The related mechanism of complete Freund's adjuvant-induced chronic inflammation pain based on metabolomics analysis

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Abstract
Chronic inflammation pain is a debilitating disease, and its mechanism still remains poorly understood. This study attempted to illuminate the metabolic mechanism of chronic inflammation pain induced by complete Freund’s adjuvant (CFA) injection, especially at spinal level. The chronic inflammation pain model was established by CFA administration. Behavioral testing including mechanical allodynia and thermal hyperalgesia was performed. Meanwhile, a liquid chromatography–mass spectrometry-based metabolomics approach was applied to analyze potential metabolic biomarkers. The orthogonal partial least squares discrimination analysis mode was employed for determining metabolic changes, and a western blot was performed to detect the protein expression change. The results showed that 27 metabolites showed obviously abnormal expression and seven metabolic pathways were significantly enriched, comprising aminoacyl-tRNA biosynthesis, arginine and proline metabolism, histidine metabolism, purine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, glutathione metabolism, and phenylalanine metabolism. Meanwhile, the results showed that the expression of arginase I and nitric oxide levels were elevated in the CFA group compared with the control group, while the argininosuccinate synthetase and argininosuccinatelyase proteins were not significantly different between the groups. These findings demonstrate that metabolic changes of the spinal cord may be implicated in neurotransmitter release and pain conductivity following CFA administration.

KEYWORDS
chronic inflammation pain, complete Freund’s adjuvant, metabolomics

1 | INTRODUCTION

Chronic pain results in dramatic decline in life quality, substantial medical expenses and a massive economic burden (Henderson & Keay, 2018). Survey data demonstrate that the prevalence of chronic pain ranges from 13.5 to 47% globally, and afflicts at least 50 million American adults (Dahlhamer et al., 2018; Tsuji et al., 2019). Generally, patients with chronic pain have symptoms of anxiety and depression, poor concentration and irritability (Gureje, Von Korff, Simon, & Gater, 1998). Some research shows that patients with chronic pain have multiple inflammatory and neuropathic conditions (Finnerup, 2013). Additionally, chronic inflammation pain, as one type...
of chronic pain, is attracting growing interest from clinicians and scientists. A previous study documented that chronic inflammation pain was derived from chemical stimuli, tissue damage or autoimmune processes. These stimuli directly caused the release of inflammatory mediators comprising prostaglandins, histamine and neurogenic factors, and elicited a series of chain reactions, thereby contributing to pain sensation by stimulating the peripheral afferent fibers (Kidd, Photiou, & Inglis, 2004). The potential mechanism regarding the chronic inflammation pain has been extensively investigated. Yet, it is of great importance in the clinical practice, while its pathogenesis has not been clarified comprehensively.

More recently, systems biology strategies such as metabolomics have been widely applied in medical fields to investigate the pathogenic mechanism, which facilitated the development of novel biomarkers for disease diagnosis and therapy (Hocher & Adamski, 2017; Yang et al., 2018; Zhang et al., 2018). Metabolism is a complex dynamic process including generating energy and producing macromolecules for sustaining cell growth and survival (Patti et al., 2012). Metabolites are downstream molecules of gene transcription and translation processes, which are closely correlated to the disease phenotype (Lains et al., 2019). Metabolic shift is identified as a hallmark of disease, and provides a noninvasive method to monitor the disease progress (Ohman & Forsgren, 2015). Furthermore, emerging evidence has revealed the relationship between inflammatory and metabolic dysregulation (Jha et al., 2015; Jiang et al., 2016; Palomer, Salvado, Barroso, & Vazquez-Carrera, 2013). A previous study revealed that aberrant metabolism may be involved in triggering inflammatory cascade reactions (O’Neill & Hardie, 2013). Notwithstanding, there are no adequate data to uncover the role of metabolism alteration in chronic inflammation pain. Therefore, the complete Freund’s adjuvant (CFA) model was established to investigate the potential mechanism for chronic inflammation pain in this study. Moreover, a metabolomics method was employed to analyze the changes in spinal metabolites. Interestingly, our results indicated that certain metabolic pathways were obviously enriched in the chronic inflammatory process, and these findings may provide new perspectives for comprehending the underlying mechanism of chronic inflammation pain.

2 | MATERIALS AND METHODS

2.1 | Animals

All experiments were performed on 8–12-week-old male C57BL/6 mice purchased from Shanghai SLAC Laboratory Animal Co. Ltd. For experiments, mice (20–35 g) were housed four or five per cage at constant room temperature (25 ± 1°C) and relative humidity (50 ± 5%) under a 12 h light/dark schedule (lights on 07:00–19:00); food and water were available ad libitum. For behavioral tests, the mice were allowed to adapt to laboratory conditions for about one week and to habituate to the testing situation for at least 15 min before experiments. The Animal Care and Use Committee of Zhejiang University approved all of the mouse protocols (approval no. 11978).

2.2 | CFA-induced chronic inflammation pain

Animals were randomly divided into two groups as follows: (a) a control group, injected with 10 μl saline (n = 10); and (b) a CFA group, injected with 10 μl 50% CFA in saline (n = 10). Chronic inflammatory pain was induced by administration of CFA as described previously (Pan et al., 2014). Briefly, an emulsion containing 10 μl of CFA with saline (proportion 1:1) was injected into the left posterior plantar of mice (n = 10). The control group received the same procedure with saline (Y. Liu et al., 2017b). Mice were allowed to acclimatize to the home cage and environment.

2.3 | Behavioral testing

2.3.1 | Mechanical allodynia

Mice were placed in individual black wood boxes without a bottom and allowed to acclimatize for at least 30 min to quantify the mechanical sensitivity of the hindpaw according to the previous literature (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). Mechanical paw withdrawal threshold in response to the stimulation of von Frey filaments was measured using the “up-down” method (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). Filaments were applied to the plantar surface of left hindpaw until they bent. A quick withdrawal or shaking of the stimulated paw or biting or licking of the paw was regarded as a positive withdrawal response, while other responses were regarded as a negative withdrawal response. A positive withdrawal response was followed by the application of a lower force filament and vice versa for a negative response until a change in behavior occurred (Zhao, Hiraoka, Ogawa, & Tanaka, 2018). The test started with the application of a 0.16 g filament. Every trial was repeated three times at 2 min intervals. According to the method described by Dixon, the 50% paw withdrawal threshold was calculated based on this assessment.

2.3.2 | Thermal hyperalgesia

To assess thermal hyperalgesia, mouse paw withdrawal latency (PWL) was measured using radiant heat (Bao et al., 2014; Bao et al., 2015). Mice were placed individually in plastic cages and allowed to acclimatize at least for 30 min. Each left hind paw received at least three stimuli with a 10 min interval between, and the average of the three values was defined as the PWL. The heat was maintained at a constant intensity and the cut-off time was set to 21 s to prevent paw damage.

2.3.3 | Sample preparation

Animals were anesthetized with 3% isoflurane on day 7 after CFA administration. Then these mice were sacrificed through decapitation. A laminectomy of L4–6 was carried out, and the spinal cord tissues
were exposed. Complete incision of L4–6 was performed and the intervening tissue was removed. Thereafter, the spinal cord was removed and stored in a liquid nitrogen box immediately for future use.

### 2.3.4 Metabolite extraction

In brief, the spinal cord tissue was homogenized in 1,500 μl methanol with water (1:1) in a 2 ml glass tissue homogenizer, and centrifuged at 15,000 g for 10 min (Tube 1). The supernatant was transferred to a 2 ml centrifuge tube, tube 2, then concentrated at room temperature in vacuum, and redissolved with 120 μl methanol–water (1:1). The solutions of centrifuge tubes 2 and 3 were mixed and then centrifuged at 15,000 g for 10 min. The culture liquid was transferred to 2 ml centrifuge tube, tube 3, which was concentrated at room temperature in vacuum, and redissolved with 120 μl methanol–water (1:1). The solutions of centrifuge tubes 2 and 3 were mixed and centrifuged once again (15,000g for 10 min). The supernatant was determined by HPLC–MS. During the study, 10 QC samples were pooled from all spinal cord samples to equilibrate the HPLC–MS system (Zhou et al., 2018).

### 2.3.5 Liquid chromatography–mass spectrometry analysis

The metabolomics data were determined using a Nexera UHPLC LC-30A system (Shimadzu, Japan), while the chromatographic separation was processed on a Waters HSS T3 (150 × 3 mm, 1.8 μm) column at 25°C, with a flow rate of 0.3 ml/min. The analysis was completed with mobile phases A (acetonitrile) and B (0.1% CH₃COOH–H₂O). The gradient program was 100% B at 0–10 min; 50% A and 50% B at 10–13 min; 95% A and 5% B at 13–14 min; 100% B at 14–15 min. All samples were kept at 4°C during the procedures.

The high-resolution MS system was performed using a TripleTOF5600 + mass spectrometer (AB SCIEX™, USA). Both positive and negative modes was used to acquire the data. Source parameters are defined as follows: scanning range, m/z 100–1,500; scanning mode, data-independent acquisition (DIA); capillary voltage, 5.000 V (positive) and 4.500 V (negative); capillary temperature, 500°C; declustering potential (DP), 60 V; collision energy (CE), 35 V; collision energy spared (CES), 15 V.

### 2.3.6 Data processing

The raw LC–MS data was imported into MS-DIAL3.96 software for preprocessing, then peak extraction, de-noise, deconvolution and peak alignment, and a 3D data matrix in CSV format was exported. The peak information was compared with metabolites from online databases including MassBank, Respect and GNPS. The three-dimensional matrix comprising sample information, retention time, mass nuclear ratio and mass spectrometry response intensity (peak area) was analyzed. Principal components analysis, partial least squares discriminate analysis and orthogonal partial least squares discrimination analysis were carried out to make multivariate statistical analysis using SIMCA-P (version 11.0, Umetrics, Umea, Sweden) software (Rezig et al., 2018).

### 2.3.7 Western blot analysis

The mouse spinal cord tissues (L4–6) were harvested and homogenized using RIPA buffer (Beyotime, P0013B) supplemented with 1x protease inhibitor cocktail (Sigma-Aldrich; P8304), phosphatase inhibitor cocktail II and III (Sigma-Aldrich; P5726). The supernatant was collected by centrifugation at 12,000g for 10 min, and the protein concentration was detected using a bicinchoninic acid protein assay kit (Beyotime, P0012S). An aliquot of 50 μg protein from each sample was separated using SDS-PAGE and transferred to a PVDF membrane, then blocked with 5% nonfat milk in TBST (pH 7.4). Thereafter, the membranes were incubated with primary antibodies including arginase I (1:1000; CST; #93668), argininosuccinate synthetase (1:1000; abcam; ab7095), argininosuccinatelyase (1:1000; abcam; ab97370) and actin (1:1000; ABclonal; AC026). After incubation with the appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (IgG, against rabbit, 1:1000; ABclonal; AS014), the immune complexes were visualized using the SuperSignal West Pico Substrate (34,077, Pierce). The digital images were quantified using densitometric measurements by Quantity-One software (Bio-Rad).

### 2.3.8 NO level detection

The spinal cord tissues (L4–6) were acquired and the level of nitric oxide (NO) was determined. Briefly, the NO detection kit (A012-1-2; Nanjing Jiancheng Biotechnology Co. Ltd; China) was purchased and the experiment protocol was performed according to the operating manual.

### 2.3.9 Statistical analysis

Data are presented as the mean ± standard deviation. An unpaired Student’s t-test was conducted using GraphPad Prism 8.0 (Graphpad, CA, USA). A value of P ≤ 0.05 was considered statistically significant.

### 3 RESULTS

#### 3.1 CFA-induced mechanical and thermal hypersensitivities

The mechanical and thermal hypersensitivities were examined on the fifth day after CFA injection. The results showed that the PWL and...
paw withdrawal threshold values were remarkably decreased in the CFA group compared with the control group (Figure 1a,b; \( P < 0.05 \)).

### 3.2 | Metabolic profiling analysis

To confirm whether chronic inflammation pain induced dramatic shifts in the metabolites in the spinal cord, an LC–MS method was applied to analyze the differences between the control and CFA groups. Principal components analysis (Figure 2a) and partial least squares discrimination analysis methods (Figure 2b) were used to detect the differences. The results showed that the two methods did not isolate differentially expressed metabolites (Figure 2a,b). Therefore, orthogonal partial least squares discrimination analysis mode was employed, and the metabolites were separated into two categories (Figure 2c). Meanwhile, the model was subjected to a parametric test, and the results indicated that the prediction rate of metabolites was 14.4%, the prediction rate of the grouping was 75.4% and the accuracy of model prediction was 72.3% (Figure 2d, e). To obtain different metabolite candidates, \( P \)-value < 0.05 and fold change > 2 were set as threshold values. The heat map and volcano plot of metabolites are separately shown in Figure 2f and g, and the details of the different metabolites are attached to Table 1.

### 3.3 | Protein expression and pathway analysis

The decrease in arginine levels may be involved in the alteration of key enzymes of the arginine–NO cycle including argininosuccinate synthetase and argininosuccinatelyase, and NO level and arginase I expression. To validate the hypothesis, the Western Blot (WB) assay was performed, and the results showed that the expression of arginase I was elevated in the CFA group compared with the control group, while the proteins of argininosuccinate synthetase and argininosuccinatelyase were not significantly different between the CFA group and the control group (Figure 3a). The NO level was obviously increased in the CFA group compared with the control group (Figure 3b, \( P < 0.05 \)). In order to screen significantly enriched pathways, the different metabolites were analyzed based on the KEGG and HMDB databases. In Table 2, metabolic pathways with raw \( P \) and impact values are listed. In addition, the impact of metabolic pathway is delineated in Figure 3c, and the pathways marked with letters were severely affected by chronic inflammation pain, with the details as follows (A–G): (A) aminoacyl-tRNA biosynthesis; (B) arginine and proline metabolism; (C) histidine metabolism; (D) purine metabolism; (E) phenylalanine; (F) tyrosine and tryptophan biosynthesis; and (G) glutathione metabolism and phenylalanine metabolism. Moreover, to provide insight into the pathobiological mechanism of chronic inflammation pain, the interaction networks among these seven metabolic pathways were generated and are presented in Figure 3d.

### 4 | DISCUSSION

Chronic inflammatory pain is universally regarded as a difficult medical problem worldwide and only partial therapy options are available. Various methods have been employed to investigate the potential mechanisms. However, the complex biochemical processes of chronic inflammatory pain remain poorly understood and little relief has been achieved in spite of the enormous efforts that have been made in basic medical and clinical research. Therefore, illuminating the underlying mechanism may provide novel strategies to alleviate pain with fewer side effects. Recently, systems biology strategies including metabolomics analysis have been widely applied to explore the pathogenic mechanism. In this study, the metabolites of CFA-induced chronic inflammation pain were analyzed based on a metabolomics method. The analysis showed that 27 metabolites were significantly altered in response to CFA injection and seven metabolic pathways were obviously enriched.

### 4.1 | The association between chronic inflammatory pain and metabolites

Inflammatory pain is a complex symptom involving multiple modulators consisting of neurotransmitters, receptors, ion channels and signaling pathways (Jiao et al., 2020). Previous studies documented that NF-\( \kappa \)B, as a ubiquitously expressed transcription factor, could effectively initiate the inflammatory response to mediate...
cell proliferation, apoptosis and metastasis (Sethi, Sung, & Aggarwal, 2008). Insulin resistance was enhanced by the NF-κB pathway to accelerate the progress of inflammatory reactions (Wang, Zhang, Wang, Wang, & Liu, 2019). Inflammatory and oxidative stress were closely correlated with the development of metabolic complications, and NF-κB signaling may promote the deterioration of non-alcoholic fatty liver disease by inducing the accumulation of triacylglycerol in the liver (Kang et al., 2017; Valenzuela & Videla, 2020). Moreover, emerging evidence has shown that metabolic disturbance may participate in regulating excitable membranes, synaptic transmission and synaptic plasticity. Surveys suggested several metabolites as biological markers that are sensitive to pain pathology induced by CFA injection. Similarly, the differentially expressed metabolites were screened, and the results showed that the expression of 26 metabolites was significantly changed in response to CFA injection. Hence, the potential regulatory network was analyzed, and a hub metabolite was sought out for developing a therapeutic method of chronic inflammation pain.

**FIGURE 2** Metabolic profiling analysis: (a) principal components analysis; (b) partial least squares discrimination analysis; (c) orthogonal partial least squares discrimination analysis; (d, e) parametric test; (f) Heat map analysis of metabolites between control group and CFA group (the color scale shows the relative metabolites expression in certain slide: blue indicates low relative expression levels; red indicates high relative expression levels; yellow indicates no change); (g) volcano plot of metabolites between control group and CFA group (red indicates the metabolites expression was significantly down/up-regulated in CFA group compared with control group; \( P < 0.05 \)). \( R^2X \) represents the prediction rate of metabolites, \( R^2Y \) represents the prediction rate of grouping, and \( Q^2 \) represents the accuracy of model prediction.
| Alignment ID | Average retention time (min) | Average Mz | Metabolite name | Adduct type | MS/MS assigned | Reference m/z | Formula | Ontology |
|-------------|-----------------------------|------------|----------------|-------------|----------------|---------------|---------|-----------|
| 44          | 3.798                       | 104.05289  | N-Methylalanine | [M + H]^+  | True           | 104.0706     | C₄H₉NO₂ | Alanine and derivatives |
| 47          | 2.967                       | 104.07158  | a-Aminoisobutyrate | [M + H]^+  | True           | 104.0706     | C₄H₉NO₂ | Alpha amino acids |
| 634         | 3.507                       | 132.101    | Isoleucine      | [M + H]^+  | True           | 132.1028     | C₉H₁₅NO₃ | Isoleucine and derivatives |
| 1006        | 4.964                       | 146.16362  | Spermidine      | [M + H]^+  | True           | 146.16518    | C₉H₁₅NO₃ | Diacylaminos |
| 1093        | 3.779                       | 150.05882  | Methionine      | [M + H]^+  | True           | 150.05832    | C₇H₁₇NO₅ | Methionine and derivatives |
| 1,149       | 4.075                       | 156.0755   | Histidine       | [M + H]^+  | True           | 156.07675    | C₉H₁₅NO₃ | Histidine and derivatives |
| 1,254       | 3.967                       | 161.12683  | l-β-Homolysine  | [M + H]^+  | True           | 161.12845    | C₉H₁₅NO₃ | β Amino acids and derivatives |
| 1,285       | 3.391                       | 162.11143  | l-carnitine     | [M + H]^+  | True           | 162.11247    | C₉H₁₅NO₃ | Carnitines |
| 1,291       | 3.753                       | 162.112    | Tyrosine        | [M + H]^+  | True           | 182.08118    | C₉H₁₅NO₃ | Tyrosine and derivatives |
| 1,572       | 3.702                       | 182.0866   | L-β-Homolysine  | [M + H]^+  | True           | 220.11795    | C₇H₁₇NO₅ | Secondary alcohols |
| 2,298       | 4.286                       | 227.11293  | L-Carnosine     | [M + H]^+  | True           | 227.11386    | C₉H₁₄NO₃ | Hybrid peptides |
| 2,657       | 1.416                       | 245.07821  | Uridine         | [M + H]^+  | True           | 245.07821    | C₉H₁₄NO₃ | Pyrimidine nucleosides |
| 2,948       | 6.998                       | 261.03534  | D-Mannose-6-phosphate | [M + H]^+  | True           | 261.03699    | C₉H₂₁O₅ | Hexose phosphates |
| 3,133       | 2.362                       | 269.08701  | Inosine         | [M + H]^+  | True           | 269.0804     | C₁₀H₁₄N₅O₄ | Purine nucleosides |
| 4,947       | 6.973                       | 364.06473  | Guanosine 5'-monophosphate | [M + H]^+  | True           | 364.06528    | C₁₀H₁₄N₅O₄ | Purine ribonucleoside monophosphates |
| 55          | 1.398                       | 115.00401  | Maleic acid     | [M - H]^−  | True           | 115.00368    | C₄H₄O₄ | Dicarboxylic acids and derivatives |
| 217         | 3.937                       | 154.06157  | His             | [M - H]^−  | True           | 154.06219    | C₉H₁₄N₂O₂ | Histidine and derivatives |
| 263         | 3.67                        | 164.07458  | L-(--)-Phenylalanine | [M - H]^−  | True           | 164.0717     | C₉H₁₄NO₂ | Phenylalanine and derivatives |
| 301         | 6.907                       | 171.00775  | Glycerophosphate(2) | [M - H]^−  | True           | 171.00639    | C₉H₂₀O₅ | Glycerophosphates |
| 312         | 1.694                       | 173.00899  | cis-Aconitate   | [M - H]^−  | True           | 173.00916    | C₉H₂₀O₅ | Tricarboxylic acids and derivatives |
| 317         | 4.026                       | 173.10483  | L-(+)-Arginine  | [M - H]^−  | True           | 173.1044     | C₉H₁₄N₂O₂ | L-α-Amino acids |
| 803         | 6.769                       | 229.0134   | D-Ribulose 5-phosphate | [M - H]^−  | True           | 229.01188    | C₉H₂₁O₅ | Pentose phosphates |
| 1,718       | 5.74                        | 322.0506   | Cytidine-3'-monophosphate | [M - H]^−  | True           | 322.04459    | C₉H₁₄N₂O₅ | Ribonucleoside 3'-phosphates |
| 1,731       | 6.877                       | 323.02869  | Uridine 5'-monophosphate | [M - H]^−  | True           | 323.02859    | C₉H₁₄N₂O₅ | Pyrimidine ribonucleoside monophosphates |
| Alignment ID | Average retention time (min) | Average Mz | Metabolite name | Adduct type | MS/MS assigned | Reference m/z | Formula | Ontology |
|--------------|-----------------------------|------------|----------------|-------------|----------------|---------------|--------|----------|
| 3,024        | 7.264                       | 476.09399  | 8-Methylthiooctyl glucosinolate | [M – H]⁻ | True | 476.10883 | C₁₆H₃₁NO₉S₃ | Alkylglucosinolates |

**TABLE 1** (Continued)

| Alignment ID | INCHIKEY | SMILES | MS1 isotopic spectrum | MS/MS spectrum | m-CON-1-1 | m-CON-1-2 | m-CON-2-1 | m-CON-2-2 |
|--------------|----------|--------|-----------------------|----------------|-----------|-----------|-----------|-----------|
| 44           | GDFAOVXKHJXLEI-VIKHMYSHEASA-N | CN[C@@H][C(C)=O] | 104.05318:10556 105.05653:4518 106.05998:904 | 58.07356:42104:1170:6:42 | 2,194 | 813 | 6,113 | 23,888 |
| 47           | FUOOLUPWFVMBKG-UHFFAOYSA-N | CC(C)(O)=O | 104.06766:5184 105.07101:4230 106.07437:2256 | 56.05714:83 580.7238:2343 | 58.11862:142 58.14444:48 | 58.21546:42 58.37488:42 | 59.05377:43 59.08196:319 | 59.1004:63 59.91335:48 | 60.08711:1660 60.11774:104 | 60.19324:42 60.48803:42 | 61.00925:63 69.03474:42 | 71.08587:42 87.05281:63104. | 104.06766:275158 | 132.0626:175158 | 133.10435:40078 | 134.10771:5289 | 53.00695:63 53.02544:42 | 55.02148:42 55.06335:83 | 56.06026:171 57.06399:150 | 57.07038:149 58.05942:83 | 58.0691:63 58.07985:63 | 62.94052:21 69.04636:63 | 69.07684:478 69.10146:102 | 69.21879:42 71.07623:42 | 72.06087:146 72.08722:42 | 72.94139:146 73.0655:63 | 74.0699:42 85.8249:42 | 86.09304:87 86.10222:2372 | 86.20303:179 86.27247:83 | 86.34325:83 86.53212:42 | 87.06313:20 87.08025:133 | 87.09867:104 89.0625:44 | 90.05686:982 90.90903:42 | 114.07255:63115:50105:6 | 311.07801:42119.07763:21 | 127.86925:43132.07666: | 840132.11559:42 | 14,260 | 715 | 869 | 106.976 |

**TABLE 1** (Continued)
| Alignment ID | INCHIKEY | SMILES | MS1 isotopic spectrum | MS/MS spectrum | m-CON-1-1 | m-CON-1-2 | m-CON-2-1 | m-CON-2-2 |
|-------------|----------|--------|-----------------------|----------------|-----------|-----------|-----------|-----------|
| 1.006       | ATHGHQPFGPMSJY-UHFFFAOYSA-N | NCCCCNCCCCN | 146.16512:4739 147.16847:688 148.17183:0 | 56.96902:21 58.07324:42 72.08226:146 72.09304:104 84.08359:6311.121149:42 | 53 | 69 | 1.045 | 3.522 |
| 1.093       | FFEARJCKVFRZRR-UHFFFAOYNA-N | CSCCC(N)(O)=O | 150.056:51970 151.05935:5422 152.06271:8131 | 53.04919:42 53.06153:42 53.06387:146 58.07324:42 60.08455:63 61.08674:21 | 991 | 2.193 | 3.376 | 7.331 |
| 1.149       | HNDVDQJCIGZPNO-UHFFFAOYNA-N | O=C(O)(N)CC1-CN=CN1 | 156.07379:27948 157.07714:3718 158.0805:3718 | 50.02816:21 54.04945:63 56.06555:147 66.04946:83 68.05582:83 71.95312:42 71.95852:42 81.04952:169 82.05473:167 82.07135:63 83.07144:83 83.06357:31 83.10729:42 86.06815:21 93.04813:366 95.05525:22 95.06213:63109.721:65110.07299:88311.05206:42 1.05206:42115.50999:21 56.07678:83 | 2.559 | 3.836 | 10.967 | 5.033 |
| 1.254       | PJDINCOFOROBQW-LURJTMIESA-N | NCCCC[C@H](N)(C)(O)=O | 161.12852:5683 162.13187:1610 163.13523:1610 | 70.07452:21 72.08102:125 8 4.08356:83 84.10168:6313 9.03064:21144.10316:63146.07782:42161.118142 | 4.751 | 1.240 | 3.089 | 56.286 |
| Alignment ID | INCHIKEY               | SMILES                              | MS1 isotopic spectrum           | MS/MS spectrum               | m-CON-1-1 | m-CON-1-2 | m-CON-2-1 | m-CON-2-2 |
|-------------|------------------------|-------------------------------------|---------------------------------|-----------------------------|-----------|-----------|-----------|-----------|
| 1.285       | PHIQHXFUZVPYII-ZCFIWIBFSAS-N | C\([N+](C)(C\@H)\)\((O)[O-][;]O\) | 162.1134:29728 163.1179:5364 164.12045:1490 | 57.03634:42 58.07023:104 59.07545:63 60.09476:104 85.03555:10410209923 104103:03878 104103:04 737:14614609703:63162 11127:503 | 137.818 | 126.043 | 189.429 | 120.297 |
| 1.291       | PHIQHXFUZVPYII-ZCFIWIBFSAS-N | C\([N+](C)(C\@H)\)\((O)[O-][;]O\) | 162.1185:524328 163.1152:89133 164.11856:11269 | 54.93892:42 55.95851:43 57.04489:61 57.0627:96 57.09255:56 58.07563:878 59.07764:378 59.35122:63 601388:48 60.08713:1754 60.11197:60 60.12213:63 60.1365:111 61.0302:125 610643:72 84.76516:85 85.03555:2327 85.0882:99 86.05917:42 97.7429:42 98.96867:42 1009148:1763 103.0430:25 444.10301:31 102.103:2264 483104.41814:42 11414:9605 242146.10208:63 161587:61 08.82161:10399:24 162146:26 125131:66 623456:62 125162:44 14545:104 | 114.299 | 2126.919 | 2391.938 | 55.919 |
| 1.572       | OUYCCASQSFEME-UHFFFAOYNA-N | O=C(O(N)\(CC\))\(=CC=CC(O)=C\) | 182.08034:80922 183.08369:11783 184.08705:2561 | 51.03576:63 53.04075:7:148 53.06729:42 55.02642:31 68.437:32 65.04722:246 67.05714:42 74.79868:21 77.04453:33 77.07794:36 79.05739:73 79.08247:21 81.03521:21 81.06955:52 88.0251:21 90.77292:42 91.0581:2809 91.10388:61 91.1683:43 93.05737:31 94.04394:42 94.7534:52 951052:211776 95.08797:26 95.10886:26 99.03855:2110 1.04308:31105:05828.941 1.06608:22107:05055:90 1.0108.0651:21109:06851:52 1.1571:05843:33118.06648:16718.66634:2119.0511 16128:119.09734:34120.0 5537:311210.06388:3122.64 | 8.060 | 623 | 884 | 226.568 |
| Alignment ID | INCHIKEY | SMILES | MS1 isotopic spectrum | MS/MS spectrum | m-CON-1-1 | m-CON-1-2 | m-CON-2-1 | m-CON-2-2 |
|--------------|----------|--------|-----------------------|----------------|-----------|-----------|-----------|-----------|
| 2.218        | GHOKWGTUZJEAOQD-ZETCQYMHSA-N | CC(C)(CO)[C@@H](O)(O)=NC CC(O)=O | 220.11549:34997 221.11884:3891 222.1222:1196 | 56.01493:42 57.07793:63 59.06138:42 60.6334:82 69.08517:42 70.03354:63 72.04784:63 77.04002:42 79.05535:42 83.05476:42 85.06741:63 87.08044:42 90.05307:85 90.05977:337 94.07184:42 95.05688:83 98.01903:42 98.02183:831 105.0196:42 109.474 | 181.066 167.154 220.827 109.474 |
| 2.298        | CQVVPNPJLQNMDC-ZETCQYMHSA-N | NCCC(O)=N[C@@H](CC1=CN=CN1)CC(O)=O | 227.1124:74781 228.11575:11134 229.11911:1465 | 55.04132:42 68.05227:42 82.05465:35 83.06734:215 83.06734:215 83.08278:64 83.96175:93 0.494 146 93.84448:43 95.0 63.235109.72236:47 110.07436:1878 110.1069 2.69110.16467:5719.075 93.63122675:36112:20 875.147136:08853:63141 1092.21446:08275:63151 0.34888:215208:218:83155 0.3369:43156:07483:71216 1.68329:21164:07979:23017 2.06091:21820:08009:6318 1.10577:125192:07181:422 100862.188210.1046:1042 24.81111:21227.10995:104 227.12697:83 | 18.611 8.659 3.375 691.174 |
| Alignment ID | INCHIKEY  | SMILES                        | MS1 isotopic spectrum | MS/MS spectrum | m-CON-1-1 | m-CON-1-2 | m-CON-2-1 | m-CON-2-2 |
|-------------|-----------|-------------------------------|-----------------------|----------------|-----------|------------|-----------|-----------|
| 2.657       | DRTQHPVMGBUCF -UHFFFAOYNA-N | O=C1N=C(O)=ON1C=O C=O | 245.07242:3838 246.07577:45224 70.07913:0 | 70.02979:42 71.02264:21 96.01913:63 97.0379:421 3.02923:65113 0.03673:5371 13.06073:42245 22159:63 | 69.991 | 42.498 | 24.696 | 39.146 |
| 2.948       | NBSCHQHZLSJFNQ -QTVWNMPRSA-N | OC1O[C@H]1O[C@H](O)[O][C@H][O][C@H][C@H][O][C@H]1O | 261.03534:50962 262.03869:4518 263.04205:1061 | 53.04903:63 57.04477:63 356.21:67 0.02851:21 69.0 | 3809.106 71.04882:42 80.9 8725.8:85 81.03551:281 85.2 | 3593.230 93.06441:21 97.0 | 4064.3:63 98.9625:42 99.3 | 2415.7:99 0.4521:104199.0 | 0723.42103:3968 44019:03 0464 725109:09969:23 | 118.19:23 | 981.21127:4094:383127:0 | 0.5207:127145:04799:61360:98993:21207:00189:42 | 2225.0 | 0177:42225:01447:104243:0 | 2344:146 |
| 3.133       | UGQMRVRMYASKQ -KQYNXCSUSA-N | OC1O[C@H]1O[C@H][O][C@H]+OC1O[C@H]1O[C@H][O][C@H][C@H][C@H][O][C@H]1O | 269.08591:112501 270.08926:17870 271.09262:3310 | 55.02217:63 55.03479:42 57.04383:63 7.06385:63 67.0 | 0.3049:42 69.03893:63 71.0 | 0211:45 73.0350:83 82.0 | 154:83 85.02518:42 85.03 | 168.63:85 0.05459:63 92.029 | 88:42 94.03973:213 95.217 | 23.21:97 0.02918:42 99.883 | 33:42103:40815:43 | 110.03706:67411054003:8 | 85118:6619:43119:03718:7 | 769120:0197:104133:047:5 | 59:83136:60722:209137:0 | 4619:23885:137.17505:16 | 82:137.26236:66731:737:3519 | 250137:39595:42137:4891 | 7.42137:55205:167137:644 | 71:125137:81856:121538:3 | 0925:13384:0051:42140:3 | 2079:42225:12384:42219 | 2112:21 |
| 4.947       | RQFCJASXICIDSA -UUXOKMHZSA-N | O[C@@H]1O[C@@H][COP (O)(O)=O][O][C@@H][C@@H]1ON1C=NC2=CN1N=CN=C2 | 364.05899:1155 36 506234:434366 | 065:787 | 110.05524:10135:02759:21149:02536:21152:05 | 627:125152:08411:631 | 6983 | 2643 | 263 | 6031 | (Continues) |
| Alignment ID | INCHIKEY          | SMILES                                      | MS1 isotopic spectrum          | MS/MS spectrum        | m-CON-1-1 | m-CON-1-2 | m-CON-2-1 | m-CON-2-2 |
|-------------|-------------------|---------------------------------------------|-------------------------------|----------------------|-----------|-----------|-----------|-----------|
| 55          | VZCY00QTPOCHFL    | O=C(O)C=cc(=O)O                            | 115.00503:9050 116.00838:577 117.01174:576 | 71.05444:21 71.05563:21 | 55,633    | 77,348    | 48,858    | 62,670    |
| 217         | HNDVDQJICIZPNO    | N[C@@H](CC1=CN=C N1)C(O)=O                 | 154.06317:5394 155.06652:6751 56.06988:630 | 80.04959:42 91.03069:21 9 | 2,130     | 584       | 2,731     | 2,805     |
| 301         | AWUCVROLDVIAJX    | O[C@@H](O)(O)(O)=O                          | 171.01015:13672 172.0135:624173 0.1666:286 | 77.99744:43 78.96368:57 9 | 193,915   | 455,002   | 195,998   | 313,002   |
| 312         | GTZCFVGUGFEME     | OC(=O)(=O)C=O                              | 173.01173:4774 174.01508:988175.018 44:188 | 85.03636:63 | 26,885    | 37,094    | 29,617    | 1902      |
| 317         | ODKSFYDXXFIFQN    | N[C@@H](CCCNC(N)=N)(N)=O                   | 173.10464:16345 174.10799:2098 175.11135:74 | 105.03072:21131 0.8583:37 | 9,483     | 93,266    | 131,938   | 8,595     |
| 803         | FNZLVKNWTIPJ      | OCC(O)C=H|O|(O)=O                                  | 229.00955:5537 230.0133:849231 0.1666:282 | 78.95767:63 78.96269:294 9 | 193,915   | 455,002   | 195,998   | 313,002   |
| 1718        | UOOOPKAINPLOPU    | OC(=O)(=O)C=O                              | 322.0498:4108 323.0515:759324:0 5651:33 | 78.96099:125 96.97363:422 | 33,820    | 128,567   | 23,557    | 160,602   |
| 1731        | DJJCFVJGTHFX      | OC(=O)(=O)C=O                              | 323.03195:4429 324.0353:820325.0386:612 | 78.96327:167 80.51807:21 96.9734:3111 0.2354:42 211.00215:6323:03466:63 | 29,233    | 149,656   | 17,963    | 202,163   |
| 3,024       | CWOJBEDMJZKAB     | CSCCCCCCCC|C|C@H|O|C@H|O|C@H|O|C@H|O|C@H|O|C@H|O|C@H|O|N=O(O)=O 10281:695 | 476.0961:3018 477.0995:479478.10281:695 | 96.98495:42357:10698:42 389.07697:104458:1270 121476.10638:167476.127 93.188 | 33,371    | 34,322    | 19,035    | 24,637    |
| Alignment ID | m-CON-3-1 | m-CON-3-2 | m-CON-4-1 | m-CON-4-2 | m-CON-5-1 | m-CON-5-2 | m-CFA-1-1 | m-CFA-1-2 |
|-------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 44          | 45,403    | 29,632    | 46,466    | 46,437    | 50,186    | 6,318     | 52,019    | 38,489    |
| 47          | 87,202    | 80,753    | 98,610    | 104,309   | 96,175    | 82,126    | 91,306    | 110,792   |
| 634         | 120,636   | 816       | 154,014   | 34,887    | 3,356     | 859       | 15,242    | 1,267     |
| 1,006       | 8,376     | 7,891     | 12,487    | 8,258     | 28,946    | 582       | 12,503    | 25,642    |
| 1,093       | 5,339     | 1925      | 248,496   | 8,429     | 224,333   | 1867      | 12,403    | 9,580     |
| 1,149       | 9,758     | 57,934    | 130,906   | 8,141     | 45,796    | 2,921     | 167,330   | 124,687   |
| 1,254       | 40,507    | 44,075    | 15,866    | 60,653    | 13,569    | 372       | 77,838    | 74,484    |
| 1,285       | 182,996   | 226,630   | 171,583   | 230,633   | 230,463   | 1,617     | 354,025   | 440,909   |
| 1,291       | 39,264    | 15,737    | 7,897     | 45,777    | 2049      | 2,570,282 | 27,836    | 16,605    |
| 1,572       | 388,549   | 282,080   | 350,601   | 507,047   | 346,685   | 2         | 433,140   | 282,783   |
| 2,181       | 147,563   | 108,886   | 56,756    | 58,130    | 89,778    | 218,550   | 133,007   | 121,659   |
| 2,298       | 7,229     | 672,318   | 676,762   | 765,788   | 534,700   | 11,967    | 994,556   | 706,764   |
| 2,657       | 37,751    | 48,829    | 31,960    | 31,420    | 14,001    | 31,382    | 9,996     | 37,357    |
| 2,948       | 220,245   | 70,522    | 88,688    | 424,738   | 442,372   | 825,961   | 540,513   | 331,605   |
| 3,133       | 1,083,286 | 1,455,366 | 1,113,897 | 1,444,050 | 899,13     | 1,494,445 | 1,074,040 | 1,458,989 |
| 4,947       | 1,126     | 1,516     | 323       | 5,970     | 5,029     | 4,968     | 1877      | 7,575     |
| 55          | 32,151    | 49,493    | 32,421    | 69,355    | 21,203    | 39,650    | 26,928    | 50,276    |
| 217         | 1,596     | 25,392    | 32,088    | 2,772     | 46,927    | 1924      | 37,518    | 31,430    |
| 263         | 77,746    | 57,819    | 75,580    | 88,057    | 81,639    | 137       | 100,927   | 61,536    |
| 301         | 258,938   | 164,165   | 293,164   | 184,300   | 291,288   | 304,236   | 128,549   | 153,126   |
| 312         | 1,439     | 1,126     | 1,095     | 1,447     | 1725      | 52,483    | 1791      | 2,320     |
| 317         | 20,461    | 7,508     | 6,163     | 15,823    | 9,093     | 60,177    | 10,077    | 6,734     |
| 803         | 86,127    | 4,758     | 31,327    | 69,766    | 35,181    | 109,452   | 14,016    | 7,827     |
| 1718        | 21,139    | 14,234    | 34,175    | 26,041    | 13,700    | 33,224    | 10,321    | 34,112    |
| 1731        | 18,277    | 21,042    | 29,042    | 20,533    | 14,499    | 28,719    | 13,747    | 23,658    |
| 3,024       | 14,148    | 28,477    | 7,809     | 21,548    | 27,341    | 22,910    | 13,362    | 10,208    |
| Alignment ID | m-CFA-2-1 | m-CFA-2-2 | m-CFA-3-1 | m-CFA-3-2 | m-CFA-4-1 | m-CFA-4-2 | m-CFA-5-1 | m-CFA-5-2 |
|-------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 44          | 45,164    | 35,816    | 72,491    | 40,667    | 48,518    | 38,682    | 43,410    | 72,211    |
| 47          | 92,627    | 107,516   | 125,306   | 114,844   | 154,446   | 108,947   | 156,350   | 139,787   |
| 634         | 1,462,686 | 8,721     | 1,404,377 | 1,314,712 | 1,672,418 | 3,525     | 1,578     | 6,252     |
| 1,006       | 29,045    | 21,045    | 21,998    | 26,846    | 70,867    | 113,281   | 56,294    | 62,733    |
| 1,093       | 203,402   | 208,821   | 281,16    | 185,274   | 234,987   | 262,779   | 342,045   | 404,916   |
| 1,149       | 40,716    | 143,928   | 67,014    | 47,851    | 26,308    | 44,872    | 318,432   | 290,404   |
| 1,254       | 15,263    | 81,451    | 31,709    | 26,618    | 22,144    | 20,078    | 22,976    | 94,105    |
| 1,285       | 1,259,888 | 305,598   | 292,446   | 335,317   | 1,768,962 | 338,206   | 430,799   | 398,995   |
| 1,291       | 9,793     | 17,451    | 1,601     | 9,851     | 14,734    | 10,385    | 11,670    | 23,891    |
| 1,572       | 263,639   | 318,859   | 309,281   | 265,574   | 326,698   | 310,226   | 493,607   | 582,405   |
| 2,218       | 44,553    | 87,255    | 76,023    | 81,978    | 30,429    | 92,765    | 114,592   | 121,395   |
| 2,298       | 307,627   | 838,688   | 664,714   | 681,011   | 67,650    | 941,312   | 717,540   | 803,866   |
| 2,657       | 14,007    | 17,556    | 2,538     | 28,768    | 2,617     | 11,325    | 1824      | 1,048     |
| 2,948       | 354,822   | 396,896   | 424,848   | 373,235   | 413,106   | 497,578   | 948,371   | 689,780   |
| 3,133       | 656,759   | 1,296,131 | 533,352   | 1,233,329 | 494,316   | 966,957   | 696,056   | 1,027,346 |
| 4,947       | 1,051     | 9,004     | 11,751    | 1790      | 17,554    | 11,665    | 7,104     | 8,026     |
| 55          | 11,637    | 51,514    | 32,987    | 25,449    | 20,385    | 39,037    | 39,291    | 29,654    |
| 217         | 44,829    | 28,002    | 50,461    | 38,582    | 49,224    | 31,520    | 50,570    | 2,223     |
| 263         | 6,396     | 65,599    | 92,867    | 78,271    | 85,666    | 57,850    | 103,083   | 116,152   |
| 301         | 10,999    | 162,820   | 9,070     | 9053      | 9,921     | 189,087   | 256,611   | 127,301   |
| 312         | 682       | 1,536     | 1,215     | 1,062     | 1,274     | 1,506     | 2057      | 2,623     |
| 317         | 1,693     | 9,333     | 6,493     | 6422      | 4,270     | 7,258     | 14,847    | 34,069    |
| 803         | 28,820    | 11,103    | 33,714    | 42,339    | 35,998    | 68,688    | 61,508    | 9,504     |
| 1,718       | 10,600    | 21,033    | 19,820    | 12,858    | 16,402    | 12,454    | 13,615    | 9,089     |
| 1,731       | 1,557     | 17,368    | 19,780    | 20,317    | 2,303     | 20,274    | 14,242    | 5,319     |
| 3,024       | 10,621    | 9,090     | 6,317     | 19,145    | 6,381     | 14,649    | 9,622     | 6,535     |
| Alignment ID | QC-1  | QC-2  | QC-3  | QC-4  | QC-5  | VIP (CFA vs. CON) | FC (CFA vs. CON) | TTEST (CFA vs. CON) |
|-------------|-------|-------|-------|-------|-------|------------------|------------------|-------------------|
| 44          | 3.369 | 2.412 | 35.398| 1967  | 37.982| 0.060775        | 1.8934438371     | 0.0041166709      |
| 47          | 20.239| 3.645 | 41.971| 67.113| 61.782| 0.10933         | 1.5250778877     | 0.0002315302      |
| 634         | 49.284| 40.378| 42.667| 43.929| 38.667| 1.4409          | 13.468032579      | 0.017904852       |
| 1.006       | 741   | 71    | 346   | 63    | 856   | 0.097503        | 6.1808252257      | 0.0010790023      |
| 1.093       | 410   | 4.593 | 2.204 | 824   | 219.231| 0.43378        | 4.2556179107      | 0.0022510742      |
| 1.149       | 3.874 | 2.741 | 3.303 | 6.566 | 4.099 | 0.26255         | 4.5763448755      | 0.0061254643      |
| 1.254       | 3.417 | 867   | 2.832 | 2.743 | 7.909 | 0.059781        | 1.9411417257      | 0.0421885982      |
| 1.285       | 1.008 | 1.020 | 117.255| 123.861| 125.978| 1.1382        | 3.6631295405      | 0.007597821       |
| 1.291       | 2.141,254| 2.090,007| 104.083| 166.766| 41.578| 1.9093        | 0.195136254       | 0.0288782904      |
| 1.572       | 230   | 1.033 | 343.879| 8.427 | 382.659| 0.38975       | 1.6987417454      | 0.0247046305      |
| 2.218       | 95.250| 291.906| 285.777| 247.110| 218.772| 0.12778       | 0.6513850084      | 0.0240294256      |
| 2.298       | 459   | 802   | 451.869| 130.45 | 501.460| 0.88068       | 1.9830595505      | 0.0156686685      |
| 2.657       | 30.913| 36.493| 47.935| 42.544| 40.265| 0.064638       | 0.3417941529      | 0.0004340064      |
| 2.948       | 426   | 6.442 | 11.014| 48.095| 52.117| 0.69733        | 2.1319580501      | 0.0090084967      |
| 3.133       | 1.380,913| 1.675,873| 1.554,619| 1.649,054| 1.345,126| 1.2935        | 0.6584421129      | 0.0042157925      |
| 4.947       | 49    | 129   | 273   | 28    | 443   | #N/A           | 2.710738302       | 0.007436557       |
| 55          | 45.173| 36.089| 41.432| 43.091| 38.137| 0.042704       | 0.6693331587      | 0.0155992248      |
| 217         | 1.664 | 3.377 | 1898  | 4.881 | 3.229 | 0.064842       | 3.063153116       | 0.0012762611      |
| 263         | 59.012| 58.157| 61.426| 63.599| 64.054| 0.075006       | 1.5859668171      | 0.036198234       |
| 301         | 125.714| 155.695| 163.806| 208.021| 204.086| 0.42234       | 0.3979411738      | 0.0003830492      |
| 312         | 24.108| 19.080| 22.670| 20.288| 20.534| #N/A          | 0.1037781846      | 0.0177163028      |
| 317         | 17.284| 17.708| 8.559 | 20.185| 9.741 | 0.069043       | 0.2791559887      | 0.0419059935      |
| 803         | 38.938| 49.583| 50.449| 52.324| 56.902| 0.0888        | 0.4823083618      | 0.0170326847      |
| 1718        | 29.710| 32.516| 39.322| 35.315| 39.940| 0.086863       | 0.3277804927      | 0.0305979778      |
| 1731        | 34.845| 38.533| 36.869| 43.415| 47.390| 0.13262       | 0.1929425542      | 0.0284777076      |
| 3.024       | 9.484 | 13.047| 20.190| 11.853| 19.247| 0.033732       | 0.4534713482      | 0.0001821421      |
4.2 The metabolic alterations elicited by chronic inflammatory pain

Several metabolites induced by chronic inflammatory pain were identified, which may be implicated in nervous impulse transmission. To clarify the metabolic process, spinal cord tissues were acquired and the regulatory process of metabolites was analyzed. Generally, arginine is susceptible to the level of guanidine compounds, and thereby results in citrullination (Wang et al., 2019). In addition, a previous study showed that arginine downregulation exacerbated the inflammatory reactions, and thereby resulted in the degradation of amino acids (Schroecksnadel et al., 2006). In this study, our results showed that the level of arginine was significantly decreased in the CFA group compared with the control group, which may directly mediate the inflammatory response and cause inflammatory pain. In addition, related documents revealed that arginine participated in the synthesis of NO neurotransmitter, which could produce anti-nociceptive natural opioids and N-methyl-D-aspartate receptor-mediated pain-promoting effect (Chen et al., 2016; Rondon et al., 2018), whereas neurotransmitter depletion derived from arginine decrease may contribute to inflammatory pain. Moreover, histidine is closely related to the inflammatory processes by regulating the synthesis of histamine.
| Total | Expected | Hits | Raw P | #name? | Holm adjust | FDR | Impact |
|-------|----------|------|-------|--------|-------------|-----|--------|
| 69    | 3.6034   | 17   | 1.81 × 10^{-8} | 17.827  | 1.48E-06   | 1.48E-06 | 0.12903 |
| 44    | 2.2978   | 10   | 4.84 × 10^{-5} | 9.9365  | 0.0039184  | 0.0019834 | 0.36034 |
| 15    | 0.78335  | 4    | 0.0060344 | 5.1103  | 0.48275    | 0.11698  | 0.24194 |
| 24    | 1.2534   | 5    | 0.0060671 | 5.0196  | 0.52196    | 0.11698  | 0.60232 |
| 68    | 3.5512   | 9    | 0.0071327 | 4.9431  | 0.55635    | 0.11698  | 0.23524 |
| 9     | 0.47001  | 3    | 0.0091513 | 4.6939  | 0.70465    | 0.12507  | 0 |
| 4     | 0.20889  | 2    | 0.015078  | 4.1945  | 1          | 0.15742  | 1 |
| 11    | 0.57445  | 3    | 0.016665  | 4.0944  | 1          | 0.15742  | 0.99999 |
| 41    | 2.1411   | 6    | 0.017278  | 4.0583  | 1          | 0.15742  | 0.1534 |
| 31    | 1.6189   | 5    | 0.019841  | 3.92    | 1          | 0.1627   | 0.26989 |
| 5     | 0.26112  | 2    | 0.024285  | 3.7179  | 1          | 0.18103  | 1 |
| 15    | 0.78335  | 3    | 0.039543  | 3.2304  | 1          | 0.26972  | 0.02041 |
| 26    | 1.3578   | 4    | 0.04276   | 3.1522  | 1          | 0.26972  | 0.44179 |
| 27    | 1.41     | 4    | 0.048266  | 3.031   | 1          | 0.2827   | 0.17491 |
| 17    | 0.88779  | 3    | 0.054848  | 2.9032  | 1          | 0.29984  | 0 |
| 42    | 2.1934   | 5    | 0.063642  | 2.7545  | 1          | 0.32333  | 0 |
| 30    | 1.5667   | 4    | 0.067032  | 2.7026  | 1          | 0.32333  | 0.15371 |
| 11    | 0.57445  | 2    | 0.10909   | 2.2156  | 1          | 0.49694  | 0.40741 |
| 37    | 1.9323   | 4    | 0.12318   | 2.0941  | 1          | 0.53164  | 0.16012 |
| 3     | 0.15667  | 1    | 0.14873   | 1.9056  | 1          | 0.60979  | 0 |
| 4     | 0.20889  | 1    | 0.19328   | 1.6436  | 1          | 0.75471  | 0 |
| 5     | 0.26112  | 1    | 0.23553   | 1.4459  | 1          | 0.86     | 0 |
| 18    | 0.94001  | 2    | 0.24122   | 1.4221  | 1          | 0.86     | 0.38709 |
| 6     | 0.31334  | 1    | 0.27559   | 1.2888  | 1          | 0.886    | 0 |
| 6     | 0.31334  | 1    | 0.27559   | 1.2888  | 1          | 0.886    | 1 |
| 20    | 1.0445   | 2    | 0.28093   | 1.2697  | 1          | 0.886    | 0.09164 |
| 21    | 1.0967   | 2    | 0.30076   | 1.2014  | 1          | 0.91341  | 0.01504 |
| 38    | 1.9845   | 3    | 0.31867   | 1.1436  | 1          | 0.93324  | 0 |
| 23    | 1.2011   | 2    | 0.34013   | 1.0784  | 1          | 0.9556   | 0 |
| 8     | 0.41778  | 1    | 0.34961   | 1.0509  | 1          | 0.9556   | 0.42857 |
| 9     | 0.47001  | 1    | 0.38377   | 0.95772 | 1          | 0.9834   | 0 |
| 9     | 0.47001  | 1    | 0.38377   | 0.95772 | 1          | 0.9834   | 0.4 |
| 11    | 0.57445  | 1    | 0.44686   | 0.80551 | 1          | 1        | 0 |
| 13    | 0.6789   | 1    | 0.50357   | 0.68603 | 1          | 1        | 0.2381 |
| 16    | 0.83557  | 1    | 0.57805   | 0.54809 | 1          | 1        | 0.2 |
| 18    | 0.94001  | 1    | 0.62147   | 0.47567 | 1          | 1        | 0.0256 |
| 19    | 0.99224  | 1    | 0.64149   | 0.44396 | 1          | 1        | 0.13815 |
| 43    | 2.2456   | 2    | 0.6888   | 0.40227 | 1          | 1        | 0 |
| 21    | 1.0967   | 1    | 0.67845   | 0.38794 | 1          | 1        | 0.15342 |
| 22    | 1.1489   | 1    | 0.6955   | 0.36313 | 1          | 1        | 0 |
| 26    | 1.3578   | 1    | 0.7552   | 0.28077 | 1          | 1        | 0 |
| 27    | 1.41     | 1    | 0.76823   | 0.26367 | 1          | 1        | 0 |
| 27    | 1.41     | 1    | 0.76823   | 0.26367 | 1          | 1        | 0 |
| 36    | 1.88     | 1    | 0.85855   | 0.15251 | 1          | 1        | 0.32601 |

(Continues)
neurotransmitters (Shell et al., 2016). The metabolomics data showed that histidine expression was enhanced following CFA injection and ultimately led to chronic inflammation pain.

4.3 | Phenylalanine and tyrosine metabolism

Phenylalanine and tyrosine are essential amino acids synthesized from phenylalanine. The accumulation of phenylpyruvate is toxic to the central nervous system (Rausell et al., 2019). Previous research found that the levels of phenylalanine and tyrosine were remarkably increased in cerebrospinal fluid of patients with regional pain syndrome (Meissner et al., 2014). Dopamine, norepinephrine, and epinephrine produced by the phenylalanine and tyrosine metabolic reactions play a critical role in the brain. Norepinephrine released from the sympathetic nerve can activate β2ARs receptors, and result in production and secretion of the proinflammatory cytokine, subsequently causing hyperalgesia of sensory neurons and increasing chronic inflammatory pain (Li et al., 2013). Interestingly, our findings indicated that the pronounced increase of phenylalanine and tyrosine may accelerate pain signal transduction by increasing the concentration of neurotransmitters in the spinal cord.

Currently, data suggest that metabolic changes are relevant to many diseases, and metabolites obtained from accessible samples such as urine or plasma may serve as potential biomarkers for diagnosis of chronic inflammatory pain in the clinic (Liu et al., 2017a; Liu et al., 2017b). The spinal cord is the primary center of transmission signals. The signals of nociceptive stimuli are transmitted to the posterior horn of the spinal cord by fine fibers, and eventually pass to the cerebral cortex after processing in the spinal cord (Descalzi et al., 2015; Meacham, Shepherd, Mohapatra, & Haroutounian, 2017).

Therefore, investigating the alteration of metabolites in spinal cord may help to illuminate the neuronal communication mechanism regarding CFA injection-induced chronic inflammation pain. Collectively, this study provides a new perspective for comprehending the pathological process of CFA-induced chronic inflammation pain, and enhancing efforts to develop new therapeutic strategies.

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TABLE 2 (Continued)

| Metabolites                                    | Total | Expected Hits | Raw P | #name? Holm adjust | FDR | Impact |
|------------------------------------------------|-------|---------------|-------|--------------------|-----|--------|
| Fatty acid metabolism                          | 39    | 2.0367        | 1     | 0.88011            | 1   | 1      | 0      |
| Tryptophan metabolism                          | 40    | 2.0889        | 1     | 0.88655            | 1   | 1      | 0.17715|
| Tyrosine metabolism                            | 44    | 2.2978        | 1     | 0.90906            | 1   | 1      | 0.14045|
| Primary bile acid biosynthesis                 | 46    | 2.4023        | 1     | 0.9186             | 0.084902 | 1 | 1 | 0.02976|

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**COMPETING INTERESTS**

The authors declare that they have no competing interests.

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