Nontargeted and targeted metabolomics approaches reveal the key amino acid alterations involved in multiple myeloma

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Purpose: Multiple myeloma (MM), a kind of malignant neoplasm of plasma cells in the bone marrow, is a refractory disease. Understanding the metabolism disorders and identification of metabolomics pathways as well as key metabolites will provide new insights for exploring diagnosis and therapeutic targets of MM. **Methods**: We conducted nontargeted metabolomics analysis of MM patients and normal controls (NC) using ultra-high-performance liquid chromatography (UHPLC) combined with quadrupole time-of-flight mass spectrometry (Q-TOF-MS) in 40 cases of cohort 1 subjects. The targeted metabolomics analysis of amino acids using multiple reaction monitoring-mass spectrometry (MRM-MS) was also performed in 30 cases of cohort 1 and 30 cases of cohort 2 participants, to comprehensively investigate the metabolomics disorders of MM. **Results:** The nontargeted metabolomics analysis in cohort 1 indicated that there was a significant metabolic signature change between MM patients and NC. The differential metabolites were mainly enriched in metabolic pathways related to amino acid metabolism, such as protein digestion and absorption, and biosynthesis of amino acids. Further, the targeted metabolomics analysis of amino acids in both cohort 1 and cohort 2 revealed differential metabolic profiling between MM patients and NC. We identified 12 and 14 amino acid metabolites with altered abundant in MM patients compared to NC subjects, in cohort 1 and cohort 2, respectively. Besides, key differential amino acid metabolites, such as choline, creatinine, leucine, tryptophan, and valine, may discriminate MM patients from NC. Moreover, the differential amino acid metabolites were associated with clinical indicators of MM patients. **Conclusions:** Our findings indicate that amino acid metabolism disorders are involved in MM. The differential profiles reveal the potential utility of key amino acid metabolites as diagnostic biomarkers of MM. The alterations in metabolome, especially the amino acid metabolome, may provide more evidences for elucidating the pathogenesis and development of MM.

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3 **Running title:** Metabolomics analysis of MM

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17   **Abstract**

18   **Purpose**: Multiple myeloma (MM), a kind of malignant neoplasm of plasma cells in the bone

19   marrow, is a refractory disease. Understanding the metabolism disorders and identification of

20   metabolomics pathways as well as key metabolites will provide new insights for exploring

21   diagnosis and therapeutic targets of MM.

22   **Methods**: We conducted nontargeted metabolomics analysis of MM patients and normal

23   controls (NC) using ultra-high-performance liquid chromatography (UHPLC) combined with

24   quadrupole time-of-flight mass spectrometry (Q-TOF-MS) in 40 cases of cohort 1 subjects. The

25   targeted metabolomics analysis of amino acids using multiple reaction monitoring-mass

26   spectrometry (MRM-MS) was also performed in 30 cases of cohort 1 and 30 cases of cohort 2

27   participants, to comprehensively investigate the metabolomics disorders of MM.

28   **Results:** The nontargeted metabolomics analysis in cohort 1 indicated that there was a

29   significant metabolic signature change between MM patients and NC. The differential

30   metabolites were mainly enriched in metabolic pathways related to amino acid metabolism, such

31   as protein digestion and absorption, and biosynthesis of amino acids. Further, the targeted

32   metabolomics analysis of amino acids in both cohort 1 and cohort 2 revealed differential

33   metabolic profiling between MM patients and NC. We identified 12 and 14 amino acid

34   metabolites with altered abundant in MM patients compared to NC subjects, in cohort 1 and

35   cohort 2, respectively. Besides, key differential amino acid metabolites, such as choline,

36   creatinine, leucine, tryptophan, and valine, may discriminate MM patients from NC. Moreover,

37   the differential amino acid metabolites were associated with clinical indicators of MM patients.

38   **Conclusions:** Our findings indicate that amino acid metabolism disorders are involved in MM.

39   The differential profiles reveal the potential utility of key amino acid metabolites as diagnostic

40   biomarkers of MM. The alterations in metabolome, especially the amino acid metabolome, may

41   provide more evidences for elucidating the pathogenesis and development of MM.

42   **Key words:** Multiple myeloma; metabolomics profiles; metabolic pathways; differential amino

43   acid metabolites; diagnostic biomarkers

44   **Introduction**

45   Multiple myeloma (MM) is a kind of malignant neoplasm characterized by the accumulation of

46   plasma cells in the bone marrow (Kocemba-Pilarczyk et al. 2018; Xiang et al. 2017). It is the

47   second most common hematologic malignancy (Tabata et al. 2020) and accounts for 1 % of all

48   cancers diagnosed in the United States (Schecter & Lentzsch 2013), with an annual incidence of

49   6.3 new cases per 100,000 individuals (Neuse et al. 2020). Due to the recurrent relapsing disease

50   course, MM is considered mostly incurable and requires various therapies (Harding et al. 2019).

51   To date, much progress has been made in the treatment strategy of MM. According to these

52   clinical trials (Fayers et al. 2011; Gay et al. 2011; Mateos 2010), the combination of diverse

53   drugs, such as proteasome inhibitors, immune-modulatory drugs, monoclonal antibodies, HDAC

54   inhibitors and individual CAR-T cell therapy, and autologous hemopoietic stem cell

55   transplantation with traditional drugs, such as corticosteroids, alkylating agents and

56   anthracyclines, has achieved significant clinical effects, but MM remains an incurable disease.

57   Despite considerable advances in treatment, the prognosis of MM is still very heterogeneous

58   (Schavgoulidze et al. 2021). Several reports have demonstrated the links between MM treatment

59   and its early diagnosis (Korde et al. 2015; Mateos et al. 2013). Therefore, a more comprehensive

60   understanding of MM features will aid in the design of an effective treatment strategy for this

61   disease.

62   Accumulating evidence has confirmed that metabolic alterations are involved in myeloma cell

63   growth and drug resistance (Maiso et al. 2015; Pinto et al. 2020). Metabolomics analysis is a

64   comprehensive method of metabolites that can dynamically monitor the intermediate and final

65   products of biochemical reactions and has been widely used in cancer diagnosis, treatment, and

66   prognosis (Armitage & Southam 2016; Cao et al. 2020; Kochanowski et al. 2021). By analyzing

67   the metabolic profiles of MM patients at diagnosis and after achieving complete remission, some

68   of the metabolic changes, such as glutamine, cholesterol, and lysine, have been observed,

69   suggesting the potential of metabolic profiles in identifying MM biomarkers or monitoring

70   response to treatment (Puchades-Carrasco et al. 2013). Studies have also revealed significant

71   alterations in amino acid, lipid and energy metabolism by analyzing the metabolomic plasma

72   profile of MM patients and NC and suggested the potential of cellular metabolic processes as

73   promising therapeutic targets in MM (Steiner et al. 2018). In addition, more studies have focused

74   on the indispensable mediator in the plasma microenvironment, amino acids. Glutamine (Cory &

75   Cory 2006; Pochini et al. 2014) metabolism has proven to have close correlations with

76   hematopoietic malignancies. Du et al. reported that 23 metabolites changed significantly in MM

77   mainly by arginine, proline and glycerol phospholipid metabolic pathways (Du et al. 2018). Zaal

78   et al. demonstrated that bortezomib resistance in MM was associated with increased serine

79   synthesis (Zaal et al. 2017). These findings have confirmed the crucial role of some metabolites

80   and metabolic pathways in the pathogenesis of MM. However, metabolism encompasses the

81   generation of energy, the synthesis and breakdown of glucose, amino acids and fatty acids, and

82   oxidative phosphorylation. The metabolic changes in MM cells include the generation,

83   accumulation and inhibition of metabolites as well as metabolic shifts (El Arfani et al. 2018).

84   Although previous findings have revealed some metabolic changes in MM, the key metabolites

85   and metabolic pathways in MM have not been fully investigated.

86   Metabolomics approaches can be nontargeted or targeted. Nontargeted metabolomics involves

87   global profiling of the metabolome and often provides more information than targeted

88   metabolomics. However, targeted metabolomics can quantify a select group of metabolites, such

89   as amino acids, which is more quantitative than nontargeted metabolomics (Zhang et al. 2016).

90   In the current study, we conducted nontargeted and targeted metabolomics of amino acids to

91   explore the metabolism disorders and identify metabolomics pathways as well as key differential

92   amino acid metabolites in MM patients. Our findings will provide new insight for elucidating the

93   possible mechanism of MM and exploring promising biomarkers or therapeutic targets for this

94   disease.

95   **Materials and methods**

96   **Study design and patient recruitment**

97   In this study, a total of 70 participants in two cohorts were recruited for metabolomic detection.

98   Patients with MM were collected from Lanzhou University Second Hospital, and NC subjects

99   were recruited through conventional physical examination in the hospital. MM patients were

100   diagnosed according to the criteria of International Myeloma Working Group (IMWG)

101   (Rajkumar. 2016). The International Staging System (ISS) was used to evaluate disease

102   progression (Loehrer. 2006). The patients with MM in the study were all newly diagnosed cases.

103   Subjects with immunodeficiency disease, medication use, smoking habits, or underlying chronic

104   disease were excluded from the study at the preliminary screening stage. All MM patients in this

105   study were not treated with radiotherapy or chemotherapy. Finally, in the cohort 1, 40

106   participants including 20 MM patients and 20 NC subjects, were selected for nontargeted- and

107   targeted-metabolomics. We also collected the information about clinical variables, including the

108   biochemical detection of serum albumin (ALB), interleukin 6 (IL-6), lactic dehydrogenase

109   (LDH), β2-microglobulin (B2-MG), globulin (GLB), and serum creatinine (SCR) using the

110   Beckman automatic biochemistry analyzer (Beckman Chemistry Analyzer AU5800, Beckman

111   Coulter, Inc., USA) and Roche electrochemiluminescence system (Cobas e 801 Analyzer, Roche

112   Diagnostics GmbH, Germany), for all the 40 participants in cohort 1. The study of cohort 1

113   mainly contained two parts: 1) all 40 participants underwent nontargeted metabolomics analysis,

114   and 2) 30 participants (including 15 MM patients and 15 NC subjects) underwent targeted

115   metabolomics analysis. In the cohort 2, another 30 cases, including 15 MM patients and 15 NC

116   subjects, were recruited as the validation group of the targeted metabolomics analysis. **Figure 1**

117   summarized the study design, and detailed information on the participants could be found in

118   **Table 1** (cohort 1) and **Supplementary Table 1** (cohort 2). This study was approved by the

119   Medical Ethics Committee of Lanzhou University Second Hospital (2018a-037). Written

120   informed consent was obtained from all participants.

121   **Nontargeted metabolomics analysis using ultrahigh-performance liquid chromatography**

122   **(UHPLC) combined with quadrupole time-of-flight mass spectrometry (Q-TOF-MS)**

123   **Sample collection and preparation**

124   Peripheral blood samples of all 40 participants in cohort 1, including 20 MM patients and 20 NC

125   subjects, were collected, and serum samples were obtained by centrifugation. The samples were

126   then stored at -80°C before further processing for UHPLC-Q-TOF/MS analysis.

127   **UHPLC-Q-TOF-MS analysis**

128   UHPLC analysis was performed with an Agilent 1290 Infinity LC UHPLC system.

129   Chromatographic separation was carried out at 25°C on a hydrophilic interaction

130 chromatography (HILIC) column with a flow rate of 0.3 mL/min. The mobile phase consisted of

131 solvent A (water + 25 mM ammonium acetate + 25 mM ammonia) and solvent B (acetonitrile).

132 Gradient elution was performed as follows: 95% solvent B with 0-0.5 min; the linear change of

133 solvent B was from 95% to 65% within 0.5-7 min, from 65% to 40% with 7-8 min, and

134 maintained at 40% for 8-9 min; then, the linear change of solvent B was from 40% to 95%

135 within 9-9.1 min and maintained at 95% for 9.1-12 min. The samples were placed in an

136 automatic sampler at 4℃ throughout the whole analysis. To avoid the influence of signal

137 fluctuation caused by instrument detection, continuous analysis of samples was carried out in

138 random order. Moreover, pooled quality control (QC) samples (generated by taking an equal

139 aliquot of all the samples included in the experiment) were run at the beginning of the sample

140 queue for column conditioning and every ten injections thereafter to assess inconsistencies that

141 are particularly evident in large batch acquisitions in terms of retention time drifts and variation

142 in ion intensity over time.

143 Q-TOF-MS analysis was conducted on an AB Triple TOF 6600 mass spectrometer (AB

144 SCIEX, Framingham, MA, USA). After HILIC chromatographic separation, the electrospray

145 ionization (ESI) source conditions were as follows: ion Source Gas1 (Gas1): 60 psi, Ion Source

146 Gas2 (Gas2): 60, Curtain gas (CUR): 30, source temperature at 600℃, ionSapary Voltage

147 Floating (ISVF): ± 5500 V (positive and negative model); TOF MS scan m/z range: 60-1000 Da,

148 product ion scan m/z range: 25-1000 Da, TOF MS scan accumulation time: 0.20 s/spectra, and

149 product ion scan accumulation time: 0.05 s/spectra. Information-dependent acquisition (IDA)

150 was used to obtain the secondary MS, as well as high sensitivity mode, declustering potential

151 (DP): ± 60 V (positive and negative modes), and collision energy: 35 ± 15 eV. IDA was set as

152 exclude isotopes within 4 Da and candidate ions to monitor per cycle: 6.

153 **Data processing and analysis**

154 The raw data were converted into. mzXML format files using the ProteoWizard converter tool

155 and then processed using XCMS software for peak alignment, retention time correction, and

156 peak area extraction. Then, the structures of metabolites were identified by comparing the

157 accuracy of m/z values (< 25 ppm) and matching second-stage spectra with the laboratory's self-

158 built database (Applied Protein Technology Co., Ltd., Shanghai, China).

159   After data preprocessing by Pareto scaling, multidimensional data analyses were carried out,

160 including unsupervised principal component analysis (PCA), supervised partial least squares

161 discrimination analysis (PLS-DA), and orthogonal partial least squares discriminant analysis

162 (OPLS-DA). Single-dimensional statistical analyses, including Student's t-test and fold change

163 (FC) analysis, were conducted. The variable importance in the projection (VIP) value (> 1) in the

164 PLS-DA model and P value analyzed by Student's t-test were combined to confirm the

165 significance of differential metabolites. Significant differential metabolites were obtained when

166 VIP > 1 and P value < 0.05, and differential metabolites were selected when VIP > 1 and 0.05 <

167 P value < 0.1. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

168 (http://www.genome.jp/kegg/) pathway enrichment analysis for significant differential

169 metabolites was conducted by the MetaboAnalyst online tool

170 (http://www.metaboanalyst.ca/MetaboAnalyst/) to investigate the metabolomics pathways.

171 **Targeted metabolomics analysis of amino acids using multiple reaction monitoring-mass**

172 **spectrometry (MRM-MS)**

173 **Sample collection and preparation**

174 Peripheral blood samples of 30 out of 40 participants in cohort 1 (including 15 MM patients and

175 15 NC subjects) and another 30 cases in cohort 2 (the validation group, including 15 MM

176   patients and 15 NC subjects) were collected for targeted metabolomics analysis. Serum samples

177   were obtained by centrifuging at 1000 ×g for 10 min and then stored at -80°C.

178   **MRM-MS analysis**

179   The analysis was performed with an Agilent 1290 Infinity LC UHPLC system. The mobile

180   phases were as follows: solvent A was 25 mM ammonium formate + 0.08% formic acid (FA) in

181   water, and solvent B was 0.1% FA-acetonitrile. The sample was placed in an automatic sampler

182   at 4°C, the column temperature was 40°C, the flow rate was 250 μL/min, and the injection

183   volume was 1 μL. Gradient elution was performed as follows: the linear change of solvent B was

184   from 90% to 70% within 0-12 min; from 70% to 50% with 12-18 min; from 50% to 40% within

185   18-25 min, from 40% to 90% with 30-30.1 min; and maintained at 90% for 30.1-37 min. In the

186   sample queue, a QC sample was also set in a certain number of experimental samples at every

187   interval to test and evaluate the stability and repeatability of the system. MS analysis was carried

188   out in positive ionization mode on a 5500 QTRAP mass spectrometer (AB SCIEX, Framingham,

189   MA, USA). The conditions of the 5500 QTRAP ESI source were as follows: ion source

190   temperature, 500°C; Gas1, 40 psi; Gas2, 40 psi; CUR, 30 psi; and ISVF, 5500 V. MRM mode

191   was then used to detect the ion pairs.

192   **Data processing and analysis**

193   The chromatographic peak area and analyte retention time were extracted using Multiquant

194   software. Based on the standards of amino acids and their derivatives, the analyte retention time

195   was corrected, followed by identification of the metabolites. PLS-DA was also conducted to

196   explore the amino acids profiling and differential amino acid metabolites between groups. Score

197   scatter plots and loading plots were generated to visualize the separation of samples and

198   metabolites. MedCalc software (v19.0.4) was utilized for receiver operating characteristic curve

199 (ROC) analysis to evaluate the diagnostic accuracy of amino acid metabolites. Moreover,

200 Spearman correlation analysis was performed with the statistical platform R package (v3.2.4).

201 KEGG pathway enrichment analysis for significant differential metabolites was also conducted

202 by the MetaboAnalyst online tool.

203 **Results**

204 **Nontargeted metabolomics analysis using UHPLC-Q-TOF-MS**

205 **Multivariate statistical analysis**

206 To analyze the metabolic changes between MM patients and NC, nontargeted metabolomics

207 analysis was carried out using UHPLC-Q-TOF-MS. As a result, the PCA score plots showed a

208 clear separation between MM patients and NC in both positive-ion and negative-ion modes

209 (**Figure 2A**). To further identify ion peaks that could possibly be used to differentiate the

210 metabolic profiles of MM patients and NC, the supervised PLS-DA model was conducted. The

211 results showed that MM patients were also separated from NC by the PLS-DA score plots in

212 both modes (**Figure 2B**). Consistent results were also obtained by the OPLS-DA score plots in

213 both modes (**Figure 2C**). Further permutation tests consisting of 200 permutations demonstrated

214 that the model was not overfitted (positive-ion model: R2 = (0.0, 0.707), Q2 = (0.0, -0.3673);

215 negative-ion model: R2 = (0.0, 0.8331), Q2 = (0.0, -0.4174); **Figure 2D**). These data indicated

216 that there was a significant metabolic change between MM patients and NC.

217 **Analysis of differential metabolites between MM patients and NC**

218 To compare differences in metabolites between MM patients and NC, differential metabolites

219 were screened out between MM patients and NC in both positive-ion and negative-ion modes

220 based on VIP > 1 in the PLS-DA model and P < 0.1 analyzed by Student's t-test. The results

221 showed that a total of 62 differential metabolites were identified between MM patients and NC

222 in positive-ion mode, of which 56 were significant (P < 0.05). Similarly, 45 differential

223 metabolites were identified between MM patients and NC in negative-ion mode, of which 38

224 were significant (P < 0.05) (**Supplementary Table 2**). These data confirmed that there were

225 significant differences in metabolites between MM patients and NC.

226 **Metabolic pathway analysis**

227 To investigate the metabolomic pathways involved in MM development, the differential

228 abundant metabolites were enriched for the related metabolic pathway analysis using the

229 MetaboAnalyst online tool. As shown in **Figure 3**, differential metabolites were significantly

230 enriched in multiple metabolic pathways, such as protein digestion and absorption (including L-

231 alanine, L-glutamine, L-tryptophan, L-histidine, L-asparagine, L-valine, and L-isoleucine), ABC

232 transporters (including L-alanine, L-glutamine, L-histidine, L-valine, and L-isoleucine), and

233 biosynthesis of amino acids (L-alanine, L-glutamine, L-tryptophan, L-histidine, L-asparagine, L-

234 valine, L-citrulline, and L-isoleucine). Notably, these pathways were associated with amino acid

235 metabolism.

236 **Targeted metabolomics analysis of amino acids using MRM-MS**

237 **Serum amino acid metabolites of MM patients and NC showed distinguished profiles**

238 Previously, the results indicated that the altered metabolic profile of MM was mainly involved in

239 amino acid metabolomics features. We further conducted a target amino acid metabolomics

240 analysis to gain more insight into MM metabolism. Generally, in the cases of cohort 1, the PLS-

241 DA two-dimensional (2D) score plot showed clearly distinguished profiles between the MM and

242 NC groups (**Figure 4A**). Moreover, scatter loading plot analysis was carried out to evaluate

243 whether these differential amino acid metabolites accounted for the distinguished profiles. MM

244 patients exhibited higher levels of choline, creatinine, glutamate, and asparagine as well as lower

245 levels of alanine/sarcosine, valine, tryptophan, and cystine than NC (**Figure 4B**). These data

246 suggested that altered amino acid profiles could discriminate MM patients from NC.

247 **Expression of differential amino acid metabolites and the KEGG functional analysis**

248 According to the metabolomics features, with the purpose of finding potential diagnostic

249 biomarkers or therapeutic targets for MM, we focused on the differential amino acid metabolites

250 in MM patients compared to those in NC. To further investigate the detailed variety degree of

251 each metabolite, we analyzed the intensity differences in a total of 28 amino acid metabolites.

252 Compared to NC, MM patients exhibited differential metabolic profiles. Overall, in the cases of

253 cohort 1, there was a total of 12 significantly differential abundant amino acid metabolites in

254 MM patients relative to NC ($P < 0.05$), including lysine, leucine, isoleucine, histidine, valine,

255 threonine, glutamine, tryptophan, choline, ornithine, creatinine, and alanine/sarcosine

256 (**Supplementary Table 3**). Furthermore, we validated these findings with the samples in cohort

257 2, and the results showed similar dysregulation trends with the findings of cohort 1, that we

258 identified 14 significantly differential abundant amino acid metabolites in MM group

259 (**Supplementary Table 4**). Specifically, the high expression levels of creatinine (FC = 2.029, P

260 < 0.01 in cohort 1; FC = 1.632, P < 0.05 in cohort 2) and choline (FC = 1.508, P < 0.001 in

261 cohort 1; FC = 1.234, P = 0.125 in cohort 2) were found in MM patients in both cohort 1 and

262 cohort 2. Besides, since glutamate signaling has been reported involved in malignant disorders

263 (Willard & Koochekpour 2013), we also examined glutamate levels and found slightly high

264 levels in MM patients (FC = 1.186, P = 0.245 in cohort 1; FC = 1.292, P = 0.346 in cohort 2).

265   To determine the pathway impact of these differential amino acid metabolites, we performed

266 pathway classifications provided by KEGG analysis. Results indicated that the 12 differential

267 abundant metabolites in cohort 1 were significantly enriched in 22 metabolic pathways, such as

268   aminoacyl-tRNA   biosynthesis,   valine/leucine/isoleucine   biosynthesis,

269   phenylalanine/tyrosine/tryptophan biosynthesis, D-glutamine/D-glutamate metabolism, and

270   histidine metabolism (**Figure 4C, D**).

271   **Differential amino acid metabolites may discriminate MM patients from NC**

272   We subsequently evaluated the performances of these differential abundant metabolites in

273   distinguishing MM from NC group. ROC analysis showed that, the amino acid metabolites

274   classifier could act as potential disease diagnostic biomarkers in both cohort 1 and cohort 2

275   (AUC > 0.7, P < 0.05, Table 2 and Supplementary Figure 1). In MM patients of cohort 1, the

276   AUC values of two representative upregulated amino acid metabolites, choline and creatinine,

277   were 0.822 and 0.822, respectively (Table 2). Similarly, the AUC of creatinine in the cohort 2

278   was 0.747 (Table 2). Meanwhile, the ROC analysis also showed good diagnostic values of the

279   downregulated amino acid metabolites in MM, such as leucine (AUC = 0.871 in cohort 1, AUC

280   = 0.920 in cohort 2) and tryptophan (AUC = 0.884 in cohort 1, AUC = 0.902 in cohort 2).

281   Specifically, we observed extremely high AUC values of the metabolite valine in both cohort 1

282   (AUC = 0.964) and cohort 2 (AUC = 0.960). The results above suggested that these key

283   differential amino acid metabolites might be regarded as potential biomarkers for the diagnosis

284   or therapeutic targets of MM.

285   **Differential amino acid metabolites were associated with MM phenotypes**

286   To investigate the correlations between differential amino acid metabolites and MM-associated

287   phenotypes, we analyzed the correlations among 6 clinical indices/variables and 12 amino acid

288   metabolites that differed significantly in abundance between MM patients and NC in the cohort 1

289   (**Figure 5**). The clinical variables included serum albumin (ALB), interleukin 6 (IL-6), lactic

290   dehydrogenase (LDH), β2-microglobulin (B2-MG), globulin (GLB), and serum creatinine (SCR).

291 Among 12 amino acid metabolites whose abundances differed in MM patients, two mainly

292 correlation clusters were obtained. The cluster of upregulated components, creatinine and choline

293 in MM patients, was positively correlated with the levels of LDH, SCR, and B2-MG. Moreover,

294 in the downregulated amino acids cluster, 10 amino acid metabolites were negatively correlated

295 with the levels of IL-6 and GLB. Among these metabolites, the relationship between GLB and

296 valine/isoleucine/tryptophan/leucine/sarcosine exhibited high correlation coefficients (adjusted P

297 < 0.01). These data suggested that the combination of clinical indices and amino acid metabolites

298 alterations facilitate a better understanding of MM progression.

299 **Discussion**

300 Metabolite disorder plays an essential role in cancer initiation and progression (La Vecchia &

301 Sebastián 2020; Whisner & Aktipis 2019). Metabolic changes in the tumor microenvironment

302 affect the effects of immunotherapy (Kouidhi et al. 2018). Therefore, a better understanding of

303 metabolic changes in the tumor microenvironment will improve the beneficial effects of

304 immunotherapy. Recently, with the development of metabolomics, more researchers have

305 focused on the clinical examination of metabolite variation, which might provide more predictors

306 and clues in disease diagnosis and treatment (Chen et al. 2019).

307  MM, as a heterogenic disease with dynamic metabolic processes in bone marrow and its

308 microenvironment, has been reported to exhibit metabolic changes (El Arfani et al. 2018; Silva et

309 al. 2020). Despite this, the specific metabolic profiles in MM patients and potential clinical

310 biomarkers remain ambiguous and need to be further clarified. Metabolomics analysis is a

311 powerful means for investigating metabolic processes, revealing metabolic mechanisms and

312 identifying crucial biomarkers responsible for metabolic alteration. In the present study, we

313 conducted nontargeted metabolomics analysis using UHPLC-Q-TOF-MS and found a significant

314   metabolic change between MM patients and NC. Differential metabolites could discriminate

315   MM patients and NC, which were significantly enriched in metabolic pathways related to amino

316   acid metabolism, such as protein digestion, and absorption and biosynthesis of amino acids.

317   These data suggested the key role of amino acid metabolism in MM. We then conducted the

318   targeted metabolomics analysis of amino acids using MRM-MS in two cohorts of participants

319   and found that serum amino acid metabolites of MM patients and NC showed distinguished

320   profiles. Compared to NC, MM patients exhibited higher levels of choline and creatinine as well

321   as lower levels of valine, tryptophan, leucine, etc. In another related study, the lipids profiles of

322   MM showed lower concentrations of phosphatidylcholine (PC), lysophosphatidylcholine (LPC)

323   and sphingomyelins (SM) (Silva et al. 2020). Besides, the concentration of essential amino acids,

324   especially tryptophan, was significantly decreased in MM cases (Silva et al. 2020), which was

325   consistent with our findings. Further ROC analysis in this study showed relatively good

326   diagnostic values of upregulated amino acid metabolites, including choline and creatinine, and

327   downregulated amino acid metabolites, such as leucine, tryptophan and valine. These results

328   suggested that the key differential amino acid metabolites could be used as promising biomarkers

329   for MM diagnosis. In addition, the clinical indicators were found to be related to altered amino

330   acid metabolomics in correlation analysis. MM patients were accompanied by elevation of

331   β2-microglobulin (β2-MG), and researchers found an inverse correlation between the

332   concentration of tryptophan and β2-MG (Silva et al. 2020). In the current study, we also

333   observed a negative correlation between the differential metabolite tryptophan and β2-MG.

334   Besides, the downregulated amino acids, including lysine, leucine, and valine, were also

335   negatively related with the high level of β2-MG. Taken together, we hypothesized that the

336   diagnostic value may be improved by combining examination of clinical indicators and amino

337   acid levels.

338   Amino acids and proteins have been shown to play central roles in cellular metabolism

339   (Rizzieri et al. 2019; Zhang et al. 2019). Amino acids are responsible for the formation of a

340   variety of components that are used for cell proliferation (Martinez-Outschoorn et al. 2017).

341   Notably, it has been reported that targeting amino acid metabolism may be a promising therapy

342   for cancer, suggesting the crucial role of amino acid metabolism in cancer (Bai et al. 2020; Boon

343   et al. 2020; Wang & Zou 2020). Moreover, protein digestion and absorption is also an enriched

344   metabolism/digestive pathway involved in mechanisms of inhibitory effect of 3-O-kaempferol-3-

345   O-acetyl-6-O-(p-coumaroyl)-β-D-glucopyranoside (HK-11, a flavonoid compound) on MM cell

346   proliferation (Hou et al. 2020), implying the potential role of protein digestion and absorption

347   pathway in MM development. Our nontarget metabolomics analysis revealed that the differential

348   metabolites between MM patients and NC were significantly enriched in metabolic pathways

349   related to amino acid metabolism. It can therefore be speculated that amino acid metabolism is

350   involved in MM. Further targeted metabolomics analysis of amino acids showed that the

351   concentration of key metabolites was altered in MM, and most of the amino acid metabolites was

352   downregulated. We highlight that these key metabolites may act as potential biomarkers for MM,

353   and that the supplementation of the disease phenotype-negatively related metabolic components

354   may help prevent or improve the prognosis of MM. Recent studies have also investigated the

355   proteomic alterations in the bone marrow interstitial fluid and serum samples, and identified

356   candidate proteins to be associated with drug resistance in MM patients (Chanukuppa et al. 2019;

357   Chanukuppa et al. 2021). The complex metabolite and lipid profiling of bone marrow plasma

358   could help differentiate patients with monoclonal gammopathy of undetermined significance

359 (MGUS) from MM (Gonsalves et al. 2020). Therefore, the metabolic and proteomic detection

360 provides more evidences for the development of improved diagnosis and treatment of MM. In

361 the current study, we also conducted the targeted metabolomics analysis involving amino acids

362 in MM patients, and it's necessary to broaden the metabolic classes to find novel biomarkers in

363 the future.

364   In conclusion, our results reveal that amino acid metabolism disorder is involved in MM. Key

365 differential amino acid metabolites, such as choline, creatinine, leucine, tryptophan, and valine,

366 may discriminate MM patients from NC. The alteration in metabolism, especially amino acid

367 metabolism, may provide evidences for elucidating the pathogenesis of MM and lay a reference

368 for clinical diagnosis and therapy of this disease. However, there are still several limitations in

369 our research. Although we summarized differential amino acid metabolites and explored their

370 diagnostic value, more functional validations *in vivo* are still needed. Moreover, the sample size

371 was relatively small to make some general conclusions. **More research is needed to**

372 **support the results and to investigate the underlying biological functions of the key amino**

373 **acid metabolites with large scale and mechanism study in the future. Besides, confounders**

374 **such as medication taken or smoking habits of the individuals can influence the metabolic**

375 **trend, comprehensive research involving the factors should be conducted in larger cohort**

376 **in future research.**

377 **Acknowledgments:** None.

378 **Funding:** This work was supported by the Cuiying Scientific and Technological Innovation

379 Program of Lanzhou University Second Hospital, CY2017-MS13

380 **Conflicts of Interest:** The authors declare that they have no conflicts of interest.

381 **Data availability statement:** The raw data of this study have been deposited in FigShare

382 (https://figshare.com/) with the link: https://doi.org/10.6084/m9.figshare.14904135.v1.

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505 **Figure legends**

506 **Figure 1. Overview of the study design and workflow.** UHPLC-Q-TOF/MS: ultra-high-
507 performance liquid chromatography (UHPLC) with quadrupole time-of-flight mass spectrometry
508 (Q-TOF-MS); MRM-MS: multiple reaction monitoring-mass spectrometry; PCA: principal
509 component analysis; PLS-DA partial-least squares discrimination analysis; OPLS-DA:
510 orthogonal partial least squares discriminant analysis; KEGG: Encyclopedia of Genes and
511 Genomes; ROC: receiver operating characteristic curve.

512 **Figure 2. Score plots of supervised PCA, PLS-DA, and OPLS-DA analysis based on the**
513 **combination of UHPLC-Q-TOF/MS data from MM patients and NC.** (A) PCA score plots
514 between MM patients and normal controls (NC) in both positive-ion and negative-ion modes. (B)
515 PLS-DA score plots between MM patients and normal controls (NC) in both modes. (C) OPLS-
516 DA score plots between MM patients and normal controls (NC) in both modes. (D) Permutation
517 tests consisting of 200 permutations demonstrated that the OPLS-DA model was not overfitted in
518 either mode.

519 **Figure 3. Metabolic pathway enrichment analysis of differential metabolites between MM**
520 **patients and NC.** Each related metabolic pathway is shown as a circle, whose size and color are
521 based on the pathway impact value and the P value, respectively.

522 **Figure 4. PLS-DA analysis based on MRM-MS data and functional pathway of key**
523 **differential metabolites between MM and NC groups.** (A, B) PLS-DA scores and loading
524 plots between MM patients and normal controls based on the MRM-MS data. (C, D) Metabolic
525 pathway enrichment analysis of differential amino acids metabolites between MM patients and
526 NC**.** In (C), each related metabolic pathway is shown as a circle, whose size and color are based
527 on the pathway impact value and the P value, respectively.

528 **Figure 5. Correlation analysis of 12 differential amino acids identified from MM patients vs.**
529 **NC with 6 clinical indicators.** Numbers in the lower left panel: value of the correlation
530 coefficient; symbols in the upper right panel: results of the significance test; *adjusted P < 0.05,
531 **adjusted P < 0.01. ALB: albumin; LDH: lactic dehydrogenase; B2-MG: β2-microglobulin.

532 **Supplementary Figure 1. ROC curve analysis of the diagnostic value of differential amino**
533 **acids for MM in cohort 1 (A) and cohort 2 (B).**

534

535

536

537

Figure 1

Overview of the study design and workflow.

UHPLC-Q-TOF/MS: ultra-high-performance liquid chromatography (UHPLC) with quadrupole time-of-flight mass spectrometry (Q-TOF-MS); MRM-MS: multiple reaction monitoring-mass spectrometry; PCA: principal component analysis; PLS-DA partial-least squares discrimination analysis; OPLS-DA: orthogonal partial least squares discriminant analysis; KEGG: Encyclopedia of Genes and Genomes; ROC: receiver operating characteristic curve.

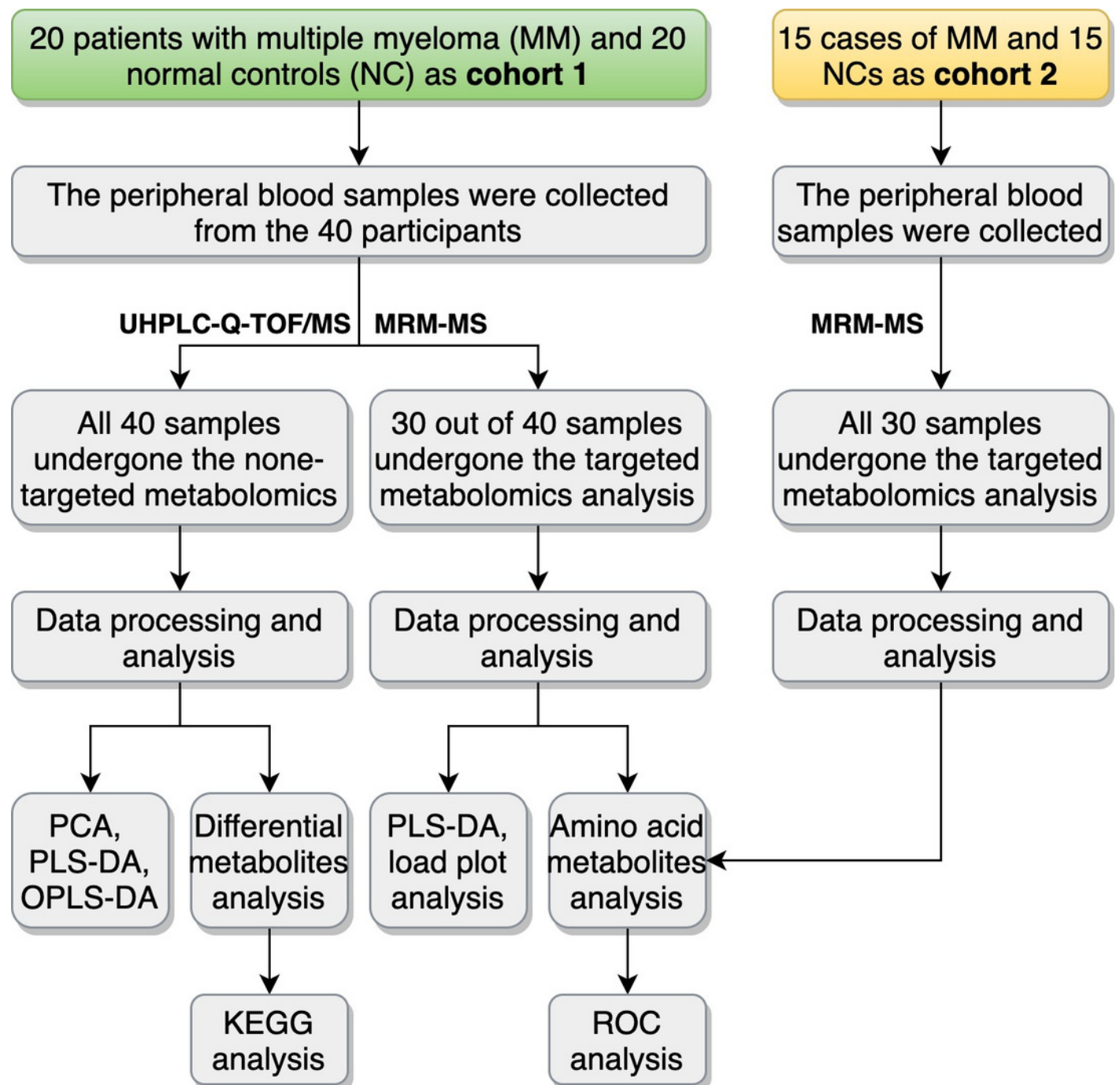
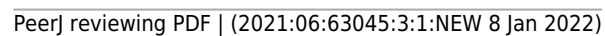



Figure 2

Score plots of supervised PCA, PLS-DA, and OPLS-DA analysis based on the combination of UHPLC-Q-TOF/MS data from MM patients and NC.

(A) PCA score plots between MM patients and normal controls (NC) in both positive-ion and negative-ion modes. (B) PLS-DA score plots between MM patients and normal controls (NC) in both modes. (C) OPLS-DA score plots between MM patients and normal controls (NC) in both modes. (D) Permutation tests consisting of 200 permutations demonstrated that the OPLS-DA model was not overfitted in either mode.

A
Scores (PCA)
Positive-ion mode

Scores (PCA)
Negative-ion mode

B
Scores (PLS-DA)
Positive-ion mode

Scores (PLS-DA)
Negative-ion mode

C
Scores (OPLS-DA)
Positive-ion mode

Scores (OPLS-DA)
Negative-ion mode

D
R2=(0.0, 0.707), Q2=(0.0, -0.3673)
Positive-ion mode

R2=(0.0, 0.8331), Q2=(0.0, -0.4174)
Negative-ion mode

Figure 3

Metabolic pathway enrichment analysis of differential metabolites between MM patients and NC.

Each related metabolic pathway is shown as a circle, whose size and color are based on the pathway impact value and the P value, respectively.

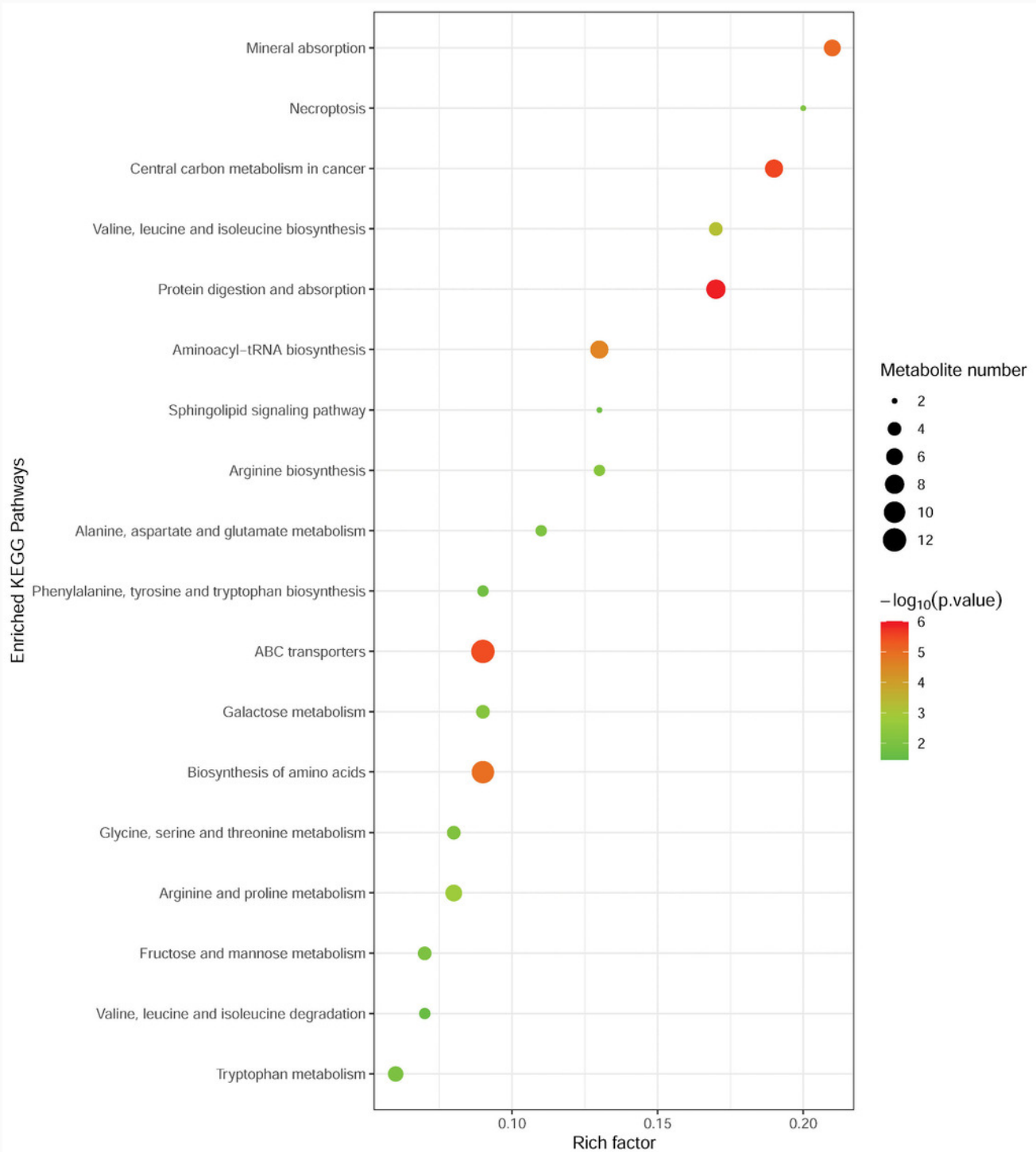



Figure 4

PLS-DA analysis based on MRM-MS data and functional pathway of key differential metabolites between MM and NC groups.

(A, B) PLS-DA scores and loading plots between MM patients and normal controls based on the MRM-MS data. (C, D) Metabolic pathway enrichment analysis of differential amino acids metabolites between MM patients and NC. In (C), each related metabolic pathway is shown as a circle, whose size and color are based on the pathway impact value and the P value, respectively.

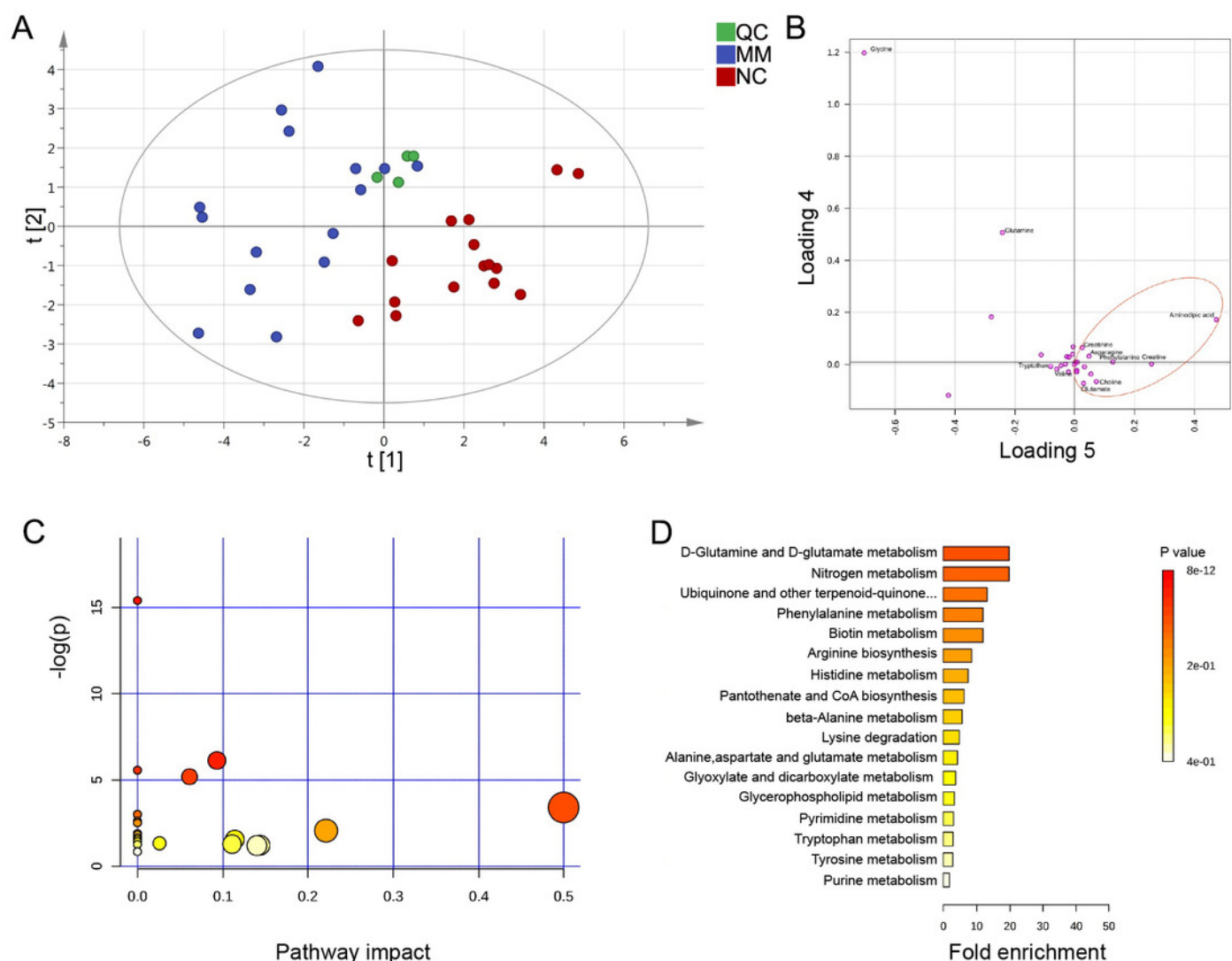



Figure 5

Correlation analysis of 12 differential amino acids identified from MM patients vs. NC with 6 clinical indicators.

Numbers in the lower left panel: value of the correlation coefficient; symbols in the upper right panel: results of the significance test; *adjusted P < 0.05 , ** adjusted P < 0.01 . ALB: albumin; LDH: lactic dehydrogenase ; B2-MG : β2-microglobulin .

Clinical characteristics of the 40 participants in cohort 1

Abbreviations: ISS: International Staging System; ALB: albumin, reference range: 40-55; IL-6: interleukin 6, reference range: 0.00-5.30; LDH: lactic dehydrogenase, reference range: 120-250; β2-MG: β2-microglobulin, reference range: 609-2366; GLB: globulin, reference range: 20-40; SCR: serum creatinine, reference range: 41.0-73.0. Compared with the NC group, [*]Significant association with $P < 0.05$, [#] Significant association with $P < 0.01$, [△]Significant association with $P < 0.001$.

1   **Table 1. Clinical characteristics of the 40 participants in cohort 1**

Group	Age	Sex	ISS	ALB (g/L)	IL-6 (pg/mL)	LDH (U/L)	β2-MG (ng/mL)	GLB (g/L)	SCR (μmol/L)
Normal control (NC, n=20)	55.10±10.43	Female (n=8) Male (n=12)	---	42.16±6.12	4.21±1.98	183.00±66.28	1037.05±423.28	27.62±6.51	67.88±33.47
Multiple myeloma (MM, n=20)	54.90±11.29	Female (n=8) Male (n=12)	ISS I (n=4) 1SS II (n=6) ISS III (n=10)	37.89±9.08	12.24±14.72*	241.20±112.80	10846.45±10615.85△	49.56±26.42△	243.75±229.60#

2   Abbreviations: ISS: International Staging System; ALB: albumin, reference range: 40-55; IL-6: interleukin 6, reference range: 0.00-

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4   reference range: 20-40; SCR: serum creatinine, reference range: 41.0-73.0. Compared with the NC group, *Significant association

5   with $P < 0.05$, # Significant association with $P < 0.01$, △Significant association with $P < 0.001$.

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Performance of differential metabolites in distinguishing MM from NC group

1 **Table 2. Performance of differential metabolites in distinguishing MM from NC group**

	cohort 1					cohort 2			
metabolites	sensitivity	specificity	AUC	P	metabolites	sensitivity	specificity	AUC	P
alanine/sarcosine	60.0	93.3	0.751	0.008	alanine/sarcosine	66.7	93.3	0.773	0.003
choline	66.7	100.0	0.822	<0.001	creatinine	73.3	86.7	0.747	0.012
creatinine	66.7	100.0	0.822	<0.001	Glutamine	66.7	86.7	0.773	0.003
Glutamine	100.0	60.0	0.778	0.002	histidine	80.0	86.7	0.907	<0.001
histidine	80.0	80.0	0.778	0.002	Isoleucine	60.0	93.3	0.840	<0.001
Isoleucine	73.3	80.0	0.782	0.001	leucine	86.7	86.7	0.920	<0.001
leucine	93.3	73.3	0.871	<0.001	lysine	40.0	100.0	0.724	0.017
lysine	40.0	100.0	0.720	0.024	ornithine	46.7	100.0	0.769	0.002
ornithine	66.7	80.0	0.782	0.001	serine	80.0	73.3	0.738	0.012
threonine	93.3	73.3	0.853	<0.001	taurine	86.7	60.0	0.764	0.003
tryptophan	73.3	93.3	0.884	<0.001	threonine	93.3	66.7	0.827	<0.001
valine	86.7	93.3	0.964	<0.001	tryptophan	73.3	93.3	0.902	<0.001
					tyrosine	80.0	66.7	0.769	0.002
					valine	86.7	93.3	0.960	<0.001