age) were included; and viii) pregnant women were also excluded. The HB met the last six criteria were recruited. All patients were recruited from the First Affiliated Hospital of Xinxiang Medical University, and provided the written informed consent before sample collection.

GC-MS acquisition

First, the included patients used the sterile cup to collect the morning urine samples (9am to 10am). Then, after quickly transferring the urine samples into the sterile tube, we sent the urine samples to our lab under low-temperature condition. After centrifugation (1500g x 10 minutes), the resulted supernatant was immediately divided into equal aliquots and stored at -80°C for subsequent analysis. Prior to GC-MS analysis, the following steps were completed: 1) mixed and vortexed 15 μl urine and 10 μl L-leucine-13C6 (0.02 mg/ml, internal standard solution); 2) added 15 μl urease into the mixed solution to degrade the urea at 37°C for 60 minutes; 3) extracted the mixture successively using 240 μl and 80 μl of ice-cold methanol; 4) vortexed the obtained mixture for 30 seconds and then conducted centrifugation (at 4°C , 14000 rpm x 5 minutes); 5) at room temperature, transferred 224 μl supernatant into a glass vial for vacuum drying; 6) derivatized the obtained dried metabolic extract using 30 μl methoxyamine (20 mg/ml) at 37°C for 90 minutes; 7) added 30 μl of N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane into the obtained dried metabolic extract; 8) heated the mixture at 70°C for 60 minutes to obtain trimethylsilyl derivatives; 9) after derivatization and cooling to room temperature, added 1.0 μl derivative into the GC-MS system. Meanwhile, the detailed information of quality control samples preparation was described in Supplementary file 1.

GC-MS analysis

The GC-MS analysis was performed mainly referring to the previous study [21]. Briefly, we injected 1 μL derivative solution into the Agilent 7980 GC system (Agilent Technologies Inc., USA). Then, we used the HP-5 MS fused silica capillary column (30 m \times 0.25 mm \times 0.25 μm , Agilent, USA) to do separation. The helium carrier gas was set a flow rate of 1 mL/minute. We set the injector temperature at 280°C ; the column temperature was initially set at 80°C (held for two minutes) and then equably (10 $^{\circ}\text{C}/\text{minute}$) increased to 320°C (held for six minutes). Subsequently, we introduced the column effluent into the ion source of an Agilent 5975 mass selective detector (Agilent Technologies Inc., USA). We set the MS quadrupole

temperature at 150°C and the ion source temperature at 230°C . Data acquisition was performed in the full scan mode from m/z 50 to 550. After the data was conversion into a NetCDF file format, we used TagFinder to process the GC-MS metabolite profiles [22]. This processing used deconvolution, alignment and data reduction to obtain a list of mass and retention time pairs with corresponding intensities for all detected peaks from each data file in the data set. The produced three-dimensional data set, including peak index (RT-m/z pair) and normalized peak area percentages, was imported into SIMCA-P 13.0 (Umetrics, Umeå, Sweden) for further analysis.

Metabolomic data analysis

If a variable had a nonzero measurement value in at least 80% of the variables within one of the two subsets, the variable was included in the data set; otherwise the variable was removed [23]. The levels of these eligible identified metabolites were normalized to creatinine, and then imported into the SIMCA-P 13.0 (Umetrics, Umeå, Sweden) to conduct metabolomic data analysis. The orthogonal partial least squares discriminant analysis (OPLS-DA) was used to visualize the discrimination between dHB and HB [24]. The three parameters ($R^2\text{X}$, $R^2\text{Y}$ and $Q^2\text{Y}$) were used to assess the quality of the built OPLS-DA model [24]. The former two parameters and last parameter were used to assess the goodness-of-fit and predictability of the model, respectively [24]. Meanwhile, a 300-iteration permutation test was further conducted to investigate whether the built model was over-fitted or not. If the values of R^2 and Q^2 from the original model were higher than their corresponding values from the permutation test, the built model was deemed valid and not over-fitted [24]. By analyzing the OPLS-DA loadings plot, the metabolites with variable importance plot (VIP) > 1 (equivalent to a p-value < 0.05) were viewed as the differential urinary metabolites contributing to sample discrimination [24].

Statistical analysis

The SPSS 19.0 was used to conduct the subsequent analysis. To obtain a simplified potential biomarker panel, the identified differential metabolites were used to conduct step-wise logistic regression analysis based on Bayesian Information Criterion (BIC). The receiver-operating characteristic (ROC) curve analysis was used to assess the diagnostic performance of this identified panel. The student's t-test, nonparametric Mann-Whitney U test and Chi-square test were performed when appropriate. The p-values were corrected using Benjamini and Hochberg False Discovery Rate method [25]. The Pearson correlation analysis was performed

in R software, using the corrplot package. The correlation analysis was used to assess the correlations between the metabolites. The online software MetaboAnalyst 3.0 was used to conduct pathway analysis and functional enrichment analysis [26]. All tests were two-sided, and a p-value<0.05 was considered to be statistically significant.

Abbreviations

Hepatitis B virus: HBV; HBV-infected patients with depression: dHB; HBV-infected patients without depression: HB; nuclear magnetic resonance: NMR; gas chromatography-mass spectrometry: GC-MS; orthogonal partial least squares discriminant analysis: OPLS-DA; receiver-operating characteristic: ROC; area under the receiver operating characteristic curve: AUC; Hamilton Depression Rating Scale: HDRS; bipolar disorder: BD; Bayesian Information Criterion: BIC.

AUTHOR CONTRIBUTIONS

LJH and XWS designed this study; LJH, XXW and YZ collected urine sample; LQ, FY and LLZ made the GC-MS analysis; LJH and XXW performed the data analysis; LJH and XWS wrote the original manuscript. All the authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPLEMENTARY MATERIAL

Please browse the links in Full Text version of this manuscript to see Data Sets.

Quality control samples

The 15 μ l urine from each samples were pooled and fully mixed. After centrifugation (1500g x 10 minutes), the resulted supernatant was immediately divided into equal aliquots (15 μ l) and stored at -80°C for subsequent analysis. In total, there were 18 quality control (QC) samples. We run the pooled QC samples alongside the sample runs for controlling batch and unwanted sources of variations. Continuous analysis of the samples was performed in a random sequence. One QC sample was set after ten experimental samples, which was used to monitor and evaluate the stability of the system and the reliability of the experimental data. The detected values of the QC samples were extracted based on the data detected by the mass spectrometry and the RSD was calculated to remove the molecules with RSD > 30%. The QC samples running alongside samples as PCA plots were showed in Figure S1A. No QC samples was 2SD from the mean (Figure S1B). These results indicated the stability of the system and the reliability of the experimental data.

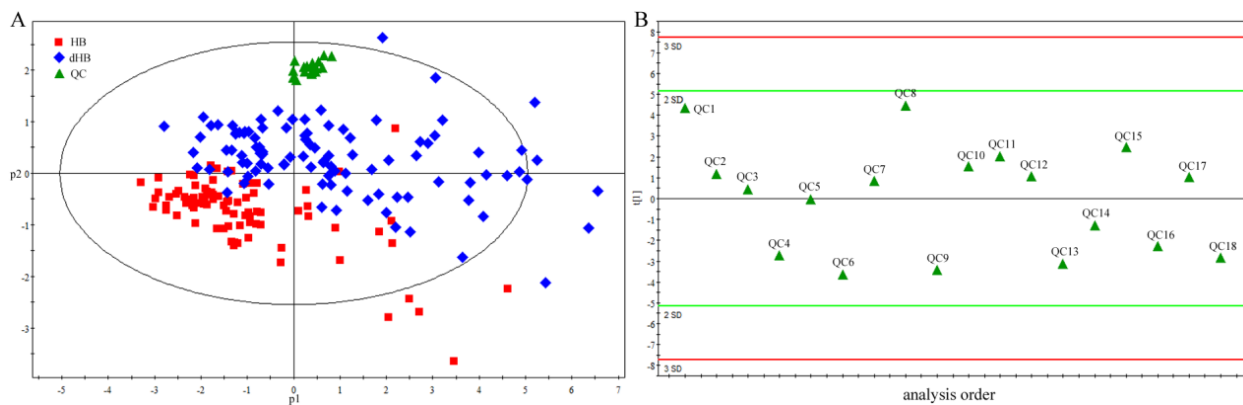


Figure S1. QC samples showed the stability of system and the reliability of the experimental data.

Medication effects on metabolites

All the included patients did not receive any antidepressant medications, but there were 11 patients in the HB group and 16 patients in the dHB group receiving medicines for treating HBV, such as telbivudine, lamivudine, interferon and adefovir dipivoxil. Previous metabolic study reported that the medications might have a non-significant effect on the urinary metabolites [1]. In this study, the OPLS-DA model built with non-medicated and medicated patients in HB group

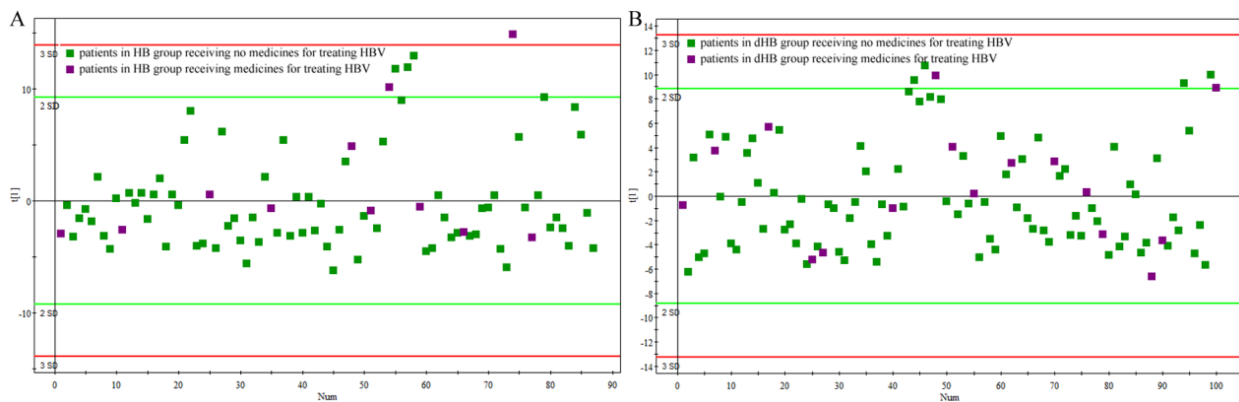


Figure S2. Non-medicated and medicated patients had similar metabolic phenotype in both groups.

showed that the non-medicated and medicated patients could not be well separated, which demonstrated that the non-medicated and medicated patients in HB group had the similar metabolic phenotype (Figure S2A). Meanwhile, the OPLS-DA model built with non-medicated and medicated patients in dHB group also showed the similar results (Figure S2B). These results indicated that the medicines for treating HBV might have little effect on the urinary metabolites. Limited by the small number of medicated samples, further studies should collect larger samples to determinate the potential influences of drugs on the urinary metabolites.

Sex-differences analysis

In order to check the sex-differences of urinary metabolites, we used the female and male patients in the HB group to build the OPLS-DA model (Figure S4A). The results showed that the female and male patients could not be well separated, which demonstrated that the female and male HB had the similar metabolic phenotype. Meanwhile, the OPLS-DA model built with female and male patients in the dHB group also showed the similar results (Figure S4B). Meanwhile, in this study, we found that these identified biomarkers had no sex specificity. The panel consisting of these biomarkers could effectively distinguish female dHB from female HB with AUC of 0.985 (95% confidence interval (CI): 0.968-1.00) (Figure S4A), and male dHB from male HB with AUC of 0.956 (95%CI: 0.914-0.998) (Figure S4B). These results demonstrated that there were no sex-differences in urinary metabolites in both HB and dHB groups.

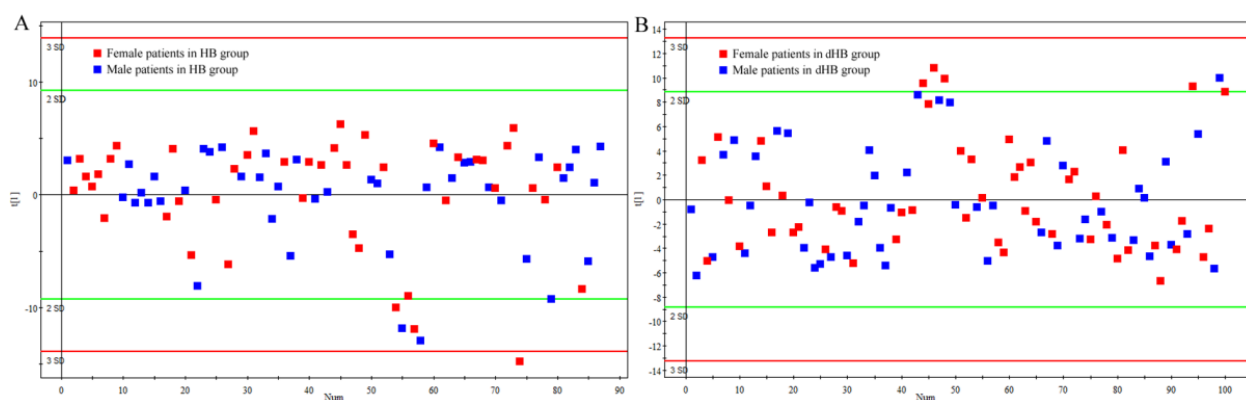


Figure S3. Female and male patients in both group had the similar metabolic phenotype.

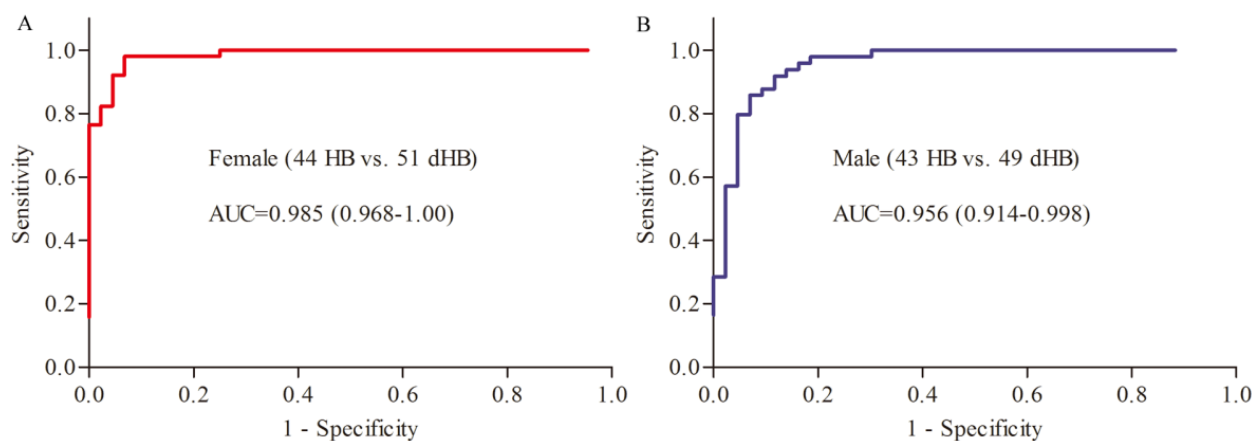


Figure S4. Diagnostic performances of the panel in diagnosing female and male dHB.

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