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Evaluation of characteristic metabolites of aromatic amino acids in patients with HIV infection at different stages of disease

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Abstract

Background: Acquired immune deficiency syndrome (AIDS), human immunodeficiency virus (HIV) infection, and antiretroviral therapy are usually associated with metabolic disorders. Screening for biomarkers to evaluate the progression of metabolic disorders is important for the diagnosis and treatment of HIV infection. This study aimed to establish and validate a method to quantify serum aromatic amino acid (AAA) metabolites as biomarkers of metabolic disorders in patients with HIV.

Methods: The AAAs and metabolites were analyzed using high-performance liquid chromatography–tandem mass spectrometry. Pearson's correlation, heatmap, and receiver operating characteristic curve analyses were used for statistical analysis.

Results: Under optimal detection conditions, the lower limits of phenylalanine (Phe), tryptophan (Trp), kynurenine (Kyn), tyrosine, phenylacetylglutamine (PAGln), and 5-hydroxytryptamine quantification reached 0.02, 0.02, 0.01, 0.02, 0.01, and 0.002 μg/ml, respectively, and the precision of intra- and inter-day was stay below 10.30%. Serum samples were stable for at least 6 months when stored at −80°C. The inter-group differences and associations between the biomarkers exhibited a particular volatility trend in PAGln, Trp, and Kyn metabolism in HIV-infected patients with metabolic syndrome.

Conclusions: The developed method can be used for rapid and sensitive quantification of the AAA metabolism profile in vivo to further appraise the process of HIV infection, evaluate intervening measures, conduct mechanistic investigations, and further study the utility of PAGln, a characteristic metabolite of AAA, as a biomarker of HIV infection coupled with metabolic syndrome.

KEYWORDS
aromatic amino acid metabolites, high-performance liquid chromatography–tandem mass spectrometry, human immunodeficiency virus, metabolic disorder, phenylacetylglutamine

Zhong-Wen Yuan and Hai-Ling Gan contributed equally to this work.

1 | INTRODUCTION

Metabolic disorders, characterized by insulin resistance (IR) and dyslipidemia, are common in patients with human immunodeficiency virus (HIV) infection. They are induced by the chronic inflammatory status of acquired immune deficiency syndrome (AIDS)/HIV and antiretroviral therapy (ART). Metabolic diseases, such as diabetes and cardiovascular diseases (CVDs), can significantly affect the lives of people with AIDS because diabetes and obesity are related to gut microbiota dysbiosis, which leads to gut microbiota metabolite changes. People with HIV infection are at a higher risk of developing CVD than those without it.[1] Therefore, research on the changes in microbial metabolites in these patients can facilitate the diagnosis and evaluation of their condition, adjustment of treatment, and improvement in the quality of their lives.

Gut microbiota plays a fundamental role in the metabolism of aromatic amino acids (AAAs) in the body. AAAs such as tryptophan (Trp), phenylalanine (Phe), and tyrosine (Tyr) are associated with IR and the host immunity.[2–4] Dysregulation of the Trp–kynurenine (Kyn) and Kyn–nicotinamide adenine dinucleotide metabolic pathways is one of the mechanisms of IR.[5] Kyn pathway metabolite levels are increased in patients with IR. Moreover, endogenous Trp metabolites (Kyn, 5-HT, and melatonin) and bacterial Trp metabolites (indole, indolic acid, skatole, and tryptamine) affect the gut microbial composition, microbial metabolism, and host immunity. Indole-3-acetic acid (IAA) was proved to be associated with the amelioration in IR, lipid metabolism, and oxidative and inflammatory stress.[6] Gut microbiome changes accompany the increase in indoleamine 2,3-dioxygenase 1 (IDO1)-dependent Trp metabolism and Kyn production, resulting in intestinal inflammation and IR.[7] Increase in serum Kyn levels correlates with the Kyn/Trp ratio in obesity, metabolic syndrome, body mass index (BMI), and blood triglycerides in patients with metabolic syndrome.[8]

Within a few weeks of HIV infection, the virus attacks the gut on a massive scale, depleting memory $CD4^+$ T cells and disrupting tight junctions in the intestinal epithelium, which may not be fully restored even after early ART. This gut destruction leads to an imbalance in the intestinal microbiota composition and microbial translocation, inducing chronic immune activation and inflammation.[9] The recovery of low $CD4^+$ T-cell counts in the peripheral blood is associated with an increase in abundance of *Enterobacteriaceae*. Numerous cross-sectional studies have indicated that the predominant gut microbiota shifts from *Bacteroides* to *Prevotella* after HIV infection.[10]

HIV infection upregulates IDO1 expression by increasing the levels of HIV-Tat, a pro-inflammatory mediator that promotes Trp degradation in the Kyn pathway and generates various intermediate metabolites, aggravating T-cell dysfunction and exhaustion, Treg differentiation, and dysbiosis of gut microbiota. Due to the critical role of Trp metabolism in HIV infection, estimating the levels of aberrant metabolites in these infectious diseases has important implications.[11]

Phe is metabolized into phenylacetic acid (oxidative pathway) and phenylpropionic acid (reductive pathway) by *Clostridium sporogenes*. Subsequently, these metabolites condense with glutamine or glycine to form phenylacetylglutamine (PAGln) or phenylacetylglycine, respectively. The enzymes involved in the reaction are encoded by *porA* or *fldH*.[12] PAGln mediates cellular events by signaling G protein-coupled receptors, including α2A, α2B, and β2-adrenergic receptors[13] and is thus a novel gut microbiota-dependent metabolite that promotes CVD. High serum PAGln levels are a strong and independent risk factor for overall mortality and CVD in patients with chronic kidney disease.[14] The correlation between HIV infection and serum PAGln levels has not yet been studied further.

The detection methods and selected AAA metabolite biomarkers in this study can be used for the clinical judgment and auxiliary diagnosis of HIV, including the diagnosis of metabolic syndrome, designing of the therapeutic schedule, and prognosis evaluation. Furthermore, the results provide a reliable experimental technology for basic and clinical research on HIV-related or other metabolic disorders. Additionally, this research provides a reference for experimental research focused on regulating lipid metabolism-related pathways in vivo and in vitro.

2 | MATERIAL AND METHODS

2.1 | Solvents and reagents

Phenylalanine (>99%, M0125A), Tryptophan (>99%, J1128AS), Kynurenine (>99%, MB5637), Tyrosine (>98%, O1204AS), and 5-Hydroxytryptamine (>98%, K1808035) were purchased from meilunbio; Phenylacetylglutamine (97.7%, 140,739–200,501) was purchased from NIFDC; L-phenylalanine-d5 (Phe-d5, SDHA-013) was purchased from CIL and used as an analytical internal standard (IS); High-performance liquid chromatography–grade methanol was purchased from Merck Millipore. Ultrapure water was obtained using a Merck Millipore water purification system.

2.2 | Samples

The project was approved by the Medical Ethics Committee of Guangzhou Eighth People's Hospital, Guangzhou Medical University (ethical approval batch number: 201913126). All participations gave written informed consent. Serum samples from HIV-infected patients were collected from the Department of Infectious Diseases (Guangzhou Eighth People's Hospital, Guangzhou Medical University), and serum samples of healthy subjects were donated by volunteers between August 2019 and February 2020.

Participants diagnosed as HIV-infected or AIDS patients according to China's AIDS Diagnosis and Treatment Guidelines (2018 edition) were divided into three groups. (I) HIV/AIDS with incipient (HIV-I, $n = 16$): patients were diagnosed with HIV/AIDS in the past month, not yet started ART; (II) HIV/AIDS patients with normal metabolism (HIV-N, $n = 30$, normal fasting blood glucose and lipid levels) and treated with two nucleoside reverse transcriptase inhibitors

(NRTIs) + efavirenz (EFV) for at least 1 year; (III) HIV/AIDS patients with hyperglycemia and/or hyperlipidemia (HIV-P, $n = 62$) and treated with two NRTIs+EFV for at least 1 year. Healthy participants ($n = 40$) with normal glucose and lipid metabolism and no medication history in for 1 month were selected as the normal control group. The demographics and baseline clinical characteristics were shown in Table 1. Fasting peripheral blood was collected and centrifuged for 15 min at 2000 × *g*. Serum samples were stored at −80°C until analysis.

2.3 | Standard solutions

Stock solutions of Phe, Trp, Kyn, Tyr, PAGln, and 5-HT were made up to 5 mg/ml in 80% methanol at the appropriate pH and stored in brown vials at −40°C. Calibrators of Phe, Trp, Kyn, Tyr, PAGln, and 5-HT were prepared by continuous dilution of the corresponding stock solutions with 10% methanol. A Phe-d5 internal standard (IS) stock solution at 100 ng/ml was prepared with methanol and stored at −20°C.

2.4 | Sample preparation for HPLC–MS/MS

Serum samples (50 μl) were prepared with 200 μl methanol (−20°C, containing 100 ng/ml Phe-d5) for protein precipitation. The samples were vortexed for 1 min, and subsequently centrifugated (CF1524R, SCILDGEX, Connecticut) at 20000 × *g* for 15 min at 4°C, and the supernatant (200 μl) was dried at 40°C with a stream of N_2. The residue was reconstituted of 100 μl of 10% methanol and centrifuged at 20000 × g for 15 min at 4°C after vortex for 1 min. Eighty microliters of supernatant were transferred to vials for HPLC–MS/MS analysis.

2.5 | HPLC–MS/MS conditions

Biomarkers were analyzed on a Sciex 3200 MD LC–MS/MS system (AB Sciex Pte. Ltd.) using a C18 analytical column (Waters UPLC® BEH C18 column, 1.7 μm, 2.1 × 50 mm). The mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic

TABLE 1 Summary of demographics and baseline clinical characteristics.

	Normal ($n = 40$)	H-I ($n = 16$)	H-C ($n = 30$)	H-P ($n = 62$)
Number	40	16	30	62
Gender				
Male	28	16	27	57
Female	12	0	3	5
Age (Years)	32.53 ± 5.51	27.5 ± 10.86	37.63 ± 9.23	40.85 ± 11.28
BMI (kg/m^2)	22.38 ± 2.50	21.74 ± 2.08	20.37 ± 1.63	22.38 ± 2.5
CD4$^+$cell count (cells/mm^3)	–	330.25 ± 145.23	459.1 ± 153.15	523.5 ± 199.01
HIV-RNA Level , (Copies/ml)	–	<20	<20	<20
Past history				
Hypertension (*n*)	0	1	1	1
Fatty liver (*n*)	0	2	3	3
Gout (*n*)	0	0	1	2
HCV Infection (*n*)	0	1	0	1
Diabetes family history (*n*)	0	0	0	1
HAART	–	–	TDF + 3TC + EFV($n = 29$) AZT/3TC + EFV ($n = 1$)	TDF + 3TC + EFV($n = 56$) AZT/3TC + EFV ($n = 6$)
Administration time (Month)	–	–	45.63 ± 20.44	45.53 ± 22.78
ALT (U/L)	16.22 ± 7.91	15.49 ± 6.00	27.95 ± 13.27	42.17 ± 33.23
AST (U/L)	17.61 ± 3.41	17.51 ± 3.57	23.16 ± 6.52	32.24 ± 28.96
FPG (mmol/L)	4.83 ± 0.63	5.00 ± 0.40	5.50 ± 0.42**	7.48 ± 2.20***$^{\#\#\#}$
TC (mmol/L)	4.13 ± 0.69	3.95 ± 1.04	4.09 ± 0.5	5.15 ± 0.96***$^{\#\#\#}$
HDL-C (mmol/L)	1.39 ± 0.28	1.06 ± 0.24	1.34 ± 0.27	1.21 ± 0.30
LDL-C (mmol/L)	3.00 ± 0.79	2.69 ± 1.05	2.63 ± 0.38	3.34 ± 0.88
TG (mmol/L)	1.01 ± 0.46	1.10 ± 0.43	1.02 ± 0.32	2.47 ± 1.76***$^{\#\#}$
UA (mmol/L)	361.42 ± 86.09	399.18 ± 56.66	331.12 ± 74.41	376.85 ± 108.33
FINS (μU/ml)	6.17 ± 1.89	6.04 ± 2.86	5.77 ± 1.95	9.99 ± 4.39***$^{\#\#}$
HOMA-β	109.62 ± 44.8	88.63 ± 50.77	59.85 ± 22.03***	57.59 ± 25.54***
HOMA-IR	1.34 ± 0.59	1.32 ± 0.6	1.42 ± 0.53	3.49 ± 2.37***$^{\#\#\#}$

Note: Versus normal group: ***$p < 0.001$; versus H-I group: $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

acid in methanol (solvent B) was supplied according to the following program: 0–2 min (5% B); 2–5.5 min (5%–40% B); 5.5–6.5 min (40%–90% B); 6.5–7.5 min (90%–80% B); 7.5–12 min (80%–5% B); 12–15 min (5% B). The injection volume was 10 μl and the flow rate equaled 0.4 ml/min. The mass spectrometry characteristics of AAAs were shown in Table 2. The following MS parameters were used: curtain gas, 35 psi; ion spray voltage, 4500 V; temperature, 430°C; ion source gas, 1 = 45 psi; ion source gas, 2 = 45 psi; interface heater, on; and collision gas, medium. The multiple reaction-monitoring parameters are shown in Table 1. All HPLC–MS/MS data were obtained using the Analyst Software (v1.6.2).

2.6 | Assay validation

The evaluation of the calibration curve, recovery, matrix effect, accuracy and precision, and stability were performed according to the "Guidance for Industry – Bioanalysis Method Validation' (Food and Drug Administration, May 2018)" "USP 1010 Analytical Data-Interpretation and treatment" and "AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals" using phosphate buffer saline (PBS).[15–18] An AAA-free serum matrix was not available, we have tried used 5% bovine serum albumin in phosphate-buffered saline as a blank matrix, but the level of Phe, Trp, and Tyr in the system were still high (The peak area counts exceeds 10^5). Therefore, the PBS was used as an alternative matrix for method validation (Precision, accuracy, stability, repeatability, and recovery).

Six fresh quality control (QC) samples at four concentrations (lower limit of quantification [LLOQ], low, medium, and high) and a set of calibration samples (seven samples) were included in each run. Precision, accuracy, and benchtop stability were evaluated using standard samples maintained at 4 or 25°C for 12 h. Autosampler stability was tested after storing the processed samples in HPLC autosampler vials at 4°C for 24 h. Long-term stability was evaluated at −80°C for 6 months, Three freeze–thaw cycles was carried out at −40°C and 25°C. Fifty-six degree Celsius stability was evaluated at 56°C for 30 min (heating serum samples at 56°C for 30 min is typically used to inactivate HIV prior to sample processing). Repeatability, recovery were assessed using QC samples at four concentrations. Furthermore, matrix effect of normal serum sample, hemolysis sample, and hyperlipidemia sample (Serum respectively from 3 different donors were mixed and prepared, 6 replicates) were also investigated in the same procedure, and the residual effect was investigated use the high concentration sample of QC.

2.7 | Data analysis

Data are expressed as mean ± SD. Graphical, Brown-Forsythe and Welch analysis of variance (ANOVA) tests were performed using GraphPad Prism 8.3 (GraphPad Software, Inc.). Pearson's correlation, heatmap, and receiver operating characteristics (ROC) analysis were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/faces/home.xhtml, Xia Lab @ McGill). Principal component analysis (PCA) was carried out using SIMCA 14.1 (Umetrics AB), and p value (Brown-Forsythe and Welch ANOVA tests) <0.05 was considered as statistically significant differences.

3 | RESULTS

3.1 | Assay validation

3.1.1 | Selectivity and calibration curves

Selectivity was assessed used QC standard samples and no interference was observed in the chromatograms of target retention times. The LLOQs of Phe, Trp, Kyn, Tyr, PAGln, and 5-HT in the human serum were 0.02, 0.02, 0.01, 0.02, 0.01, and 0.002 μg/ml, respectively. The correlation coefficients of the calibration curves exceeded 0.999, proclaiming an excellent linearity over the concentration range employed. The regression equations of the calibration curve and the corresponding correlation coefficients are presented in Table 3.

3.1.2 | Precision, accuracy, recovery, repeatability

The precision and accuracy of the within- and between-run assay variations in the PBS are shown in Table 4. The accuracy varied between 92.36% and 103.94% (within-run), and 92.52% and

TABLE 2 Mass spectrometry characteristics of AAAs.

	Q1 Mass (Da)	Q3 Mass (Da)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Phe-D5 (IS)	171.1	125.2	16.51	10.47	19.14	3.01
Phenylalanine	166.1	120.1	39.26	3.74	19.12	2.53
Tryptophan	205.2	188.1	38.16	3.12	15.78	3.25
Kynurenine	209.1	192.1	37.55	3.04	15.89	3.25
Tyrosine	182.1	136.2	32.4	4.22	16.94	2.46
Phenylacetylglutamine	265.3	130.0	44.05	4.44	18.03	2.41
5-hydroxytryptamine	177.1	160.1	29.85	4.18	14.05	3.87

TABLE 3 Calibration parameters of analytes.

	Standard curve	R^2	Range
Phenylalanine	y = 1.6016x + 0.0086	0.9998	0.02–20
Tryptophan	y = 0.609x + 0.0726	0.9995	0.02–20
Kynurenine	y = 0.1674x + 0.0083	0.9994	0.01–10
Tyrosine	y = 0.1646x + 0.006	0.9998	0.02–20
Phenylacetylglutamine	y = 0.5235x − 0.0054	0.9997	0.01–10
5-hydroxytryptamine	y = 0.6977x + 0.0017	0.9999	0.002–2

TABLE 4 Precision and accuracy for AAA analysis in PBS ($n = 3$).

	Conc	Intra-day		Inter-day	
	(μg/ml)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Phenylalanine	0.02	97.76 ± 4.77	4.88	98.29 ± 7.11	7.23
	2.50	95.87 ± 5.61	5.86	95.16 ± 5.75	6.05
	5.00	97.09 ± 3.12	3.22	99.42 ± 4.55	4.58
	10.00	98.30 ± 4.46	4.53	96.89 ± 5.73	5.92
Tryptophan	0.02	95.78 ± 8.56	8.93	96.30 ± 7.77	8.07
	2.50	97.03 ± 6.10	6.29	96.10 ± 6.49	6.75
	5.00	98.17 ± 4.90	4.99	96.29 ± 4.92	5.11
	10.00	97.76 ± 3.64	3.73	97.39 ± 4.64	4.76
Kynurenine	0.010	94.07 ± 6.45	6.85	92.91 ± 7.19	7.74
	0.125	99.52 ± 8.05	8.08	97.98 ± 7.36	7.51
	0.250	99.68 ± 5.08	5.09	97.63 ± 6.06	6.21
	0.500	95.78 ± 5.01	5.23	97.77 ± 4.44	4.55
Tyrosine	0.02	99.11 ± 4.72	4.76	97.95 ± 4.37	4.46
	2.50	98.08 ± 4.45	4.54	97.25 ± 4.29	4.41
	5.00	96.19 ± 5.29	5.50	98.20 ± 6.02	6.13
	10.00	98.98 ± 3.06	3.10	97.71 ± 3.25	3.33
Phenylacetylglutamine	0.010	92.36 ± 7.67	8.31	96.18 ± 9.31	9.68
	0.125	99.43 ± 3.39	3.41	98.82 ± 5.21	5.27
	0.250	93.70 ± 5.65	6.03	96.36 ± 6.12	6.35
	0.500	101.15 ± 1.64	1.62	100.56 ± 5.72	5.68
5-hydroxytryptamine	0.002	98.10 ± 10.10	10.30	94.48 ± 8.22	8.70
	0.025	103.94 ± 7.02	6.76	103.33 ± 5.75	5.56
	0.050	100.84 ± 4.68	4.64	99.18 ± 3.98	4.02
	0.100	93.42 ± 3.40	3.64	92.52 ± 2.65	2.87

103.33% (between-run). The relative standard deviations (RSDs) of within- and between-run precision were below 10.30% and 9.68%, respectively.

According to the guiding principles of USP 1010 Analytical Data-Interpretation and Treatment and AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals, the evaluation requirements for repeatability were different for different determined compounds, and all RSDs (Between 1.87% and 9.96%) were lower than the corresponding standard request (Table 5). The recovery of six endogenous metabolites was between 91.99% and 105.41%, and all RSDs were below 10% (Table 5).

3.1.3 | Matrix effect and residual effect

The IS-normalized matrix effects of six endogenous metabolites were between 86.58% and 112.91%, and all RSDs were below 15% (Table 6). More importantly, there is no matrix effect for hemolysis and hyperlipidemia sample. The residual effect of Phe, Trp, Kyn, Tyr, PAGln, and 5-HT in the blank sample after the QCH sample were 4.35 ± 0.68%, 7.61 ± 1.68%, 6.80 ± 2.43%, 8.29 ± 1.34%, 2.43 ± 0.72%, 3.73 ± 0.69%, respectively; not more than 20% of the LOQ samples. The residual effect of the internal standard (Phe-d5) was 0.05% and much <5%. The results of residual effect were meeting the requirements of methodological investigation.

TABLE 5 Recovery and repeatability for AAA analysis in PBS ($n = 3$).

	Conc. (μg/ml)	Recovery (%, x ± SD)	Recovery (RSD%)	Repeatability (RSD%)
Phenylalanine	0.02	100.26 ± 5.4	5.38	7.53
	2.50	103.70 ± 6.06	5.84	2.50
	5.00	91.99 ± 4.82	5.24	3.50
	10.00	103.21 ± 6.46	6.25	2.69
Tryptophan	0.02	105.41 ± 6.49	6.16	6.39
	2.50	97.63 ± 6.77	6.94	2.91
	5.00	99.77 ± 3.16	3.16	3.12
	10.00	97.85 ± 3.65	3.73	1.87
Kynurenine	0.010	94.31 ± 4.42	4.69	8.11
	0.125	98.15 ± 2.14	2.18	5.28
	0.250	99.26 ± 7.65	7.71	6.37
	0.500	100.62 ± 2.42	2.40	2.38
Tyrosine	0.02	104.49 ± 7.88	7.54	6.87
	2.50	93.71 ± 4.20	4.48	2.11
	5.00	102.85 ± 5.24	5.10	3.33
	10.00	101.32 ± 6.16	6.08	2.39
Phenylacetylglutamine	0.010	98.59 ± 4.45	4.51	9.39
	0.125	99.76 ± 8.75	8.77	4.11
	0.250	95.15 ± 6.70	7.04	5.36
	0.500	99.07 ± 4.45	4.49	3.95
5-hydroxytryptamine	0.002	103.3 ± 8.12	7.86	9.96
	0.025	99.74 ± 6.30	6.32	7.75
	0.050	95.13 ± 4.37	4.59	2.98
	0.100	98.30 ± 3.19	3.24	3.70

TABLE 6 Matrix effect for AAA analysis in serum ($n = 6$).

		Hemolysis serum		Hyperlipidemia serum		Normal serum	
Cpd.	Add Conc. (μg/ml)	Matrix effect (%, x ± SD)	RSD%	Matrix effect (%, x ± SD)	RSD%	Matrix effect (%, x ± SD)	RSD%
Phenylalanine	2.50	86.58 ± 6.57	7.59	100.29 ± 9.9	9.87	98.38 ± 10.07	10.24
	5.00	98.83 ± 7.35	7.43	97.15 ± 8.15	8.39	96.95 ± 9.30	9.59
	10.00	104.88 ± 4.58	4.36	98.32 ± 7.25	7.38	97.94 ± 4.60	4.69
Tryptophan	2.50	101.15 ± 8.43	8.33	100.68 ± 9.05	8.99	99.87 ± 9.66	9.67
	5.00	94.72 ± 8.96	9.45	93.75 ± 7.61	8.12	93.57 ± 7.95	8.49
	10.00	98.29 ± 10.12	10.30	98.66 ± 9.09	9.22	94.08 ± 9.21	9.79
Kynurenine	0.125	93.56 ± 6.69	7.15	94.33 ± 11.35	12.0	98.58 ± 8.02	8.14
	0.250	97.95 ± 4.78	4.89	95.82 ± 5.91	6.16	95.49 ± 8.52	8.93
	0.500	93.93 ± 6.15	6.55	93.68 ± 8.26	8.82	103.05 ± 10.76	10.44
Tyrosine	2.50	101.18 ± 9.31	9.20	95.62 ± 7.82	8.18	96.80 ± 10.18	10.51
	5.00	94.81 ± 6.91	7.29	101.71 ± 7.71	7.58	103.03 ± 7.60	7.38
	10.00	99.64 ± 9.04	9.07	103.48 ± 11.39	11.01	98.37 ± 12.49	12.70
Phenylacetylglutamine	0.125	96.99 ± 7.83	8.08	97.44 ± 6.23	6.40	105.09 ± 11.21	10.66
	0.250	101.21 ± 7.99	7.89	104.15 ± 6.57	6.31	104.73 ± 2.53	2.42
	0.500	93.22 ± 6.58	7.06	97.24 ± 8.82	9.07	97.99 ± 9.74	9.94
5-hydroxytryptamine	0.025	112.91 ± 8.48	7.51	107.7 ± 13.84	12.86	106.20 ± 9.84	9.26
	0.050	96.15 ± 7.17	7.45	93.65 ± 7.03	7.51	100.67 ± 5.96	5.92
	0.100	95.46 ± 6.93	7.26	91.29 ± 5.47	5.987	105.15 ± 8.77	8.34

3.1.4 | Stability of QC samples

Three concentrations of QC samples (high, medium, and low) were used for the evaluation of stability at different conditions (Table 7). According to the results, all the mean accuracy ranged from 88.99% to 106.02%. The obtained data showed that six endogenous metabolites remained extraordinary stable at least 6 months at −80°C, 12 h at 4°C and 25°C, 30 min at 56°C, and three freeze–thaw cycles. These results demonstrate that serum samples can be handled under normal laboratory conditions without significant loss of biomarkers.

TABLE 7 Stability of QC samples under different conditions ($n = 3$).

Cpd.	Conc. (μg/ml)	Accuracy ($\bar{x} \pm$ SD %)					
		Autosampler	Bench-top 4°C	Bench-top RT	Freeze–thaw	56°C	Long-term
Phenylalanine	2.50	93.73 ± 9.22	95.29 ± 6.56	90.33 ± 5.75	99.75 ± 7.59	90.89 ± 5.64	101.07 ± 5.54
	5.00	98.94 ± 8.63	97.03 ± 5.91	99.40 ± 10.67	97.32 ± 10.53	98.68 ± 8.10	104.54 ± 5.84
	10.00	94.30 ± 1.06	93.13 ± 4.54	88.99 ± 1.55	96.92 ± 3.02	96.47 ± 2.24	89.78 ± 1.74
Tryptophan	2.50	97.74 ± 8.26	92.92 ± 3.24	96.59 ± 4.46	95.56 ± 2.19	95.22 ± 6.49	98.83 ± 11.81
	5.00	101.75 ± 4.59	91.35 ± 4.60	103.77 ± 1.54	103.48 ± 2.18	100.88 ± 6.66	90.71 ± 1.78
	10.00	95.75 ± 5.83	97.91 ± 4.84	93.36 ± 1.71	97.31 ± 6.31	98.97 ± 8.98	93.15 ± 2.01
Kynurenine	0.125	102.27 ± 8.76	97.28 ± 3.42	100.91 ± 4.71	97.67 ± 2.90	99.64 ± 6.74	94.45 ± 4.30
	0.250	104.75 ± 5.61	101.42 ± 1.7	98.83 ± 8.80	95.91 ± 3.73	102.61 ± 7.18	98.59 ± 7.34
	0.500	106.02 ± 3.02	98.95 ± 8.62	104.14 ± 2.11	99.40 ± 3.06	99.73 ± 4.55	100.74 ± 3.69
Tyrosine	2.50	97.74 ± 8.10	92.95 ± 3.34	96.66 ± 4.28	95.59 ± 2.34	92.93 ± 4.83	90.5 ± 4.16
	5.00	104.43 ± 4.17	95.22 ± 7.48	94.96 ± 9.75	91.84 ± 3.02	95.68 ± 6.38	98.43 ± 4.33
	10.00	93.61 ± 2.72	93.30 ± 4.37	92.88 ± 2.84	93.49 ± 6.93	89.79 ± 2.66	92.85 ± 2.95
Phenylacetylglutamine	0.125	102.18 ± 8.71	97.17 ± 3.40	100.92 ± 4.55	99.64 ± 1.74	99.51 ± 6.91	94.47 ± 4.47
	0.250	101.05 ± 4.42	93.13 ± 1.81	98.56 ± 8.06	99.05 ± 6.91	100.31 ± 6.68	97.05 ± 8.50
	0.500	100.91 ± 5.62	103.55 ± 4.68	103.14 ± 3.34	99.49 ± 3.83	99.81 ± 3.12	100.34 ± 4.63
5-hydroxytryptamine	0.025	102.22 ± 4.21	89.09 ± 2.04	94.74 ± 2.69	99.69 ± 6.32	99.90 ± 5.33	97.63 ± 9.13
	0.050	103.66 ± 4.53	95.79 ± 1.90	103.12 ± 5.21	101.91 ± 7.34	103.15 ± 7.11	99.87 ± 8.54
	0.100	101.78 ± 5.56	104.63 ± 5.09	104.08 ± 3.31	100.55 ± 4.11	100.82 ± 3.19	101.25 ± 4.62

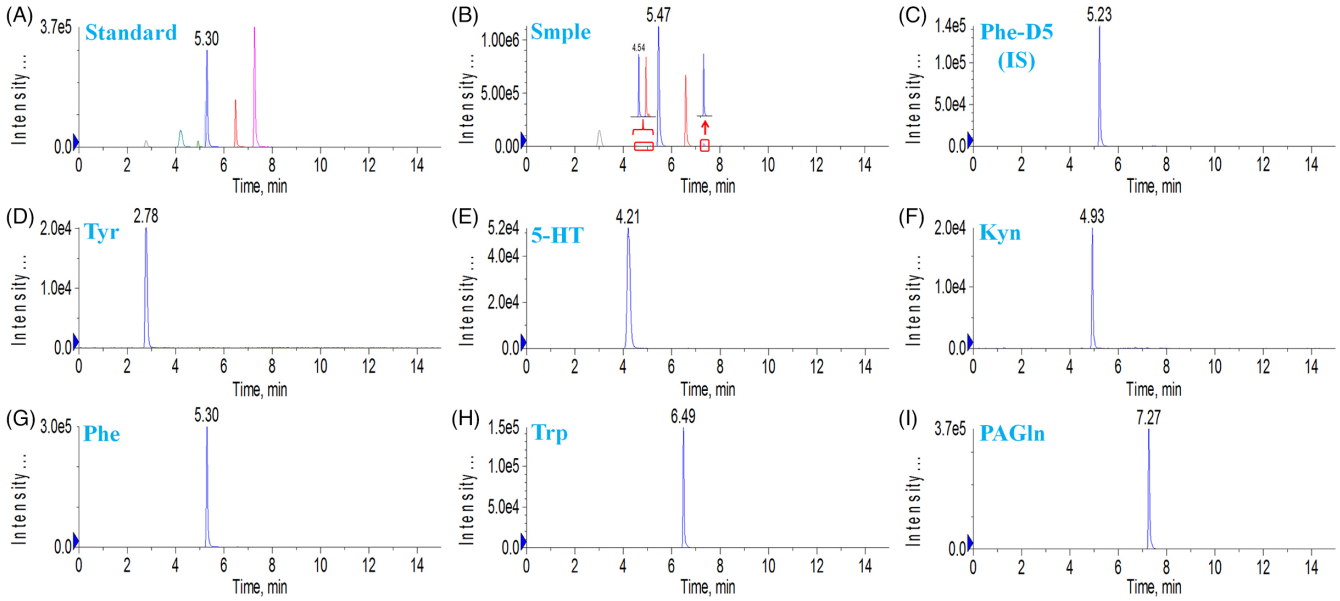

FIGURE 1 Representative HPLC–MS/MS chromatograms of AAA biomarkers (Human serum sample). Representative HPLC–MS/MS chromatograms of AAA in (A) PBS spiked with analytes and (B) a human serum specimen. (C–I) Representative HPLC–MS/MS chromatograms of Phe-D5, Tyr, 5-HT, Kyn, Phe, Trp, and PAGln.

3.2 | Determination of biomarker levels in serum using HPLC–MS/MS

The levels of six endogenous metabolites in the serum were evaluated based on the regression equation with a weighting factor (1/*x*). Typical chromatograms are shown in Figure 1. Absolute quantitative analysis was carried out using analytical standards for Phe, Trp, Kyn, Tyr, PAGln, and 5-HT.

The Brown-Forsythe and Welch ANOVA tests were employed to compare the six endogenous metabolites levels in healthy volunteers and patients with particular types of HIV infection (Figure 2). The concentrations of the metabolites in patients with HIV infection were significantly different from those in healthy volunteers. The levels of 5-HT, Tyr, and Trp were significantly decreased in patients with HIV infection, especially in those with glucose and lipid metabolic disorders, whereas the levels of Kyn, Phe, PAGln, Kyn/Trp, and Phe/Tyr were significantly increased in these patients, compared to the control group; however, there was no significant change in the PAGln/Phe levels.

The levels of PAGln were significantly increased in the patients treated with ART for at least 1 year compared to those who were incipient; furthermore, patients with hyperglycemia and/or hyperlipidemia demonstrated a higher PAGln level than those without these disorders.

3.3 | Biomarker analysis in patients with HIV infection and metabolic disorders

The heatmap (showing only group averages) shows the overall trend of AAA metabolic substances in patients with HIV infection at different stages of disease. The levels of 5-HT, Tyr, and Trp were

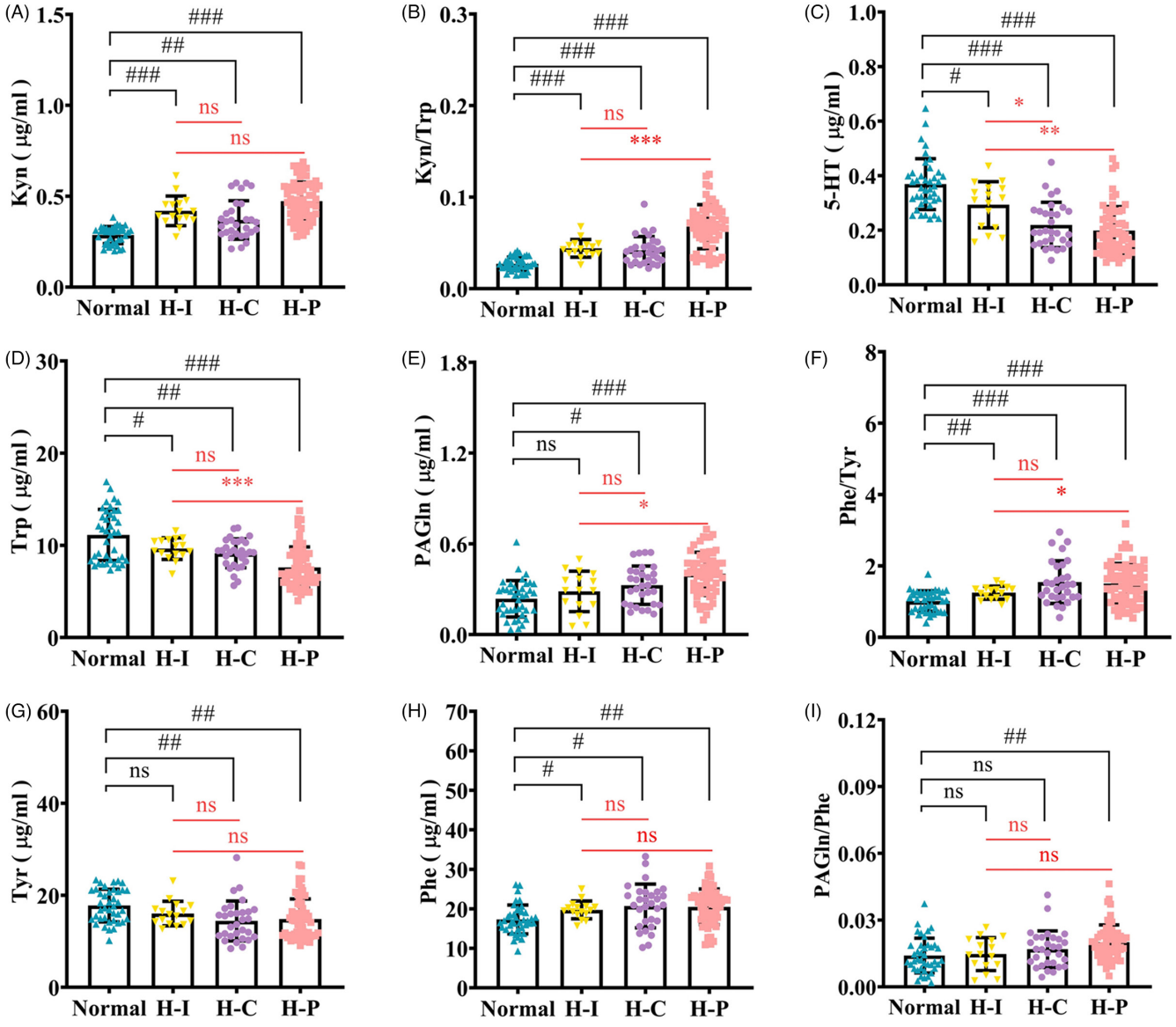

FIGURE 2 Serum levels of AAA biomarkers in HIV-infected patients with/without metabolic disorders versus normal: #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; versus H–I group: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

significantly decreased, contrarily; the levels of Kyn, Phe, PAGln, Kyn/Trp, and Phe/ Tyr were significantly increased in these patients (Figure 3A). PCA was used to appraise the contribution of AAA metabolic profiling to divide the participants into different groups of healthy, HIV/AIDS with incipient, HIV/AIDS with normal metabolism, and HIV/AIDS with hyperglycemia and/or hyperlipidemia subjects , and to identify more AAA biomarkers that can be used to detect metabolic disorders. Healthy volunteers and patients at different stages of the disease were separated based on serum AAA metabolic profiling in orthogonal partial least squares discriminant analysis score plots (Figure 3B).

Homeostasis model assessment for insulin resistance (HOMA-IR) is the primary indicator of glucose and lipid metabolism disorders. In this study, to explore the correlation between HOMA-IR and AAA metabolic biomarkers, we determined the relationship between serum AAA metabolic biomarkers using Pearson's correlation (Figure 3C). The r in the Pearson correlation showed that HOMA-IR was positively correlated with Kyn/Trp (0.33), Kyn (0.33), and PAGln (0.31), whereas a negative correlation with 5-HT (−0.30) was observed.

The area under the curve (AUC) was used for various biomarker analyses in order to distinguish the HIV infection at different stages of disease (Figure 4). The AUC values for Kyn, 5-HT, and Kyn/Trp were 0.96, 0.92, and 0.949, respectively, which indicated high accuracy (AUC > 0.9). The AUC values for PAGln and Trp were 0.82 and 0.847, respectively, which indicated a medium accuracy. The AUC values for Phe metabolic biomarkers acquired from ROC analysis and the levels of Kyn, 5-HT, Kyn/Trp, PAGln, and Trp can be determined in future studies and applied clinically.

4 | DISCUSSION

Aromatic amino acids refer to amino acids with benzene ring structure in the molecular structure, mainly including Tyr, Phe, and Trp. Kyn and 5-HT were the main metabolites of Trp. In addition, the intestinal flora was able to break down Trp and Phe, and generate indole, indolic acid, skatole, tryptamine, phenylpyruvic acid, and phenylacetic acid. Phenylacetic acid can be converted to PAGln under the action of Gln PA-transferase in liver, which have been confirmed closely related to cardiovascular disease. This study also attempts to explore the level changes of AAA metabolic, especially PAGln.

Metabolic disorders and IR are common complications of AIDS/HIV infection combined with ART and affect the treatment outcome of these patients. Phe, Trp, and Tyr are AAAs that affects the immune, metabolic, and neural responses. High plasma levels of AAAs have been reported in patients with IR.[19] Phe is an essential amino acid that can be converted into the neurotransmitters norepinephrine and epinephrine. Moreover, Phe is a Tyr precursor. A previous study found significant increases in the plasma concentrations of arginine, Phe, and glutamate in patients with HIV infection.[20] Phe metabolism is associated with the pathology of HIV-1 infection. Reportedly, elevated Phe/Tyr levels are associated with lower $CD4^+$ cell counts and higher levels of HIV-RNA and are significantly

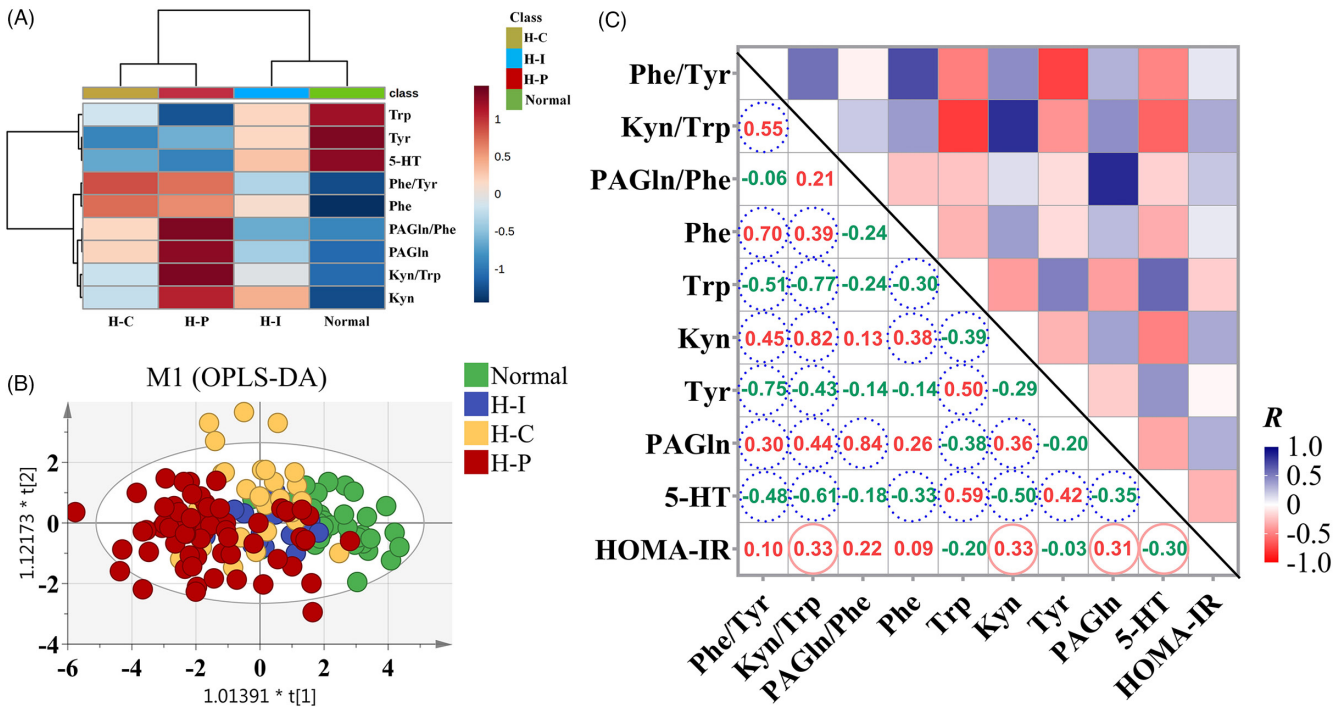

FIGURE 3 Analysis of potential AAA biomarkers. (A) Heatmap based on the serum AAA profiles of HIV-infected patients and healthy controls. (B) OPLS-DA score plots in view of the serum AAA profiles of HIV-infected patients and healthy controls. R2X (CUM) 0.804, R2Y (CUM) 0.293, and Q2 (CUM) 0.242. (C) AAA biomarkers correlations with HOMA-IR.

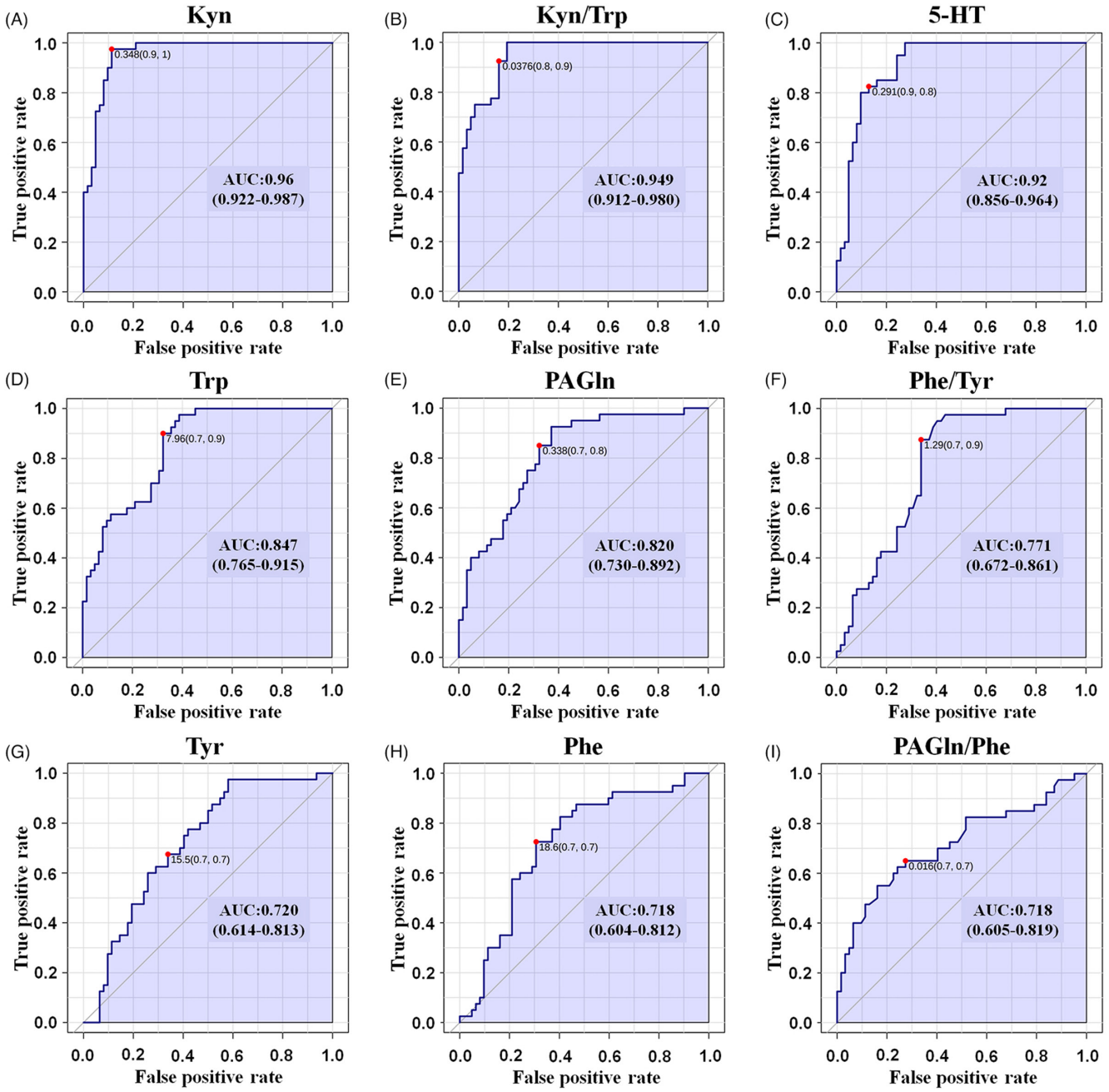

FIGURE 4 Combined prediction abilities of AAA biomarks for discriminating HIV-infected patients with metabolic disorders from healthy controls. (A) Kyn, AUC 0.960; (B) Kyn/Trp, AUC 0.949; (C) 5-HT, AUC 0.920; (D) Trp, AUC 0.847; (E) PAGln, AUC 0.82; (F) Phe/Tyr, AUC 0.771; (G) Tyr, AUC 0.72; (H) Phe, AUC 0.718; (I) PAGln/Phe, AUC 0.718.

associated with a higher degree of immune activation,[21] suggesting that oxidative stress induced by inflammation and immune activation may be involved in Phe metabolic disorders. In addition, Phe levels are positively correlated with the risk of prediabetes and type 2 diabetes mellitus (T2DM).[22] T2DM patients with a high level of Phe, PAGln, p-cresol than non-T2DM participants.[23] These data provide a link between Phe and glucose metabolism in patients with HIV infection.

There is a strong relationship between the Trp metabolic pathway and gut microbiota. For example, *C. sporogenes* can decarboxylate Trp to produce the neurotransmitter tryptamine.[24] Trp metabolism plays a critical role in infectious diseases as a regulator of the immune response. Over 95% of free Trp is catabolized via the kynurenine pathway (KP). Trp is converted to Kyn by IDO1, which has potent immunosuppressive activity that may lead to worsening of immune dysfunction in HIV-infected patients. HIV-1 utilizes the immunosuppressive activity of IDO1 to establish chronic infection.[25] Trp metabolism through the KP is implicated in gut barrier disruption in HIV infection.[26] Trp-related metabolite levels are significantly reduced in the plasma of patients with advanced atherosclerosis.[27] Kyn is the ligand for the aromatic hydrocarbon receptor. Various KP intermediates can inhibit synthesis, secretion, and signaling of

insulin. Increased levels of kynurenic acid and xanthurenic acid have been found in the urine of patients with T2DM. A cross-sectional study showed that the plasma levels of Kyn and the Kyn/Trp ratio were significantly elevated in individuals with metabolic syndrome. Moreover, Kyn/Trp ratio has been demonstrated to predict major coronary events and all-cause mortality in patients with coronary artery disease.[28] Additionally, Trp is the precursor of the neurotransmitter 5-HT; tryptophan hydroxylase catalyzes the production of 5-hydroxytryptophan (5-HTP) from Trp, and 5-hydroxytryptophan is then converted to 5-HT by 5-HTP decarboxylase. The neurotransmitter 5-HT is also an inflammatory mediator and vasoactive amine that can affect the immune system function. Studies have shown that 5-HT can inhibit HIV replication.[29,30] In addition, circulating 5-HT levels are decreased in patients with metabolic syndrome and are negatively correlated with BMI and body fat.[31] Thus, Trp metabolism may play a key role in glucose and lipid metabolism in HIV-infected patients.

On the research, to evaluation of the key endogenous substances of AAA metabolic levels for clinical, we developed a robustness HPLC–MS/MS technique. Even at trace analyte concentrations, the approach allowed the unequivocal identification of PAGln and other key endogenous substances of AAA metabolic pathway with high sensitivity and accuracy. In addition, ROC analysis, Pearson's correlation were conducted for in-depth researches of clinical diagnosis and applications, in order to screen out potential markers. The research will provide a new perspective for PAGln and the key endogenous substances of AAA of HIV infection, especially with metabolic disorder.

5 | LIMITATIONS

In this study, we focused on AAA metabolism disorder progression since we have previously shown that HIV infection at different stages of disease is associated with risk of metabolic disorder. However, we only recruit a few individuals are HIV/AIDS with incipient, and then restrict sample capacity. Next, we will expand the sample size to get a more certainty conclusion. Besides, we did not determine the relationship between AAA metabolism disorder and symptom improvement of HIV infection on account of the small sample size.

6 | CONCLUSIONS

An innovative scheme based on HPLC–MS/MS was established for the quantification of six endogenous metabolites related to AAA metabolism. In addition, ROC analysis was performed to evaluate their clinical diagnostic value. With these methods, we identified the serum biomarkers related to Trp and PAGln metabolism that can be used to distinguish between patients with HIV infection with and without metabolic disorders, and healthy participants. To the best of our knowledge, no systematic research on the PAGln levels in patients with HIV infection, who are at different stages of the disease, has been conducted. The developed methodology is suitable for quantifying PAGln and other biomarkers.

AUTHOR CONTRIBUTIONS

Z.-W. Yuan: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing—original draft, Writing—review and editing; H.-L. Gan: Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review and editing; J. Zhang: Formal analysis, Funding acquisition, Investigation, Methodology, Writing—original draft, Writing—review and editing; H.-L. Jin: Investigation, Formal analysis, Writing—original draft, Methodology; X.-Y. Feng: Investigation, Methodology, Writing—original draft; M. Wang: Formal analysis, Writing—original draft, Writing—review and editing; H.-P. Zhou: Investigation, Methodology; All authors have read and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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