

# <sup>1</sup>H NMR spectroscopy-based serum metabolomics analysis of iron deficiency anemia

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**Abstract:** Iron deficiency anemia is a prevalent type of anemia globally, caused mainly by the deficiency of iron, which is an essential component of various metabolic pathways. In this study, we aimed to investigate the changes in metabolite profiles resulting from alterations in these interconnected pathways. We selected 48 patients with IDA and 48 healthy participants based on diagnostic criteria for iron deficiency. NMR spectroscopy was utilized for metabolite profiling. Chenomx, MetaboAnalyst, and R were employed for data analysis. NMR analysis identified and quantified 119 metabolites. Significant metabolites between the two groups were determined using fold change (FC) analysis and t-test results. The FC values of the metabolite group ranked the following compounds in descending order: Acetoin, 2-Hydroxy-3-methylvalerate, Homogentisate, 3-Methylxanthine, Desaminotyrosine, Succinate, Methylguanidine, Guanidinosuccinate, 3-Methylglutarate, Ethanol, Carnosine, SN-Glycero-3-phosphocholine, and Homocysteine. The IDA patients had increased levels of Acetoin, 2-Hydroxy-3-methylvalerate, Homogentisate, 3-Methylxanthine, Succinate, Guanidinosuccinate, Ethanol, Carnosine, and SN-Glycero-3-phosphocholine, while decreased levels of Desaminotyrosine, Methylguanidine, and 3-Methylglutarate were observed. Our findings provide valuable insights into the different metabolites and pathways associated with IDA. Further molecular research is needed to validate these findings.

**Keywords:** Iron deficiency anemia; metabolomics; nuclear magnetic resonance; metabolites; 3-methylxanthine. © 2023 ACG Publications. All rights reserved.

## 1. Introduction

Iron deficiency anemia (IDA) continues to be a public health challenge in developing countries, with an estimated prevalence ranging from 2 to 5%. Inadequate production of hemoglobin due to iron deficiency impairs the oxygen-carrying capacity of the blood, resulting in symptoms such as fatigue, dyspnea, weakness, and tachycardia. However, IDA may also cause severe complications such as impaired cognitive development in children and prematurity with low birth weight during pregnancy

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which necessitates policymakers to implement national prevention programs. The World Health Organization (WHO) advocates for cost-effective prevention programs that include iron supplementation guidelines and nutrition-enrichment strategies to combat IDA. These initiatives aim to eliminate IDA and its adverse effects on human health, emphasizing the importance of improving iron intake and raising public awareness about the condition. Overall, implementing evidence-based interventions for IDA prevention and treatment remains a critical priority in global health policy [1].

Efforts to address iron deficiency anemia (IDA) have focused on developing optimal therapeutic approaches for patients with varying needs. Fine-tuning treatment regimens is critical due to the potential risks of both iron deficiency and iron overload, as iron is a vital component of redox systems that generate mild oxidative stress in cellular environments [2]. The establishment of convenient therapies requires first-line identification and treatment of the underlying cause of IDA along with supplementation. In this respect, our study is designed to build a starting point for a better understanding of individual metabolic profiles and their mechanistic implications in a small group of IDA patients as well as to introduce novel targets for further research. Setting out to assess serum metabolomes of adult iron deficiency anemia patients for the first time, the study integrates the strengths of an established analytical tool in the metabolomics field,  $^1\text{H}$  nuclear magnetic resonance (NMR) for global evaluation.

Thriving further in pharmaceuticals, the metabolomic search has proved its potential in clinical usage for early detection, personalized therapies, and eliciting obscure roots in numerous pathologies. It is a promising approach for providing crucial data to other 'omics' fields detecting low-molecular-weight metabolites in biospecimens simultaneously in an easy-to-implement way. One of the major instruments of metabolomics, NMR proved to have the most repeatable and high-throughput results among other techniques [3]. The most eminent benefit over other techniques involves that it does not require an internal standard for each metabolite of interest and elaborate preparation steps, and yields highly reproducible results. Yet, with micromolar level sensitivity and the ability to cover primarily small metabolites, NMR may not provide the full range of metabolite spectrum separately. To overcome sensitivity problems, the rapidly growing field of NMR has been enhanced via the utilization of new techniques such as dynamic nuclear polarization [4].

In order to define the serum metabolomic status of patients with IDA using NMR for the first time, this preliminary research was conducted. Despite the major obstacle of multiple interfering factors that affect the immediate metabolic profiles of participants, we aimed to establish an initial assessment of blood metabolome under iron-deficient conditions and to identify the adaptation mechanisms that counterbalance the insufficiency of this crucial element. The findings of this study may provide insight into new metabolic interactions related to iron deficiency and contribute to a better understanding of the wide range of symptoms associated with IDA.

## 2. Experimental

### 2.1. Study Design, Sample, and Clinical Data Collection

48 patients and 48 healthy subjects were chosen according to power analysis using the G power program (3.1.9.4 version). Patients were diagnosed with IDA according to low serum ferritin level (Fer,  $<30$  ng/L), low hemoglobin (Hb,  $<7.7$  mmol/L in men and  $7.4$  mmol/L in women), low serum iron (Fe,  $<7.1$   $\mu\text{g/L}$ ), and a high total iron-binding capacity (TIBC,  $>13.1$   $\mu\text{mol/L}$ ). Exclusion criteria included pregnancy, smoking, hypertension, diabetes mellitus, malnutrition, tumor, and any chronic disease presence. Samples were collected from volunteers during their initial visit to the hematology department at Bezmialem Vakif University Hospital, prior to the administration of any supplements. The biochemical analysis was done in Bezmialem Vakif University Biochemistry laboratory on the day the serums were collected (Abbott ARCHITECT ci16200 clinical chemistry analyzer). The serums were then aliquoted and stored at  $-80^\circ\text{C}$  for the NMR analysis. The clinical research ethics committee of Bezmialem Vakif University approved the study on 27.07.2021 (Number: 2021/286).

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### 2.2. Metabolomic Analysis

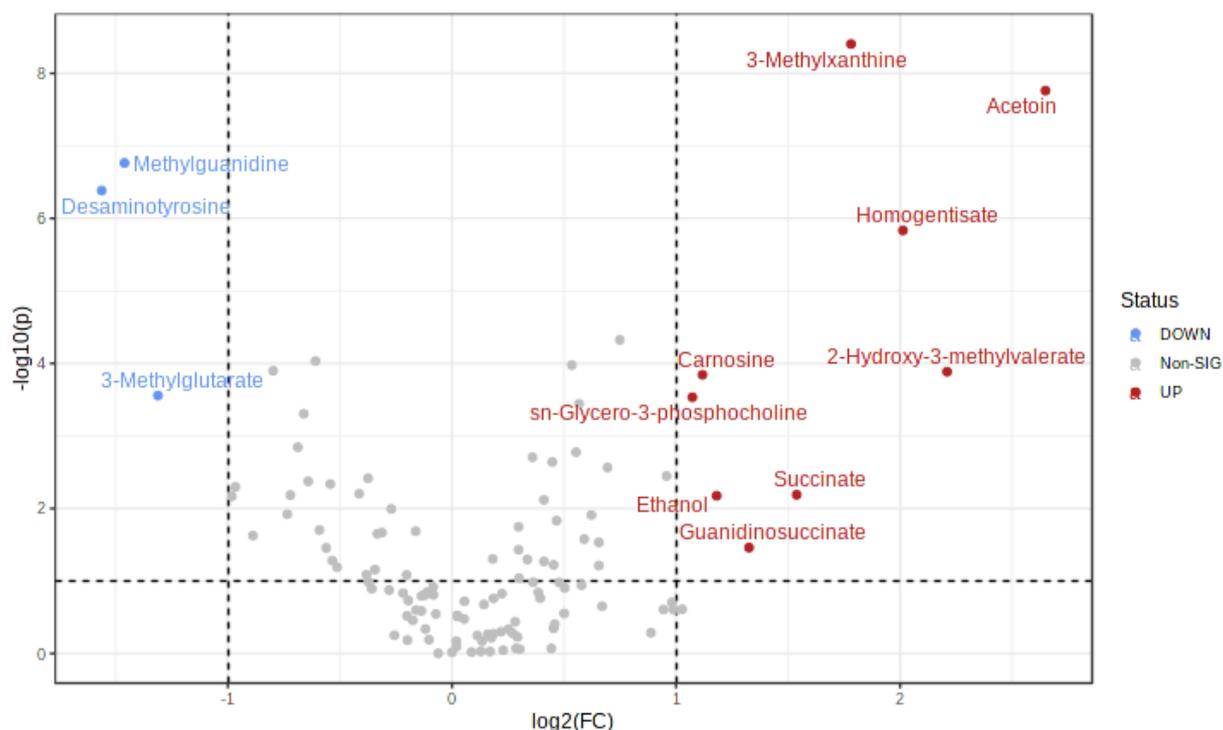
Serums were analyzed with the NMR method described in an earlier report with slight changes [5]. After thawing at room temperature, 900  $\mu$ L of each sample were mixed with 100  $\mu$ L of internal standard fumaric acid (so that the final concentration is 100  $\mu$ M) and then with 2:1 of methanol. They were centrifuged at 10,000  $\times$  g for 1,5 hours at 4  $^{\circ}$ C. The supernatants were collected and concentrated using the Eppendorf™ Vacufuge™ concentrator at the 60  $^{\circ}$ C alcohol application mode. Protein-debris precipitated serum remnants were dissolved in 500  $\mu$ l of phosphate buffer (pH= 7.2, 60 mM) and 100  $\mu$ L of 1.75 mM trimethylsilylpropanesulfonate (DSS) that dissolved in pure deuterium oxide (D<sub>2</sub>O) was added to each. In a 500 MHz NMR instrument, <sup>1</sup>H 1D (one dimensional) experiments were employed with nuclear Overhauser effect spectroscopy (NOESY) and Carr-Purcell-Meiboom-Gill (CPMG) sequences (Bruker Avance, MA). The raw data were processed with TopSpin software (4.1.3, Bruker). Metabolite identification and quantitative analysis were done using Chenomx NMR Suite software (according to NOESY sequence results for the quantification step). The NMR spectra were obtained with 4 seconds of acquisition time (AQ) and the spectral width of 12 ppm (SW). The following parameters included delay time (D1) as 5 sec., mixing time (D8) as 0.2 sec., number of dummy scans (DS) as 8, number of scans (NS) as 128, the center of the spectrum (O1P) as 4.7 ppm, receiver gain (RG) as 101. Fourier transformation, auto phasing, water suppression, and baseline corrections were carried out with TopSpin (Bruker, MA). The chemical shifts were calibrated referencing DSS ( $\delta$  0.0 ppm), and the quantification was done depending on the control concentration (100  $\mu$ M fumarate) in each sample (Chenomx Inc., Canada). As a consequent step, samples were analyzed with a CPMG pulse sequence to suppress the broad signals and to validate the NOESY experiment results. Typical spectra of an IDA patient and control are shown in Supplementary File (with NOESY and CPMG sequences, Figure S1). The two distinct experiment results were similar and correlated, therefore the statistical analyses were conducted preferring NOESY data.

### 2.3. Statistical Analysis

The routine and demographic parameters were evaluated in both groups. Descriptive statistical analysis was performed using R. Data were expressed as mean with standard error and median with median absolute deviation (MAD). After peak matching and quantification in the NMR platform, normalization was done by sample median, cubic root transformation, and autoscaling. Univariate analysis for group-specific differences was employed using a two-sample t-test (MetaboAnalyst, 5.0) for normalized values. Multivariate data analysis was then performed (MetaboAnalyst, 5.0) with the exploratory analyses (principal component (PCA) and sparse partial least squares discriminant analysis (sPLS-DA). The metabolite set enrichment (MSEA) and pathway impact analyses were done to assess a set of metabolites that are biologically meaningful for certain pathways. The receiver operating characteristics (ROC) analysis was finally carried out to identify the global disease biomarker panel.

## 3. Results and Discussion

The socio-demographic data comparing the two groups showed no difference in age, gender, body mass index (BMI), serum fasting glucose, albumin, creatinine, urea, and total protein levels ( $p > 0.05$ ). The iron deficiency panel included serum iron (Fe), ferritin (Fer), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), red cell distribution width (RDW), total and unsaturated iron-binding capacity (TIBC, UIBC) and the patient results were consistent with the diagnostic criteria (Supplementary File 1, Table S1).

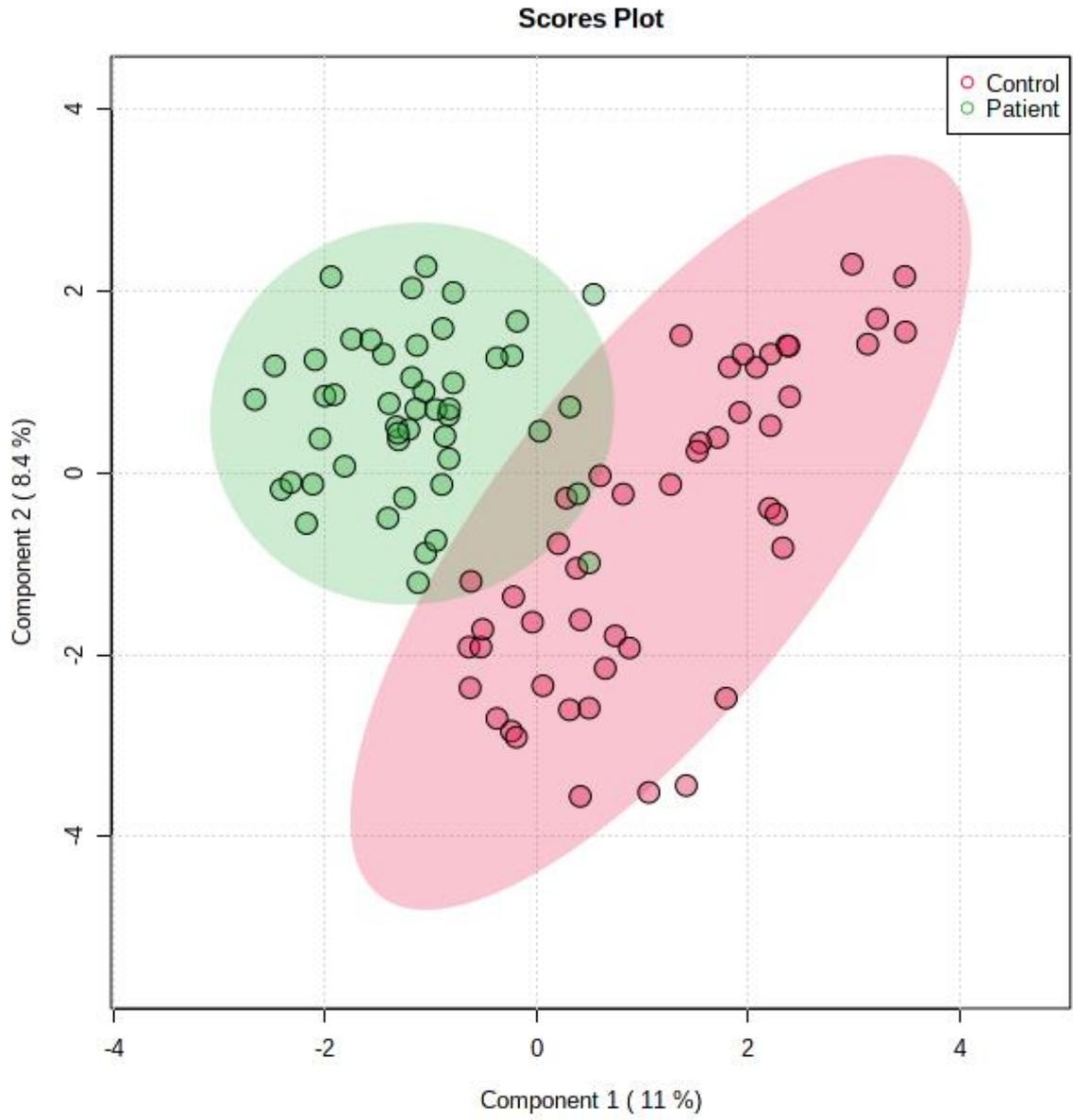


**Figure 1.** Important features selected by volcano plot with FC and t-tests (FC values log scaled, p values transformed by  $-\log_{10}$ , the red circles above the threshold, the blue circles below the threshold)

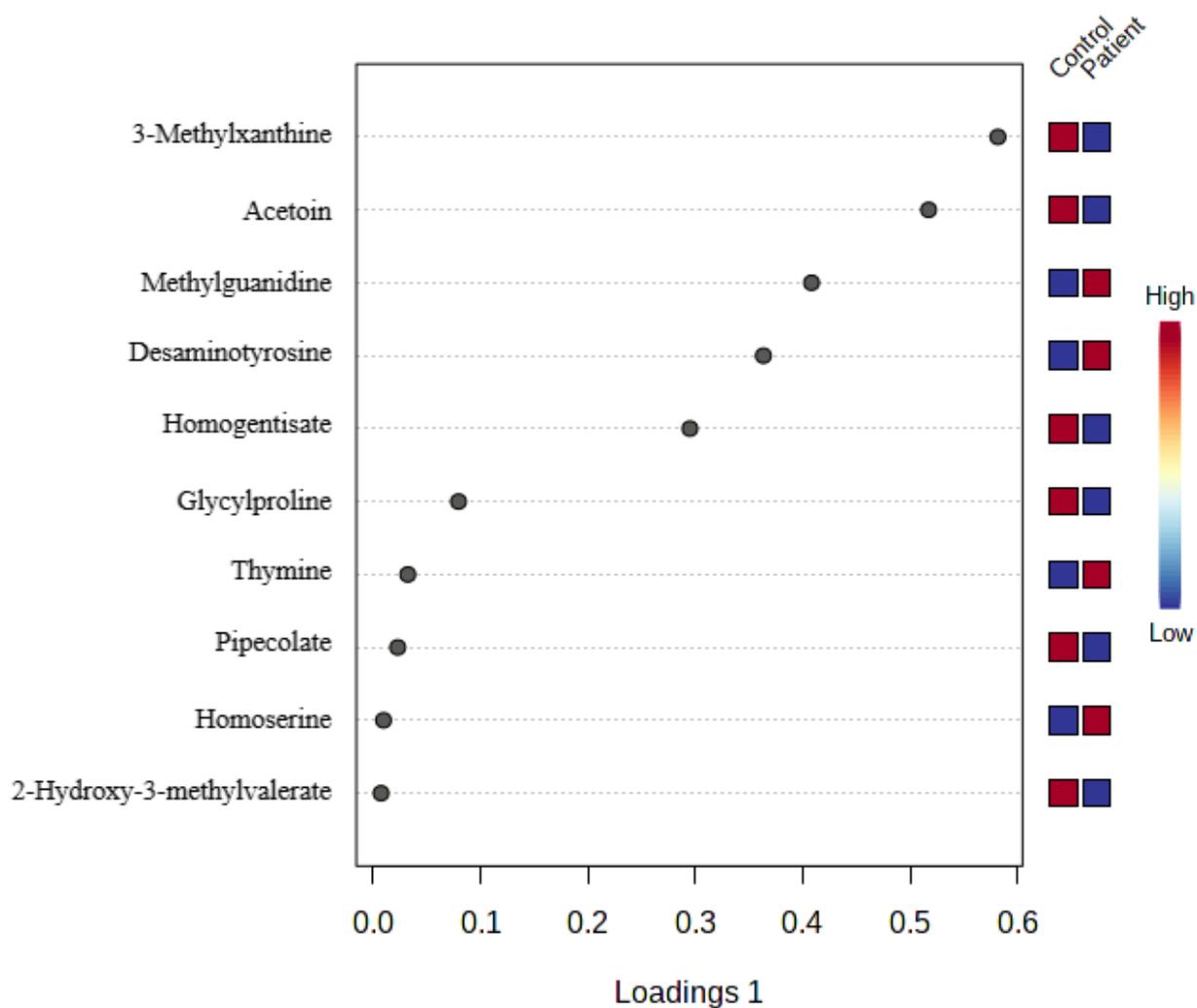
Initially, a total of 119 compounds were identified and quantified through NMR analysis. The median values for each compound, grouped according to their respective categories, are presented in Table 3. Significant metabolites were selected based on their fold change (FC) analysis, with a threshold of 2, and t-test ( $p < 0.05$ ), which allowed for the classification of distinct groups (Figure 1). The metabolite group included Acetoin, 2-Hydroxy-3-methylvalerate, Homogentisate, 3-Methylxanthine, Desaminotyrosine, Succinate, Methylguanidine, Guanidinosuccinate, 3-Methylglutarate, Ethanol, Carnosine, sn-Glycero-3-phosphocholine, and Homocysteine, ranked in descending order based on their respective FC values (Table 1).

**Table 1.** Important metabolites selected by fold-change (FC) analysis and t-test r results (FC values log scaled, p values transformed by  $-\log_{10}$ )

Metabolites	$-\log_{10}(p)$	$\log_2(FC)$
Acetoin	7.7612	2.6489
2-Hydroxy-3-methylvalerate	3.8852	2.2099
Homogentisate	5.8344	2.0128
3-Methylxanthine	8.4042	1.7811
Desaminotyrosine	6.3836	-1.5635
Succinate	2.1895	1.5385
Methylguanidine	6.7640	-1.4615
Guanidinosuccinate	1.4606	1.3259
3-Methylglutarate	3.5574	-1.3122
Ethanol	2.1754	1.1811
Carnosine	3.8430	1.1180
SN-Glycero-3-phosphocholine	3.5339	1.0729



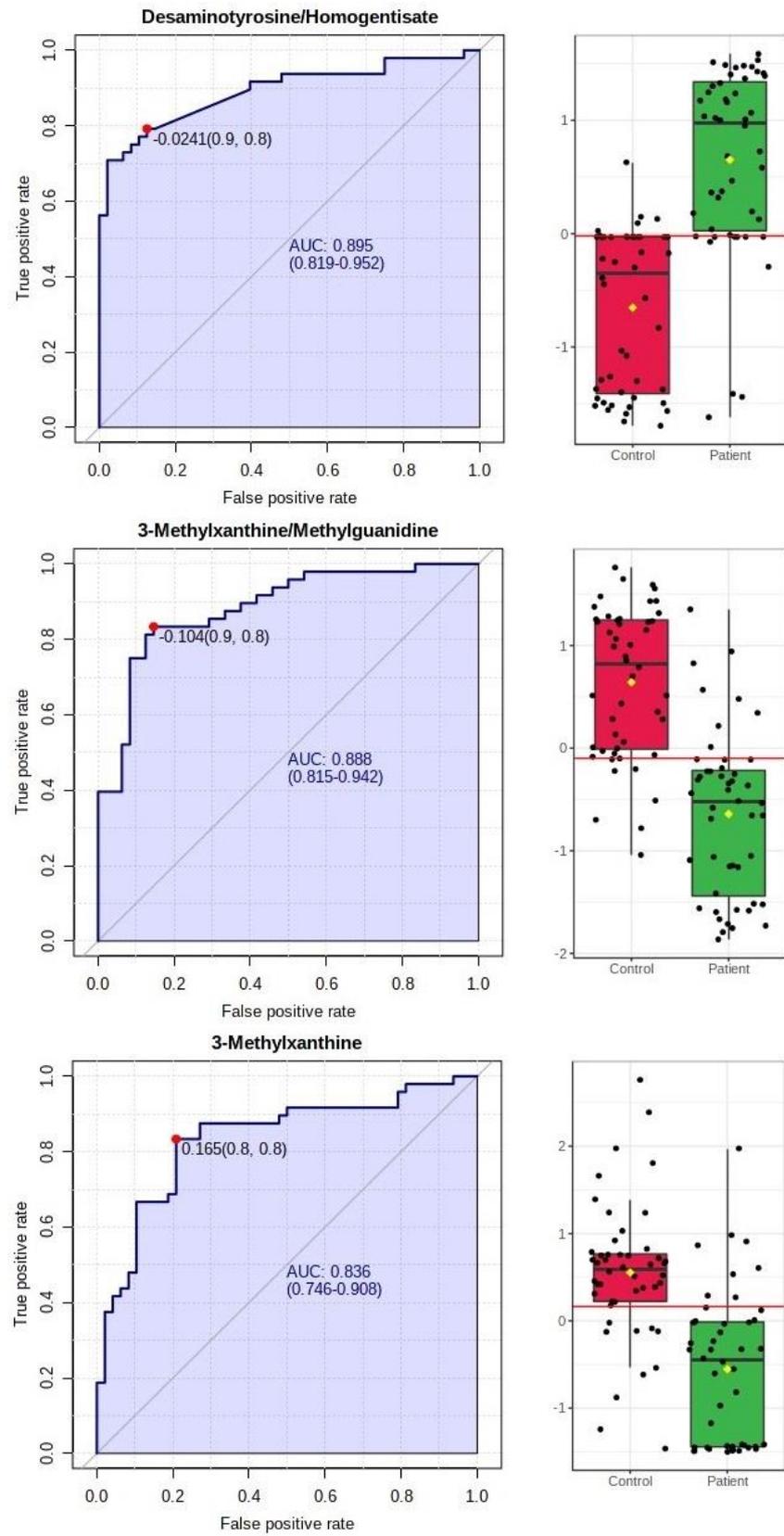
**Figure 2.** sPLS-DA score plot of all metabolites



**Figure 3.** sPLS-DA loading plot of the top ranked metabolites

sPLS-DA analysis was performed to recognize the metabolites associated with IDA (Figure 2). As a result, it was observed that 3-Methylxanthine, Acetoin, Methylguanidine, Desaminotyrosine, Homogentisate, Glycylproline, Thymine, Pipecolate, Homoserine, 2-Hydroxy-3-methylvalerate were top-ranked metabolites (Figure 3).

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**Figure 4.** The ROC curve of a metabolite or metabolite ratio pair

As a result of the receiver operating characteristic (ROC) test, 22 metabolite or metabolite ratio pairs as a biomarker with an area under curve (AUC) result above 0.8 were observed (Table 2). Desaminotyrosine/Homogentisate metabolite pair ratio was the highest AUC value of 0.891. As the single metabolite, 3-Methylxanthine had the highest AUC value of 0.835 among other metabolites (Figure 4).

**Table 2.** AUC results for metabolite or metabolite ratio pairs

<b>Metabolites</b>	<b>AUC</b>
Desaminotyrosine/Homogentisate	0.891
3-Methylxanthine/Methylguanidine	0.884
Acetoin/Anserine	0.879
3-Methylxanthine/Desaminotyrosine	0.868
Homogentisate/Methylguanidine	0.862
3-Methylxanthine/Arabinitol	0.856
Acetoin/Desaminotyrosine	0.852
3-Methylxanthine/Anserine	0.851
Homogentisate/Homoserine	0.846
Acetoin/Caprato	0.845
Acetoin/Methylguanidine	0.842
3-Methylxanthine/Thymine	0.841
3-Methylxanthine/Mannitol	0.840
3-Methylxanthine/Trimethylamine	0.839
2-Aminobutyrate/3-Methylxanthine	0.838
3-Methylxanthine	0.835
Desaminotyrosine/SN-Glycero-3-phosphocholine	0.832
Homogentisate/Mandelate	0.829
Homogentisate/Thymine	0.829
3-Methylxanthine/Mandelate	0.821
Acetoin	0.803
Glycylproline	0.780

**Table 3.** Mean  $\pm$  SE, median and MAD values of the compounds are presented studied groups

<b>Metabolites</b>	<b>Control (<math>\mu</math>M)</b>			<b>Patient (<math>\mu</math>M)</b>		
	<b>Mean <math>\pm</math> SE</b>	<b>Median</b>	<b>MAD</b>	<b>Mean <math>\pm</math> SE</b>	<b>Median</b>	<b>MAD</b>
3-Diaminopropane	126.27 $\pm$ 5.3	120.30	33.06	106.15 $\pm$ 9.8	100.10	48.48
3-Dimethylurate	26.01 $\pm$ 1.8	25.50	11.12	23.71 $\pm$ 2.1	23.25	7.34
7-Dimethylxanthine	15.29 $\pm$ 1.2	15.80	5.49	15.48 $\pm$ 2.1	14.60	10.08
Aminobutyrate	58.95 $\pm$ 5.9	48.40	37.29	49.91 $\pm$ 5	45.50	28.24
Hydroxy-3-methylvalerate	44.07 $\pm$ 6	39.60	36.03	99.22 $\pm$ 8.9	100.25	36.47
Hydroxyisobutyrate	53.82 $\pm$ 3.1	55.80	18.98	36.53 $\pm$ 3.6	32.50	20.39
Hydroxyisocaproate	40.17 $\pm$ 3.1	38.60	20.76	46.47 $\pm$ 3.4	46.25	23.80
Oxobutyrate	54.67 $\pm$ 4.5	57.20	26.02	41.31 $\pm$ 4.6	37.80	19.13
Oxoglutarate	21.24 $\pm$ 2.5	18.70	17.49	26.1 $\pm$ 4.2	21.10	16.75
Phenylpropionate	18.8 $\pm$ 1.3	16.15	8.67	19.12 $\pm$ 1.9	16.30	11.79

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Table 3 continued..

Metabolites	Control (μM)			Patient (μM)		
	Mean ± SE	Median	MAD	Mean ± SE	Median	MAD
4-Dihydroxybenzeneacetate	59.73 ± 4.1	58.35	21.28	61.6 ± 5.8	58.25	33.73
Hydroxy-3-methylglutarate	61.32 ± 4.5	60.90	25.57	39.78 ± 3.5	35.55	21.57
Hydroxyisobutyrate	29.42 ± 1.6	27.05	11.19	30.13 ± 2.7	27.25	16.53
Hydroxyisovalerate	16.84 ± 1.2	15.00	9.19	20.64 ± 3.1	15.30	12.90
Hydroxykynurenine	20.32 ± 2.4	16.15	8.52	24.67 ± 5.5	18.60	15.42
Methylglutarate	44.32 ± 6.2	39.90	37.51	26.72 ± 6.4	22.70	20.46
Methylxanthine	13.76 ± 3.6	7.10	4.60	22.28 ± 4.8	17.90	8.52
Aminobutyrate	27.58 ± 2.1	25.20	15.57	25.32 ± 2.8	21.20	18.53
Carboxyglutamate	117.91 ± 14.7	100.55	91.55	130.95 ± 17.2	103.60	67.83
Guanidinobutanoate	160.44 ± 10	145.90	62.27	130.36 ± 10.6	117.00	47.89
Hydroxyphenylacetate	16.48 ± 1.7	14.80	7.12	16.7 ± 2.2	16.40	13.64
6-Dihydrothymine	186.46 ± 10.5	166.20	54.71	193.27 ± 19.3	144.55	99.33
6-Dihydrouracil	54.19 ± 4.5	46.00	16.90	51.46 ± 4	45.30	20.90
Hydroxylysine	124.13 ± 11.5	114.15	86.29	121.93 ± 15.4	108.20	88.81
Hydroxytryptophan	22.64 ± 3.3	17.60	17.72	19.13 ± 3	14.00	10.38
Acetamide	29.35 ± 3.9	26.40	24.02	27 ± 3.5	22.20	16.61
Acetate	252.78 ± 24.8	200.10	119.87	192.75 ± 14	156.25	67.46
Acetoacetate	21.2 ± 2.2	22.30	12.90	25.06 ± 1.8	24.65	11.64
Acetoin	4.12 ± 0.6	3.80	1.93	11.91 ± 1.5	9.90	9.64
Agmatine	57.48 ± 6.1	52.00	37.29	47.93 ± 7.6	36.50	43.96
Alanine	433.6 ± 21.8	397.20	134.55	357.8 ± 32.3	318.90	225.80
Alloisoleucine	52.25 ± 6.1	42.80	36.62	48.55 ± 5.1	53.20	28.91
Anserine	30.32 ± 2.8	28.50	19.57	27.13 ± 3.7	21.70	20.31
Arabinitol	107.38 ± 4.7	104.00	30.99	84.89 ± 7.3	75.00	31.88
Arginine	71.52 ± 6.9	59.60	26.17	74.16 ± 13.8	50.25	44.55
Caprate	156.49 ± 10.3	144.30	56.86	105.4 ± 10	89.50	53.97
Caprylate	64.5 ± 6.5	55.95	34.69	55.66 ± 7.5	49.90	43.44
Carnitine	41.44 ± 2.8	39.55	20.90	32.03 ± 2.4	33.20	15.79
Carnosine	67.08 ± 8.2	56.20	34.69	96.71 ± 8.5	85.80	50.85
Cellobiose	102.41 ± 9.7	93.25	50.41	113.88 ± 12	101.60	98.74
Cholate	27.9 ± 2	27.95	11.71	29.92 ± 1.7	30.00	10.82
Citrate	20.02 ± 2.2	16.70	12.01	19.85 ± 3.1	14.90	18.53
Creatinine	22.91 ± 2.3	22.70	10.82	29.24 ± 4.1	21.00	13.05
Cystine	107.27 ± 9.5	103.90	70.13	103.55 ± 11.2	91.70	72.94
D-Threitol	88.85 ± 8.5	87.75	55.67	55.3 ± 10.5	43.35	25.57
Desaminotyrosine	18.17 ± 2	15.30	13.94	14.74 ± 1.6	15.20	6.08
Dimethyl.sulfone	10.31 ± 2.4	7.65	5.19	19.69 ± 12.1	5.85	6.23
Dimethylamine	6.09 ± 0.7	5.10	2.67	5.92 ± 0.8	4.85	5.11
Erythritol	51.88 ± 4.9	47.50	32.32	46.33 ± 5.2	45.20	33.36

Table 3 continued..

Metabolites	Control ( $\mu\text{M}$ )			Patient ( $\mu\text{M}$ )		
	Mean $\pm$ SE	Median	MAD	Mean $\pm$ SE	Median	MAD
Ethanol	135.79 $\pm$ 18.7	132.80	127.36	224.01 $\pm$ 33.5	179.90	154.78
Ethylene.glycol	12.04 $\pm$ 1.4	10.15	6.08	15.36 $\pm$ 1.6	15.45	7.86
Ethylmalonate	54.71 $\pm$ 4.6	57.45	23.50	51.58 $\pm$ 9.2	26.00	32.47
Fucose	169.69 $\pm$ 13.6	158.70	106.30	191.16 $\pm$ 25.5	176.20	168.65
Galactitol	44.59 $\pm$ 4	39.10	22.24	36.1 $\pm$ 2.7	33.90	14.38
Glucitol	33.02 $\pm$ 4.2	31.20	22.83	32.5 $\pm$ 4.8	30.10	24.02
Glucuronate	116.59 $\pm$ 12.2	103.65	81.25	71.55 $\pm$ 11	56.45	60.79
Glutamate	160.04 $\pm$ 8.6	153.45	46.03	209.39 $\pm$ 16.2	199.95	128.24
Glutamine	83.04 $\pm$ 7.9	73.90	46.18	156.87 $\pm$ 18.8	144.70	97.26
Glycerol	77.13 $\pm$ 6.7	75.40	46.41	61.32 $\pm$ 10.9	36.80	33.21
Glycine	212.54 $\pm$ 24.8	221.10	132.10	196.73 $\pm$ 25.1	214.45	174.35
Glycocholate	39.57 $\pm$ 2.7	39.40	17.20	42.86 $\pm$ 4.3	38.40	26.84
Glycylproline	101.26 $\pm$ 6.8	100.15	47.59	140.59 $\pm$ 7.1	142.25	46.26
Guanidinosuccinate	17.94 $\pm$ 3.1	13.40	11.49	33.57 $\pm$ 4.8	28.00	16.90
Histidine	48.27 $\pm$ 4.2	45.10	31.36	53.29 $\pm$ 5.3	44.70	38.84
Homocystine	19.7 $\pm$ 2.7	18.10	12.60	27.59 $\pm$ 5.4	20.60	24.61
Homogentisate	11.9 $\pm$ 2.1	13.25	10.53	15.84 $\pm$ 1.8	14.80	9.79
Homoserine	82.58 $\pm$ 7.2	76.65	45.44	102.82 $\pm$ 15.4	83.45	53.30
Homovanillate	6.72 $\pm$ 1	5.70	5.56	10.27 $\pm$ 1	9.70	6.08
Indole-3-lactate	39.74 $\pm$ 4.1	38.80	21.94	31.37 $\pm$ 5.3	19.05	24.39
Isocitrate	86.56 $\pm$ 9.2	78.70	73.54	92.79 $\pm$ 10.8	101.30	85.99
Isoleucine	75.39 $\pm$ 4.1	72.65	28.61	86.39 $\pm$ 9.2	71.50	42.25
Isopropanol	69.88 $\pm$ 8.7	46.10	28.47	75.54 $\pm$ 10	54.65	28.61
Kynurenine	23.46 $\pm$ 3.9	21.30	18.90	28.13 $\pm$ 5.7	19.00	11.86
Lactate	1983.89 $\pm$ 113.4	1908.70	674.36	2247.2 $\pm$ 212.4	1953.30	938.34
Leucine	94.4 $\pm$ 5.6	90.55	45.89	97.13 $\pm$ 8.9	84.25	66.42
Mandelate	24.68 $\pm$ 1.7	25.50	12.97	32.54 $\pm$ 3.3	32.65	18.16
Mannitol	63.18 $\pm$ 4.4	64.55	23.57	44.83 $\pm$ 3.4	38.20	20.68
Methionine	17.74 $\pm$ 1.9	19.90	10.45	17.71 $\pm$ 1.7	16.90	10.23
Methylamine	12.37 $\pm$ 1.4	9.90	5.78	14.41 $\pm$ 1.7	14.30	8.67
Methylguanidine	7.4 $\pm$ 0.7	6.20	3.71	5.5 $\pm$ 1.3	3.20	3.41
Methylmalonate	139.45 $\pm$ 12.7	125.30	75.98	117.13 $\pm$ 18.8	57.60	57.15
Methylsuccinate	30.52 $\pm$ 2.9	27.90	19.64	36.5 $\pm$ 3.2	31.85	20.46
NN-Dimethylformamide	9.81 $\pm$ 1.3	7.60	6.82	10.7 $\pm$ 2	8.60	7.86
N-Acetylaspartate	17.79 $\pm$ 2.5	12.50	13.34	18.18 $\pm$ 3	15.70	16.75
N-Acetylcysteine	32.28 $\pm$ 2	31.90	13.49	29.31 $\pm$ 3.7	26.90	24.91
N-Acetylglucosamine	124.11 $\pm$ 5	128.20	34.62	127.51 $\pm$ 5.8	124.35	23.05
N-Carbamoyl $\beta$ -Alanine	38.42 $\pm$ 3.1	35.10	24.09	34.64 $\pm$ 3.4	30.25	21.72
N-Carbamoylaspartate	109.5 $\pm$ 10.5	104.40	75.54	97.64 $\pm$ 13.5	73.30	70.28

Table 3 continued..

Metabolites	Control (μM)			Patient (μM)		
	Mean ± SE	Median	MAD	Mean ± SE	Median	MAD
N-Nitrosodimethylamine	20.65 ± 3.2	18.50	13.05	17.04 ± 2.1	14.10	9.56
N-Phenylacetylphenylalanine	20.72 ± 2.3	19.10	9.79	14.33 ± 2.1	12.00	5.04
N-6-Acetyllysine	29.76 ± 1.9	29.90	13.64	24.2 ± 3	21.00	20.46
<i>o</i> -Phosphoethanolamine	213.83 ± 14.8	218.30	114.61	217.09 ± 24.1	163.45	164.72
Pantothenate	32.02 ± 2.5	30.45	15.57	28.95 ± 2.9	29.55	20.90
Pipecolate	97.98 ± 7	91.40	45.22	109.15 ± 7.7	100.55	47.67
Proline	446.41 ± 19.7	445.20	113.57	417.7 ± 20.9	377.45	82.36
Pyridoxine	16.28 ± 1.5	14.80	11.27	14.08 ± 2.2	11.65	11.12
Pyroglutamate	189.41 ± 17.6	154.90	103.63	143.1 ± 14.2	135.80	88.81
<i>S</i> -Sulfocysteine	38.88 ± 3.9	36.50	25.95	44.74 ± 5.3	38.90	31.06
Saccharopine	160.88 ± 8.1	155.05	55.67	157.89 ± 12.3	141.75	64.49
Succinate	10.97 ± 1.2	10.75	4.37	13.87 ± 1.6	12.35	9.27
Sucrose	30.83 ± 3.3	27.20	20.90	34.72 ± 3.6	34.35	22.16
Taurine	157.15 ± 9.4	153.35	42.25	159.99 ± 8.2	160.20	60.12
Threonine	140.6 ± 9.8	136.40	57.01	109.78 ± 9.5	101.15	70.42
Thymine	8.12 ± 0.7	7.40	4.74	10.51 ± 1.2	9.55	7.19
Trimethylamine	2.88 ± 0.6	2.15	1.63	2.29 ± 0.4	1.85	1.41
Trimethylamine N-oxide	55.79 ± 3.4	55.80	19.87	58.91 ± 4.9	53.90	10.23
Tryptophan	25.6 ± 3.1	23.75	23.43	32.83 ± 5.4	19.50	16.61
Tyrosine	36.99 ± 3.1	34.20	22.09	42.09 ± 4.6	43.65	41.44
Uracil	12.9 ± 1.2	12.25	6.67	13.07 ± 1.6	14.05	10.45
Urea	166.66 ± 33.4	120.40	67.46	182.98 ± 16.7	165.15	110.38
Valine	58.34 ± 5.3	50.60	38.10	67.46 ± 9.2	45.60	32.47
Xylitol	79.1 ± 7.4	84.40	37.51	67.21 ± 6.5	69.65	39.73
Xylose	153.2 ± 10.3	148.25	83.03	179.04 ± 15.8	171.50	74.13
<i>myo</i> -Inositol	64.24 ± 8.6	49.30	28.02	66.14 ± 4.9	61.10	25.06
SN-Glycero-3-phosphocholine	43.91 ± 6	32.15	24.69	43.11 ± 5.4	45.40	22.39
<i>trans</i> -4-Hydroxy-L-proline	116.48 ± 7.6	111.10	44.48	153.27 ± 10.2	155.00	61.90
β-Alanine	73.6 ± 8.1	75.45	47.29	63.39 ± 7.8	41.95	30.99
Γ-Glutamylphenylalanine	44.2 ± 2.7	43.15	16.38	59.37 ± 4.3	60.20	27.13
3-Methylhistidine	22.31 ± 4	12.65	12.31	19.45 ± 2	19.80	13.94

The present study aimed to investigate the chemical profiles of IDA patient serums in order to identify metabolic perturbations and their potential implications in explaining disease-related symptoms. The discussion of different metabolite compositions with significantly altered amounts between patients and healthy individuals focused on the individual evaluation of root causes rather than a diagnostic perspective. As IDA can have various causes, the use of metabolomic tools to phenotype prominent groups within this heterogeneous population could provide valuable information for tailored treatment strategies that meet the needs of each patient.

While there are no human studies on iron deficiency anemia in the field of metabolomics in the current literature, there are few studies investigating the animal metabolome. What these studies

commonly found was the alteration of the neurochemical profile, especially the pathways associated with energy metabolism. Cerebrospinal fluid (CSF) analysis of infant monkeys revealed that citrate to pyruvate, citrate to lactate, and pyruvate to glutamine ratios were significantly different in iron deficiency which was then improved with dietary iron [6]. Dendritic architectures of hippocampal pyramidal neurons in IDA piglets turned out to be altered with iron accumulation due to over-supplementation [7].

The significant findings of 3-Methylxanthine, Methylguanidine, and Anserine in metabolites that are commonly used as biomarkers for various gastrointestinal disorders, particularly Eosinophilic esophagitis, suggest that diet-induced iron deficiency may be a contributing factor [8-10]. Lower levels of carnosine (beta-alanyl-L-histidine), a dipeptide that acts as a potent scavenger of reactive oxygen species and helps stabilize muscles, may indicate impaired muscle function under iron-deficient conditions. This suggests that carnosine could be a valuable target for alleviating muscle-related symptoms in individuals with IDA. Additionally, decreased levels of trans-4-hydroxy-L-proline (4-hydroxyproline), a major component of collagen and elastin, suggest a decelerated collagen metabolism in IDA. Proline is converted to 4-hydroxyproline by prolyl 4-hydroxylase, which requires iron as a cofactor (with ascorbate as a coenzyme). Iron deficiency may lead to lower serum levels of 4-hydroxyproline, which confirms the absence of muscle damage in IDA [11].

When ROC analysis was performed on quantitative NMR metabolite data, significant results were obtained in the 23 metabolites or metabolite pairs ratio but were insufficient. Considering more valuable biomarkers were identified previously (such as the transferrin/ferritin ratio [12]), the significant metabolites and their implemented pathways contribute more to a better understanding of pathophysiological background and phenotyping by referring to the individual diversity rather than early detection.

Due to the limited sample size of this work, we rigorously refrain from setting exact assumptions about the findings of significantly altered pathways. However, it was clear that amino acid-related pathways were the most affected ones, assumably on the ground of shifted energy metabolism through the non-oxidative pathways. NMR metabolomics data identified several metabolites and related pathways that were differential among groups and as the in-depth analysis confirmed. The serums of IDA patients displayed increased levels of Acetoin, 2-Hydroxy-3-methylvalerate, Homogentisate, 3-Methylxanthine, Succinate, Guanidinosuccinate, Ethanol, Carnosine, and sn-Glycero-3-phosphocholine while decreased levels of Desaminotyrosine, Methylguanidine, and 3-Methylglutarate. The pathway perturbances were evaluated in the common ground of hypoxia-induced changes.

Major drawbacks of the current study included problems that may exist in any other metabolomic research. These involve the interindividual variations over time regarding metabolite levels and lifestyle differences (diet, smoking, exercise) potentially affecting results. Thus, we suggest future studies consider these problems and apply time-specific measurements. Another downside of the quantitative serum metabolome analysis was that certain tissue-originated metabolites (biomarker candidates) are found in lower amounts and are diluted within the bloodstream. The detection of these small effect-size biomarkers needs robust technologies and an integrated approach. This limitation underlines the importance of the comparison between group levels. With these, to take an integral metabolic picture of circulating serum status, complementary techniques such as genomics and proteomics are required. Besides, the sample set included a limited number of individuals to make strong assumptions about current findings. With these impediments, our study still opens novel research inquiries as a preliminary step in IDA metabolomics.

#### 4. Conclusions

The primary aim of this study was to employ metabolomic tools to gain mechanistic insights into individual disease-driving forces, with the goal of enabling personalized care for IDA patients. Given that genetic predispositions and nutritional habits are specific to societies, we assert that a population-based evaluation of IDA is necessary. In this study, we utilized quantitative NMR metabolomics analysis to comprehensively evaluate patient sera and identified new target metabolites and their associated pathways as an initial step. Perturbed amino acid levels and their enriched pathways were among the most significant findings. Although the underlying mechanisms for these alterations remain unknown,

<sup>1</sup>H NMR spectroscopy for diagnosis of iron deficiency anemia

metabolomics shows promise in providing valuable information on low-molecular-weight compounds in biospecimens, which can be integrated with complementary ‘omics’ data.

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### Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/journal-of-chemical-metrology>

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