Supplementary Information

Targeted Metabolomics of Nucleotide Intermediates for Biomarker Discovery in Acute Kidney Injury

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Text

Liquid chromatography conditions for purine and pyrimidine determination

Targeted analysis of purines:

Mobile phase: A consists of ACN-H_2O (95:5, v/v) with 10 mmol/L ammonium formate and 0.4% acetic acid; B consists of ACN-H_2O (50:50, v/v) with 20 mmol/L ammonium formate and 0.1% FA.

Mass spectrometry parameters: positive ion mode, ion spray voltage: 5000 V, and the ion source temperature was set at 600°C. The pressure of the nebulizer gas and the heated gas: both 50 psi; curtain gas: 30 psi; the pressure of collision-induced dissociation (CID) gas: 10 psi.

Targeted analysis of pyrimidines:

Mobile phase: A consists of ACN-H_2O (95:5, v/v) with 10 mmol/L ammonium formate and 0.1% ammonia solution; mobile phase B consists of ACN-H_2O (50:50, v/v) with 20 mmol/L ammonium formate and 0.1% ammonia solution.

Mass spectrometry parameters: positive ion mode, ion spray voltage: 5500 V, and the ion source temperature was set at 550°C. The pressure of the nebulizer gas and the heated gas: 40 and 55 psi, respectively; curtain gas: 20 psi; the pressure of CID gas: 9 psi.

The elution gradients were as follows: The flow rate was 0.3 mL/min. The elution gradients were as follows: 0–5 min, 6% B; 5–7 min, 6–40% B; 7–9 min, 40–57% B; 9 –9.5 min, 57–95% B; 9.5 –10.5min, 95% B; 10.5–11.5min, 95-6% B; and 11.5 – 14.5min, 6% B. The column temperature was set at 30 °C. The autosampler was set at 6 °C and the injected volume was 5 μL.

Table S1 MS parameters of the purine analytes and ISs in MRM mode

No.	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)	Dwell Time (msec)
1	Adenine	136.0	119.1	88	13	30	12	15
2	Guanine	152.0	135.1	74	4	27	12	15
3	Xanthine	153.0	110.1	86	14	27	9	15
4	Hypoxanthine	136.9	110.2	104	14	28	10	15
5	Adenosine	268.1	136.2	74	13	60	12	15
6	Guanosine	284.2	152.2	84	11	15	13	15
7	Inosine	269.1	137.3	66	13	13	12	15
8	Xanthonsine	285.1	153.1	80	11	15	13	15
9	Deoxyadenosine	252.2	136.2	70	9	17	12	15
10	Deoxyguanosine	268.1	152.1	70	7	15	12	15
11	Deoxyinosine	253.1	137.2	60	10	12	11	15
12	AMP	348.0	136.3	70	14	25	12	15
13	GMP	364.2	152.2	66	8	20	13	15
14	IMP	349.0	137.3	70	9	17	11	15
15	XMP	365.2	97.3	68	10	25	8	15
16	cAMP	330.1	136.3	92	11	35	12	15
17	IS^1(4-Cholorophrenoalanine)	200.0	154.0	62	6	18	14	15
18	IS^2(Hypoxanthine-$^{15}N_4$)	141.0	123.1	92	11	28	9	15

Table S2 MS parameters of the pyrimidine analytes and ISs in MRM mode

No.	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)	Dwell Time (msec)
1	Uracil	113.0	70.1	106	12	23	12	25
2	Cytosine	112.1	95.0	68	9	28	5	25
3	Thymine	127.1	110.1	70	3	23	12	25
4	Uridine	245.0	113.0	54	6	13	6	25
5	Cytidine	244.0	112.1	38	7	17	9	25
6	Thymidine	243.0	127.0	46	4	13	12	25
7	Deoxyuridine	229.1	113.0	46	4	13	10	25
8	Deoxycytidine	250.0	134.1	68	10	17	9	25
9	UMP	325.0	97.2	60	7	20	5	25
10	CMP	324.0	112.1	60	4	17	11	25
11	TMP	323.0	81.1	60	5	25	7	25
12	Dihydrouracil	115.0	73.0	110	10	17	13	25
13	Dihydrothymine	129.0	69.1	122	4	20	12	25
15	IS^3(Cytidine-$^{15}N_3$)	247.0	115.1	42	4	17	10	25
16	IS^2(Hypoxanthine-$^{15}N_4$)	141.0	123.1	92	11	28	9	25

Table S3 Linear range, recovery rate of purine analytes

No.	Compound	Linear range		Recovery (%)		
		Plasma	Urine	QC level	Plasma	Urine
1	Adenine	10-2000	8-400	LQC	102.9	107.2
				MQC	90.8	100.2
				HQC	110.1	92.5
2	Guanine	2.5-8000	10-800	LQC	97.4	108.8
				MQC	99.9	95.3
				HQC	106.5	104.6
3	Xanthine	90-14400	4000-40000	LQC	101.6	99.6
				MQC	94.1	91.4
				HQC	101.0	90.7
4	Hypoxanthine	90-36000	100-40000	LQC	101.4	96.2
				MQC	108.9	110.2
				HQC	100.8	94.1
5	Adenosine	15-6000	120-12000	LQC	95.1	108.9
				MQC	95.7	108.7
				HQC	98.9	91.1
6	Guanosine	6-600	200-4000	LQC	102.2	99.9
				MQC	94.6	92.9
				HQC	108.0	94.1
7	Inosine	10-4000	400-8000	LQC	106.3	104.9
				MQC	101.0	100.3
				HQC	106.9	100.2

				LQC	N.A.	96.9
8	Xanthonsine	*N.A.*	2400-12000	MQC	N.A.	100.7
				HQC	N.A.	103.2
				LQC	94.9	110.7
9	Deoxyadenosine	2.5-1000	6.67-400	MQC	99.9	92.1
				HQC	105.1	87.4
				LQC	97.8	99.9
10	Deoxyguanosine	6-600	20-1200	MQC	95.5	96.1
				HQC	105.9	111.7
				LQC	105.5	100.2
11	Deoxyinosine	50-5000	100-4000	MQC	89.1	93.3
				HQC	109.7	109.2
				LQC	101.0	107.8
12	cAMP	10-1000	200-8000	MQC	98.6	87.5
				HQC	89.7	101.6

Note: *N.A.* represented not applicable. AMP, GMP, CMP, and IMP were undetectable.

Table S4 Linear range, recovery rate of pyrimidine analytes

No.	Compound	Linear range		Recovery (%)		
		Plasma	Urine	QC level	Plasma	Urine
1	Uracil	60-12000	24-12000	LQC	101.2	99.8
				MQC	102.8	102.5
				HQC	97.2	102.6
2	Cytosine	3-750	0.6-1500	LQC	96.3	100.1
				MQC	100.9	104.1
				HQC	91.4	92.7
3	Thymine	24-6000	4.8-12000	LQC	94.9	98.6
				MQC	100.1	105.7
				HQC	87.8	92.6
4	Uridine	2.4-3000	2.4-1200	LQC	98.7	99.6
				MQC	101.4	109.2
				HQC	94.5	92.2
5	Cytidine	1.2-3000	1.2-300	LQC	100.6	99.8
				MQC	99.1	98.5
				HQC	94.6	96.8
6	Thymidine	0.6-750	0.6-60	LQC	100.0	98.7
				MQC	107.0	96.5
				HQC	96.3	100.9
7	2’-Deoxyuridine	2-1000	0.2-250	LQC	98.5	99.3
				MQC	105.1	113.2
				HQC	91.8	100.8

8	2’-Deoxycytidine	5-1000	5-1000	LQC	114.1	91.7
				MQC	104.4	109.1
				HQC	85.4	95.3
9	Dihydrouracil	1200-60000	1200-60000	LQC	97.2	93.2
				MQC	92.8	105.1
				HQC	94.7	88.5
10	Dihydrothymine	1000-30000	300-12000	LQC	98.8	102.1
				MQC	100.3	109.7
				HQC	90.6	96.7

Note: CMP, UMP, and TMP were undetectable.

Table S5 Total mediation effects of metabolites on AKI.

Independent variable	Mediation variable	ACME (97.5% CI)	p mediation effect	ADE (97.5% CI)	p direct effect	Total Effect (97.5% CI)	p total effect
P. Hypoxanthine	AST	-0.019 (-0.079, -0.000[1])	0.012	-0.007 (-0.034, 0.037)	0.486	-0.026 (-0.053, -0.000[1])	0.018
P. Thymidine	AST	-0.058 (-0.163, -0.008)	0.016	-0.072 (-0.251, 0.061)	0.300	-0.130 (-0.312, -0.005)	0.046
U. Adenine	eGFR	-0.045 (-0.071, -0.009)	<0.001	-0.024 (-0.038, 0.019)	0.274	-0.068 (-0.072, -0.017)	0.004
U. Xanthine	eGFR	-0.060 (-0.118, -0.022)	<0.001	-0.035 (-0.065, 0.031)	0.206	-0.096 (-0.123, -0.043)	<0.001
U. Hypoxanthine	eGFR	-0.083 (-0.179, -0.042)	<0.001	-0.042 (-0.087, 0.075)	0.320	-0.124 (-0.169, -0.082)	<0.001
U. Adenosine	eGFR	-0.055 (-0.122, -0.013)	0.010	-0.040 (-0.073, 0.028)	0.306	-0.095 (-0.143, -0.049)	<0.001
U. 2'-Deoxyadenosine	eGFR	-0.037 (-0.076, -0.018)	<0.001	-0.034 (-0.055, 0.001)	0.058	-0.072 (-0.095, -0.043)	<0.001
P. Hypoxanthine	eGFR	-0.043 (-0.079, -0.007)	0.002	-0.001 (-0.013, 0.040)	0.940	-0.044 (-0.058, -0.007)	0.014
U. Cytidine	eGFR	-0.063 (-0.106, -0.031)	0.002	-0.017 (-0.052, 0.041)	0.456	-0.080 (-0.117, -0.032)	0.004
U. Guanine	Scr	-0.045 (-0.071, -0.019)	<0.001	-0.016 (-0.030, -0.000[1])	0.054	-0.060 (-0.086, -0.027)	<0.001
U. Xanthine	Scr	-0.076 (-0.098, -0.024)	<0.001	-0.018 (-0.035, 0.016)	0.346	-0.094 (-0.104, -0.031)	<0.001
U. Hypoxanthine	Scr	-0.093 (-0.127, -0.036)	<0.001	-0.028 (-0.048, 0.012)	0.218	-0.121 (-0.145, -0.054)	<0.001
U. Cytidine	Scr	-0.056 (-0.089, -0.027)	<0.001	-0.010 (-0.033, 0.022)	0.496	-0.066 (-0.102, -0.029)	0.004
P. Thymidine	Scr	-0.137 (-0.284, -0.019)	0.010	-0.051 (-0.125, 0.053)	0.270	-0.189 (-0.344, -0.047)	0.010
P. Thymidine	UA	-0.102 (-0.187, -0.035)	0.004	-0.083 (-0.258, 0.072)	0.292	-0.185 (-0.359, -0.032)	0.016
U. Uridine	Urea	-0.032 (-0.064, -0.005)	0.020	-0.024 (-0.060, 0.017)	0.246	-0.056 (-0.096, -0.012)	0.018

Note: ACME: Average Causal Mediation Effect. ADE: Average Direct Effect. If confidence interval and its value were less than 0.001, they were represented by NA. -0.000[1]: value less than -0.001; 0.000[2]: value less than 0.001

Table S6 Partial mediation effects of metabolites on AKI.

Independent variable	Mediation variable	ACME (97.5% CI)	p mediation effect	ADE (97.5% CI)	p direct effect	Total Effect (97.5% CI)	p total effect	Prop.Mediated
U. Guanine	eGFR	-0.046 (-0.079, -0.019)	<0.001	-0.028 (-0.047, -0.005)	0.020	-0.074 (-0.104, -0.037)	<0.001	57%
U. Adenosine	Scr	-0.038 (-0.116, -0.010)	0.016	-0.026 (-0.060, -0.000[1])	0.050	-0.064 (-0.146, -0.035)	<0.001	59%
U. 2'-Deoxyadenosine	Scr	-0.030 (-0.052, -0.009)	<0.001	-0.024 (-0.042, -0.005)	0.028	-0.054 (-0.081, -0.020)	<0.001	48%
U. Guanine	UA	-0.013 (-0.027, -0.002)	<0.001	-0.065 (-0.085, -0.027)	<0.001	-0.078 (-0.099, -0.032)	<0.001	13%
U. Cytidine	UA	-0.026 (-0.049, -0.004)	0.030	-0.061 (-0.089, -0.006)	0.032	-0.087 (-0.108, -0.032)	0.004	28%
P. Cytosine	UA	0.039 (0.010, 0.082)	0.002	0.169 (0.085, 0.268)	<0.001	0.208 (0.125, 0.303)	<0.001	19%
U. Adenine	Urea	-0.010 (-0.031, -0.000[1])	0.026	-0.019 (-0.047, -0.002)	0.006	-0.029 (-0.061, -0.003)	<0.001	24%
U. Guanine	Urea	-0.016 (-0.031, -0.005)	0.006	-0.052 (-0.077, -0.025)	<0.001	-0.069 (-0.093, -0.037)	<0.001	19%
U. Xanthine	Urea	-0.044 (-0.069, -0.015)	<0.001	-0.047 (-0.074, -0.012)	0.006	-0.091 (-0.113, -0.046)	<0.001	43%
U. Adenosine	Urea	-0.032 (-0.090, -0.004)	0.028	-0.061 (-0.119, -0.013)	0.022	-0.093 (-0.160, -0.052)	<0.001	33%
U. 2'-Deoxyadenosine	Urea	-0.021 (-0.040, -0.005)	0.002	-0.042 (-0.063, -0.011)	0.002	-0.063 (-0.083, -0.023)	<0.001	28%
P. Cytosine	Urea	0.118 (0.064, 0.191)	<0.001	0.099 (0.007, 0.211)	0.038	0.217 (0.142, 0.326)	<0.001	56%

Note: ACME: Average Causal Mediation Effect. ADE: Average Direct Effect. If confidence interval and its value were less than 0.001, they were represented by NA.

-0.000[1]: value less than -0.001; 0.000[2]: value less than 0.001

Table S7 Reverse mediation effects of metabolites on AKI.

Independent variable	Mediation variable	ACME (97.5% CI)	p mediation effect	ADE (97.5% CI)	p direct effect	Total Effect (97.5% CI)	p total effect	Prop. Mediated
eGFR	U. Guanine	NA	0.014	NA	<0.001	NA	<0.001	12%
eGFR	U. 2'-Deoxyadenosine	NA	0.036	NA	<0.001	NA	<0.001	9%
Scr	U. Guanine	NA	0.014	NA	<0.001	NA	<0.001	5%
Scr	U. Adenosine	NA	0.012	NA	<0.001	NA	<0.001	2%
Scr	U. 2'-Deoxyadenosine	NA	0.004	NA	<0.001	NA	<0.001	5%
UA	U. Guanine	NA	<0.001	NA	0.004	NA	<0.001	33%
UA	P. Cytosine	NA	<0.001	NA	<0.001	NA	<0.001	24%
Urea	U. Adenine	0.002 (0.000^2, 0.006)	0.028	0.020 (0.002, 0.031)	<0.001	0.022 (0.002, 0.034)	<0.001	7%
Urea	U. Guanine	0.003 (0.000^2, 0.007)	0.004	0.017 (0.002, 0.029)	<0.001	0.020 (0.002, 0.034)	<0.001	12%
Urea	U. Xanthine	0.002 (0.000^2, 0.006)	0.006	0.016 (0.001, 0.029)	<0.001	0.018 (0.002, 0.033)	<0.001	8%
Urea	U. 2'-Deoxyadenosine	0.003 (0.000^2, 0.010)	0.002	0.018 (0.002, 0.029)	<0.001	0.021 (0.002, 0.036)	<0.001	13%
Urea	P. Cytosine	0.003 (0.000^2, 0.009)	0.038	0.014 (0.002, 0.027)	<0.001	0.017 (0.002, 0.033)	<0.001	13%

Note: ACME: Average Causal Mediation Effect. ADE: Average Direct Effect. If confidence interval and its value were less than 0.001, they were represented by NA. -0.000^1: value less than -0.001; 0.000^2: value less than 0.001

Table S8 Mortality and inpatient days comparison between NAKI and AKI groups (n = 116).

□	NAKI	AKI
Death, n (%)	6 (10.3%)	8 (13.8%)
Inpatient days[#] (mean ± SEM)	22.0 ± 2.5	23.7 ± 2.7

Note: # (Inpatient days) were calculated from the date of AKI onset (for AKI patients) or from the study enrollment date (for non-AKI controls) until hospital discharge. For patients with a fatal outcome, the time of death is considered the time of discharge.

Table S9 Logistic regression analysis of renal function recovery at d3 and metabolite concentrations at AKI onset.

Metabolite	Model	OR (95% Cl)	*p*
U.Xanthine (ng/mL)	Model 1	0.953 (0.917 - 0.991)	0.016
	Model 2	0.952 (0.914 - 0.991)	0.018
	Model 3	0.951 (0.912 - 0.992)	0.021
	Model 4	0.956 (0.914 - 1.000)	< 0.05
U.Hypoxanthine (ng/mL)	Model 1	0.937 (0.882 - 0.995)	0.035
	Model 2	0.936 (0.879 - 0.996)	0.038
	Model 3	0.934 (0.873 - 1.000)	0.049
	Model 4	0.950 (0.884 - 1.021)	0.166

Notes: d3: three days after AKI-onset. Model 1: Adjusted by metabolites concentration. Model 2: Adjusted by gender, age, metabolites concentration. Model 3: Adjusted by susceptibility factors (tumor, CKD, DM), gender, age, metabolites concentration. Model 4: Adjusted by exposure factors (shock, sepsis, trauma), susceptibility factors (tumor, CKD, DM), gender, age, metabolites concentration. OR: Odds ratio.

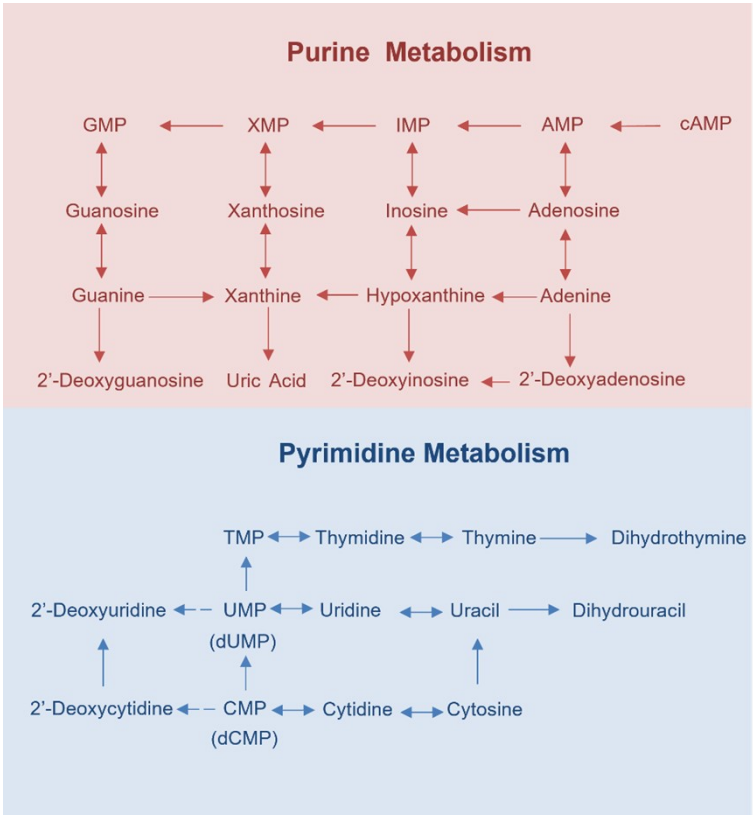

Figure S1 The schematic view of purine and pyrimidine metabolism

Note: cAMP, adenosine cyclic 3′,5′-monophosphate; GMP, guanosine 5′-monophosphate disodium salt; IMP, inosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate sodium salt; AMP, 5′-monophosphate disodium salt; UMP, uridine 5′-monophosphate disodium salt; CMP, cytidine 5′-monophosphate; TMP, thymidine 5′-monophosphate sodium salt

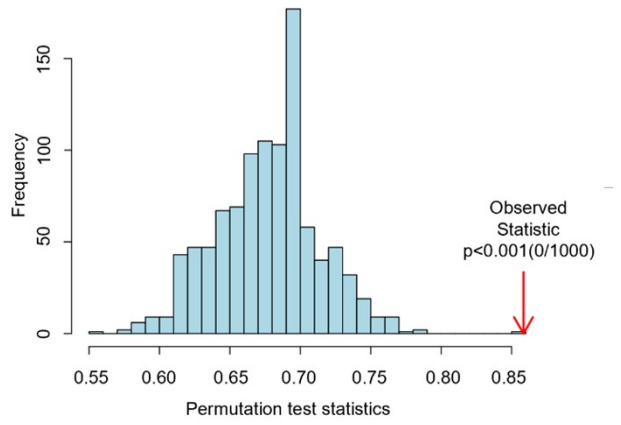

Figure S2 Permutation test of PLS-DA score plot

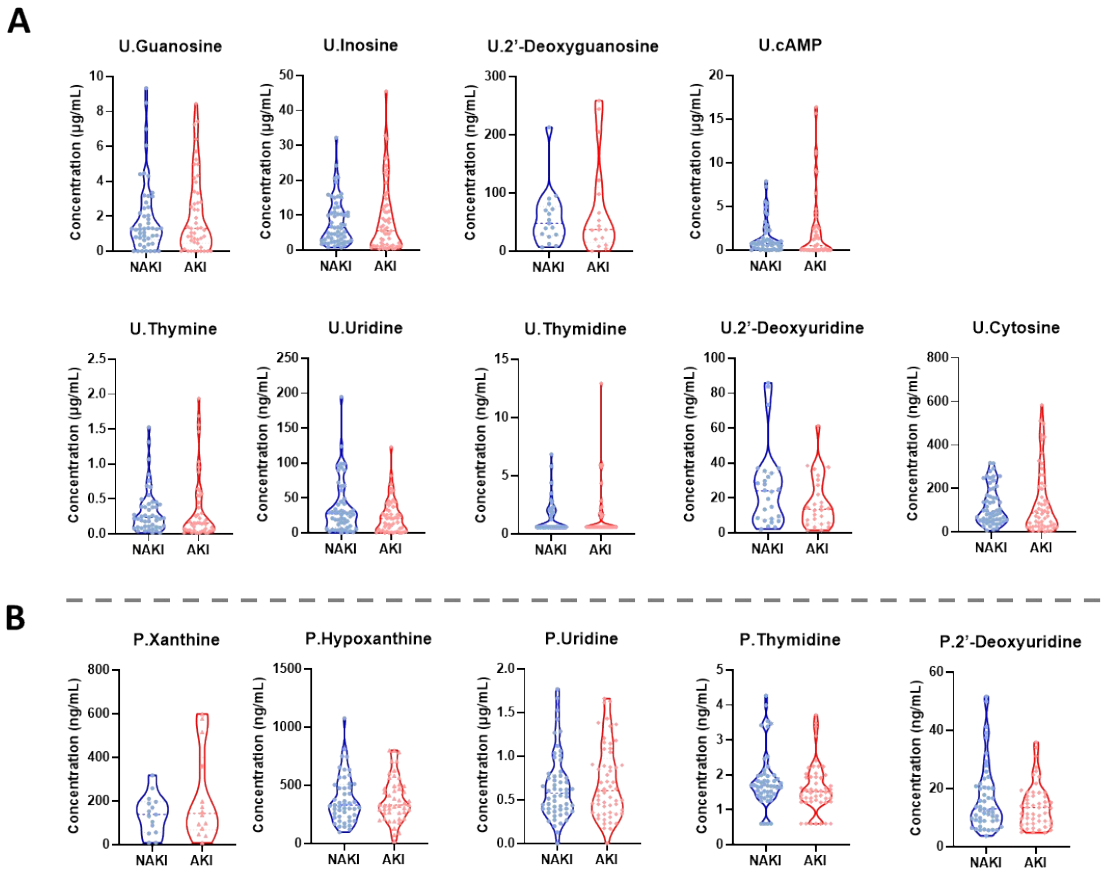

Figure S3 Purines and pyrimidines differences in urine (A) and plasma (B) samples of NAKI and AKI groups

U. represents urinary, P. represents plasma

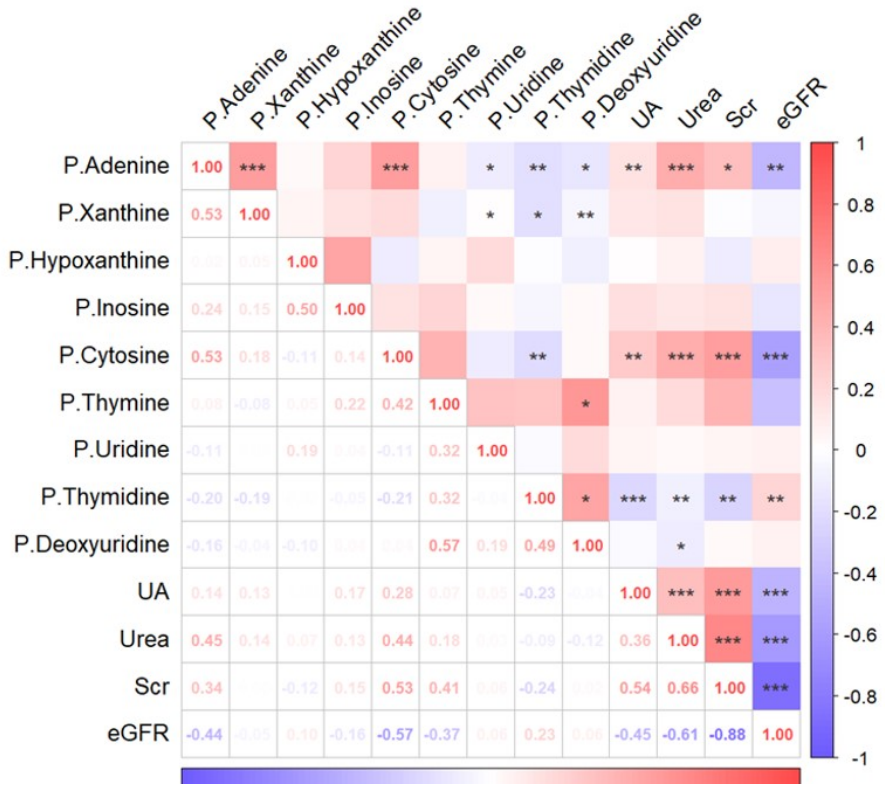

Figure S4 Correlation hot plot between metabolites and kidney function indicators in plasma

Note: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. P. represents plasma.

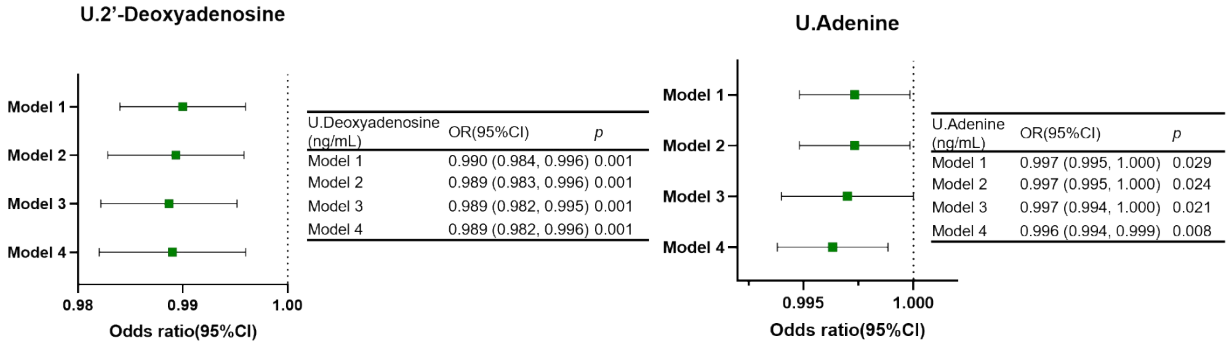

Figure S5 Binary logistic regression models of risk factors for AKI occurrence in urine and plasma

Notes:

Model 1: Adjusted by metabolites concentration.

Model 2: Adjusted by gender, age, metabolites concentration.

Model 3: Adjusted by susceptibility factors (tumor, CKD, DM), gender, age, metabolites concentration.

Model 4: Adjusted by exposure factors (shock, sepsis, trauma), susceptibility factors (tumor, CKD, DM), gender, age, metabolites concentration.

OR: Odds ratio.

Based on the KDIGO criteria, patients in the AKI group were stratified into two subgroups: KDIGO Stage 1 and a combined KDIGO Stage 2/3 group. Stages 2 and 3 were merged due to lower patient numbers in these advanced stages compared with Stage 1. After normality transformation, the data were compared between these two subgroups using a two-sample t-test. The resulting p-values were adjusted for multiple comparisons via the False Discovery Rate (FDR) method, with a significance threshold of 0.05. The outcomes of this analysis were shown in Figure S6.

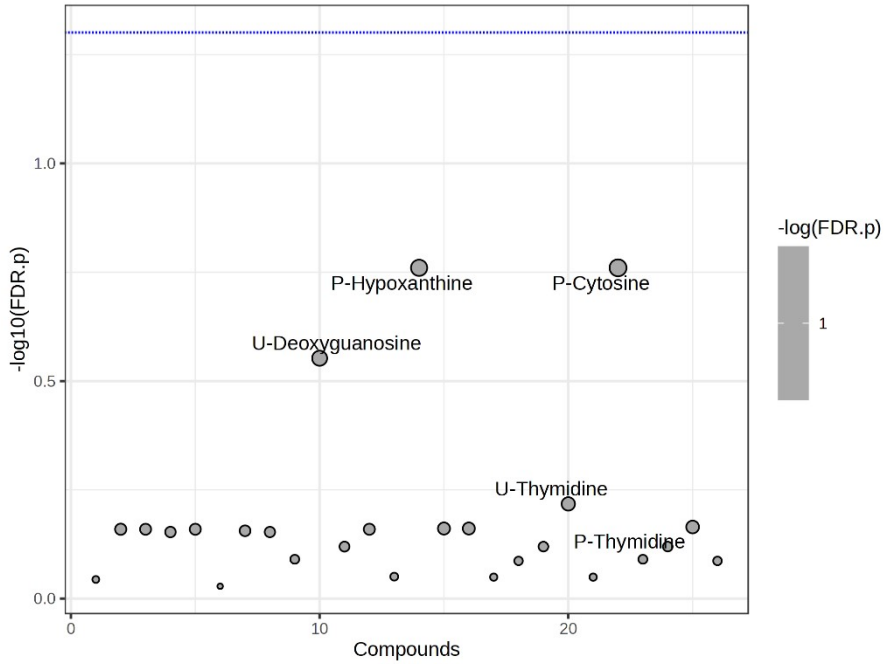

Figure S6 Comparison of nucleotide metabolite levels between KDIGO stage 1 and combined stages 2/3