Urinary metabolomics of complete Freund's adjuvant-induced hyperalgesia in rats

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Abstract

To demonstrate the differences of metabolomics changes in a hyperalgesia model and find potent biomarkers of hyperalgesia. Seven rats were placed in metabolic cages. An emulsion containing 500 μg of Complete Freund’s adjuvant (CFA) was used to induce hyperalgesia. Urine samples were collected prior to the injection of CFA and on post-injection days 1, 3, and 7. Ultraperformance liquid chromatography, coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF/MS) was used for a quantitative analysis of urinary metabolic changes in the CFA-induced hyperalgesia model. Differences between the metabolic profiles of the rats in the four groups were analyzed using partial least squares discriminant analysis (PLS-DA). Thirty-four potential urine metabolite biomarkers were identified, which changed in a trend similar with pain threshold. These potential biomarkers were involved in 11 metabolic pathways, as follows: alanine, aspartate, and glutamate metabolism; ascorbate and aldarate metabolism; glycerolipid metabolism; glycerophospholipid metabolism; histidine metabolism; phenylalanine metabolism; sphingolipid metabolism; tryptophan metabolism; tyrosine metabolism; valine, leucine, and isoleucine biosynthesis; and vitamin B6 metabolism. These results may improve our understanding of hyperalgesia and provide a basis for the clinical diagnosis of hyperalgesia.

Keywords: metabolomics; UHPLC-Q-TOF/MS; hyperalgesia; complete Freund's adjuvant; urine
Introduction

Hyperalgesia refers to increased sensitivity to pain. Although the nociceptive system provides protection against injuries, it brings years of torture to patients with chronic pain to suffer from. Chronic pain, which affects 20% of the population, is a disease with or without cause of other disorder (Breivik et al., 2006). Long-lasting and severe hyperalgesia can lead to alterations in organisms, which may decrease the alerting function and become a disease state (Costigan et al., 2009). Interactions between the immune system and peripheral nervous system play an important role in hyperalgesia via the release of pain-related chemicals and cytokines (Marchand et al., 2005). Pain intensity and relief are currently the focus of clinical practice and research (Jensen et al., 2014). Recent studies showed the hypoxia and bioenergetics dysfunction caused by neural injury may lead to hyperalgesia in neuropathic pain (Lim et al., 2015). There may be some other metabolic changes as the result of disturbance of respiratory. Metabolic profiling may provide a noninvasive method to monitor the progression of pain and detect changes in the internal environment, complementing the information grasped by the doctors to facilitate diagnosis and treatment (Ohman et al., 2015).

Complete Freund’s adjuvant-induced adjuvant arthritis is a classical model of autoimmune arthritis, well applied in the experimental model of rheumatoid arthritis (Alvarado-Vazquez et al., 2015). CFA-induced arthritis is also well recognized as a hyperalgesia model (Auh et al., 2012). Several studies have investigated molecular mechanisms underlying the origin and conduction of hyperalgesia. One of these studies revealed that Src homology 2 domain-containing tyrosine phosphatase 1-mediated dephosphorylation of the transient receptor potential vanilloid 1 alleviated hyperalgesia induced by CFA (Xiao et al., 2015). Another study demonstrated that large conductance calcium-activated potassium channels expressed in sensory neurons inhibited sensory input.
using a similar rodent model (Lu et al., 2014).

Metabolomics is the study of systematic changes in small molecule metabolites present in biological samples and is an effective tool for understanding disease phenotypes (Fujita et al., 2015). There have been a number of recent metabolomics studies of pain. One study compared inflammation-induced metabolic changes in the brain in experimental autoimmune encephalomyelitis and an adjuvant arthritis model (Lutz et al., 2013). Other studies examined the changing metabolites in patients with different kinds of pain, (e.g., neuropathic, musculoskeletal, and dysmenorrhea) (Livshits et al., 2015 Patti et al., 2012 Hadrévi et al., 2013 Caboni et al., 2014 Su et al., 2013). Recently, a study was carried out to explore the mechanisms of adjuvant-induced arthritis from the perspective of metabolomics (Jiang et al., 2016). However, no data have shown the relationship between metabolic alterations and hyperalgesia/pain intensity. In present study, we used an omics method to test the changes of metabolites in murine urine, aiming to demonstrate the differences of metabolomics changes between in a hyperalgesia model and other kind of pain, and find potent biomarkers of hyperalgesia.

**Experimental**

**Chemicals and reagents**

Chromatographic-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Fluka (Buchs, Switzerland). Ultrapure water was obtained using a Milli-Q water purification system (Millipore Corp., Billerica, MA, USA).
Animal experiments and sample collection

All the animal studies adhered to the National Institutes of Health guide for the Care and Use of Laboratory Animals and were approved by the Ethical Committee for the Experimental Use of Animals at Second Military Medical University (Shanghai, China). SD rats aged 12–13 weeks were purchased from Shanghai SLAC laboratory Animal Co., Ltd. The animals were housed in groups of four at a constant temperature of 23±1°C and allowed access to food and water ad libitum.

After conditional housing for one week, seven rats were placed in metabolic cages to collect urine samples. An emulsion containing 500 μg of CFA (Sigma-Aldrich, Saint Quentin, Fallavier, France) was injected into the left posterior plantar of each rat. Urine samples were collected just prior to the injection of CFA and post-injection days 1, 3, and 7. All the urine samples were immediately stored at -80° C until tested in the analysis.

Assessment of hyperalgesia

In each animal, the activity, in addition to redness and swelling of the left posterior limb, was monitored to evaluate inflammation of the articulation. The intensity of pain was assessed by the hind paw withdrawal latency processed before the collection of the urinary samples, which was well recognized by the previous study (Werdehausen et al., 2015).

Sample preparation

A 100 μl aliquot of urine sample was thawed at 4° C. Then, 300 μl of a methanol solution was added to the sample to precipitate the proteins, and 5 μg/ml of L-2-chlorophenylalanine within the methanol solution played a role of internal standard. The resulting solution was spun at 13,000 rpm for 15 min at 4° C. The clear supernatant (150 μl) was injected into a sampling vial for UHPLC–MS analysis. A QC sample was pooled aliquots.
from all the urinary samples collected in the course of the study.

**UHPLC-Q-TOF/MS profiling analysis**

The metabolomics data were profiled on an Agilent 1290 Infinity LC system, equipped with an Agilent 6538 Accurate Mass Quadrupole Time-of-Flight mass spectrometer (Agilent, CA, USA). Chromatographic separations were processed on an ACQUITY UPLC HSS T3 column (2.1 mm×100 mm, 2.5 μm, Waters, Milford, MA, USA). The column temperature was 25°C, and the flow rate was 0.4 ml/min, with an injection volume of 2 μl. The RRLC binary solvent system consisted of mobile phases A (0.1% formic acid) and B (ACN modified with 0.1% formic acid). The gradient elution UPLC conditions were 0–2 min, 5% B; 2–8 min, 5–15% B; 8–10 min, 15–30% B; 10–13 min, 30–95% B; and 13–15 min, 95% B, and the post-time was 5 min for equilibrating the system. The autosampler was refrigerated at 4°C.

An electrospray ionization source (ESI) in both positive and negative modes was used. The mass spectrometer operating parameters were as follows: a capillary voltage of 4 kV in the positive mode and 3.5 kV in the negative mode, drying gas flow of 11 L/min, gas temperature of 350°C, nebulizer pressure of 45 psig, fragmentor voltage of 120V, and skimmer voltage of 60 V. The electron ionization mass spectra were collected in the profile mode from m/z 100 to 1100.

To validate the stability of the system, the QC samples were randomized in sequence. The stability was then assessed based on the clustering of the QC samples in a PCA score plot of all the tested samples. This process was slightly modified from that used in our previous study (Gao et al., 2015).
**Data processing and statistical analysis**

Common data format (.mzdata) files were transformed from the UHPLC–MS raw data by Agilent MassHunter qualitative software. Interference from isotopes was eliminated, and the threshold was set to 0.1%. To generate a visual data matrix, peak extraction, alignment, and integration were done using XCMS (Smith et al., 2006) (http://metlin.scripps.edu/download/). After filtering the ions based on the 80% rule (Smilde et al., 2005), all the detected ions in each sample were standardized to the sum of the peak area of L-2-chlorophenylalanine to calculate the relative intensity of the metabolites. The resulting three-dimensional data matrix, which included the sample names, retention times, m/z pairs, and normalized ion intensities, was analyzed using the SIMCA-P (version 11.0, Umetrics, Umea, Sweden) program with multivariate statistical analysis after mean-centering and Pareto scaling.

Biochemistry data are presented as the mean±SD. The SPSS 17.0 program (IBM, New York, USA) was used, with a one-way ANOVA and Tukey’s posthoc test employed to test the statistical significance of the mean values. The significance level was set at 0.05. A heat map of the different metabolites was processed by the MetaboAnalyst platform (http://www.metaboanalyst.ca). GraphPad Prism 6.0 (Graphpad, CA, USA) was used to produce box plots of the normalized amounts of marker metabolites.

**Results**

**Nociceptive changes after CFA injection**

To test the nociceptive effect of CFA in vivo, CFA was injected into the hind paw of each rat. Von Fry filaments were used to test the mechanosensitivity to CFA, and a pain threshold curve was drawn based on a 50% hind paw withdrawal threshold. As shown in Figure 1A, the pain threshold curve decreased significantly on CFA post-injection day 1.
curve continued to slope downward, but no significance was observed. An obvious swelling on the rat’s left lower limb was observed after the CFA injection. Figure 1 B–E shows the plantars of the rats before the injection and on post-injection days 1, 3, and 7, respectively. The most severe swelling occurred on day 1 following the application of CFA. The swelling subsided over the course of the following several days.

**Metabolic profiling analysis of urine**

Typical total ion current (TIC) chromatograms of the urine samples were obtained from the rats in the four groups in both the ESI positive and ESI negative mode. Representative total ion chromatograms from UHPLC-QTOF/MS were shown in Figure 2. There were significant differences in the constituent and proportion seen from the chromatograms directly. Firstly, we screened and selected the metabolites that changed in a similar trend with the pain threshold. Supervised multivariate data analysis (PCA) was used to find the outliers. Then, partial least squares discriminant analysis (PLS-DA) was performed to visualize the trends in the data of all the four groups. The QC sample features were tightly clustered, with a marked difference between the pre- and post-injection CFA groups, as shown in Figure 3. The stability of this proposed method was satisfied, which was demonstrated from PCA result. The PLS-DA plot revealed a distinct trend of time-varying changes, in common with the trends observed in both the positive and negative modes. When 2 components were calculated in the positive mode, the cumulative $R^2_X$, $R^2_Y$ and $Q^2$ were 0.812, 0.863 and 0.707, respectively, while the cumulative $R^2_X$, $R^2_Y$ and $Q^2$ in the negative mode were 0.862, 0.728 and 0.58, respectively. No over-fitting was observed in either ESI positive or ESI negative according to the results of the permutation test in Figure 3. A one-way ANOVA and Tukey’s post hoc test were performed to test the significance of the difference between the four groups. Using the proposed approach, thirty-four metabolites were identified as potential biomarkers of hyperalgesia (Table 1).
Identification of potential biomarkers

The online database Metlin (http://metlin.scripps.edu/) and the Human Metabolome Database (HMDB, http://www.hmdb.ca/) were used to search for metabolite candidates based on the exact masses of the quasi-molecular ions detected. The metabolites were unambiguously characterized by reviewing references or comparing local metabolomics data with those in the online databases. A series of 34 metabolites, with changes in trends that were similar to those observed in the pain threshold, was identified as a potential biomarker cluster. The names, m/z values, retention times, and formulas of the metabolites are listed in Table 1.

Metabolome variation

Thirty-four metabolites were identified as potential biomarkers of hyperalgesia. Figure 4 shows show a heat map that was generated to visualize the metabolomics changes in hyperalgesia. Figure 5 presents a box plot detailing the changes in each metabolite. A range of metabolites was altered after the induction of hyperalgesia. Most of the metabolites were significantly decreased in the first day, and ranges in non-significant pattern in the following days. Interestingly, L-xylo-Hexulonolactone developed an opposite alteration, increasing to the maximum on post-injection day 7. The pathways included those involved in the metabolism of amino acids, lipids, and vitamins, which are listed in Table 1.
Pathway

The pathways of each metabolite were obtained from the online HMDB database and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/). As shown in Table 1, the pathways detected included alanine, aspartate, and glutamate metabolism; ascorbate and aldarate metabolism; glycerolipid metabolism; glycerophospholipid metabolism; histidine metabolism; phenylalanine metabolism; sphingolipid metabolism; tryptophan metabolism; tyrosine metabolism; valine, leucine, and isoleucine biosynthesis; and vitamin B6 metabolism. Seven of the metabolites were involved in the tyrosine metabolism pathway. Five potent biomarkers were detected in the tryptophan metabolism pathway. Six metabolites were associated with lipid metabolism. The other 16 potential biomarkers were associated with alanine, aspartate, and glutamate metabolism; ascorbate and aldarate metabolism; histidine metabolism; phenylalanine metabolism; sphingolipid metabolism; valine, leucine, and isoleucine biosynthesis; and vitamin B6 metabolism. The metabolic relationships between these 34 potential biomarkers and the 11 metabolic pathways are presented in Figure 6.

Discussion

Similar with previous findings (Lutz et al., 2013), the nociceptive threshold decreased after the administration of CFA in the present study, illustrating that CFA induced hyperalgesia in a rat model. As a result of PCA process, the tightly scattering of QC sample features indicates a stable experimental process. In the present work, 34 metabolites were identified as potential biomarkers of hyperalgesia. Based on information obtained from the HMDB and KEGG databases, hyperalgesia seemed to be associated with changes in 11 key metabolic pathways as we have mentioned above (Table 1). These metabolic pathways played an important role in CFA-induced hyperalgesia. The results indicate that metabolomics is an
effective supplementary technique to evaluate systemic changes induced by hyperalgesia, thereby greatly enhancing understanding of the mechanism of hyperalgesia.

In this study, the induction of hyperalgesia markedly influenced many amino acids, including arginine, proline, phenylalanine, tryptophan, and tyrosine, or their metabolites. Thus, these amino acids’ metabolic pathways have a close relationship with hyperalgesia as an outcome or initiator.

**Phenylalanine and tyrosine metabolism**

Tyrosine can be partially synthetized from phenylalanine, which is a type of essential amino acid. Previous metabolomics research on regional pain syndrome revealed a pronounced increase in the levels of tyrosine and phenylalanine in the cerebrospinal fluid of patients (Meissner et al., 2014). Interestingly, the acute depletion of phenylalanine and tyrosine was reported to have no effect on nociception, indicating that these two amino acids were intermediate products of the pain response (Becker et al., 2013). Concordant with this study, a lower amount of tyrosine was found in pain patients’ excretion (Aghabeigi et al., 1993). That trace of L-dopa significantly facilitates pain may explain the inhibition of tyrosine metabolism in a self-protective pattern.

**Tryptophan metabolism**

Tryptophan is an essential amino acid and glucose-generating amino acid. For the sake of producing energy under the stress environment, tryptophan may resolve into carbohydrates. In chronic pain syndrome, the tryptophan level was substantially decreased before analgesic treatment and increased after the therapy (Aghabeigi et al., 1993). In addition, a previous study showed that neural injury increased the production of monoamine by aromatic L-amino acid decarboxylase (Wienecke et al., 2014). Thus, significant suppression of nearly all
metabolic pathways in aromatic amino acid metabolism can be detected in hyperalgesia models, except the anabolism of monoamines. The present study also found significant decreases in the aforementioned pathways. As the result of the increase in gluconeogenesis, inhibitions in other amino acid metabolisms were also observed after the induction of hyperalgesia. This result is in accordance with that reported in earlier studies of trapezius myalgia and chronic musculoskeletal pain (Hadrévi et al., 2015 Hadrévi et al., 2013). We consider that the tryptophan-related metabolites found in this study may act as potent biomarkers of pain intensity in humans.

**Ascorbic acid metabolism**

Ascorbic acid, well known as vitamin C, is an essential reducing organic compound in the body. In vivo, ascorbic acid plays an important role as a cofactor for different enzymes required for the synthesis of amino acids, neurotransmitters, and neuropeptide hormones (Carr et al., 2013). Ascorbic acid interacts with four amino acid units (histidine 263, serine 282, phenylalanine 264, and valine 283) in the active site of the enzyme to inhibit the activity of tyrosinase (Senol et al., 2014). A previous study showed that intravenous administration of vitamin C relieved pain in patients in comparison to patients who did not receive vitamin C (Gunes-Bayir et al., 2015). Vitamin C is easy to consume in pain-derived oxidative stress, and it is a sensitive indicator of the initiation of pain. The administration of vitamin C may protect patients from some complications by terminating oxidative stress.

**Vitamin B6 metabolism**

Vitamin B6 works as a coenzyme to transfer amino in amino acid biosynthesis and metabolism. A previous study reported that pain scores in carpal tunnel syndrome patients improved more than other measured parameters following vitamin B6 treatment (Gunes-Bayir
et al., 2015). Another study reported that circulating vitamin B6 levels were inversely correlated with clinical indicators, including the disability score, duration of morning stiffness, and degree of pain, and with biochemical markers, including the erythrocyte sedimentation rate and C reactive protein levels (Chiang et al., 2005). These studies demonstrated that vitamin C and B6 not only participate in pain modulation but also serve as useful biomarkers of the pain stage. Decreases in levels of vitamin C and B6 in urine may be caused by the requirement for biochemical reactions and the mechanism of reducing excretion.

**Lipid metabolism**

In the present study, hyperalgesia had a pronounced effect on lipid metabolism. Lipids have two functions. On the one hand, the decomposition of lipids provides a large part of the energy required in metabolism. Consuming a diet rich in omega-3 fatty acids was reported to result in similar remission in hyperalgesia (Figueroa et al., 2013). Catabolism enhances when an organism is under stress. On the other hand, lipids, especially phospholipids, act as components of the cell structure. LysoPCs were reported to be potential biomarkers in the pathogenesis of fibromyalgia (Caboni et al., 2014). There was an obvious decrease in lipids in this study, in accordance with the findings of previous study (Lutz et al., 2013). As a result, appropriate supply of lipid diet to the hyperalgesia patients may bring remission to themselves.

**Sphingosine metabolism**

Sphingomyelin, a type of phospholipid mainly found in myelin, is synthesized from sphingosine (Patti et al., 2012). A previous study, which used a rat model of neuropathic hyperalgesia to detect metabolomics changes in the dorsal horn, reported conspicuous increases in sphingosine and its metabolites (Patti et al., 2012). Schwann cell autophagy was
reported to play an important role in counteracting the onset and chronification of pain (Marinelli et al., 2014). The latter may explain the increased levels of sphingosine. Based on Patti’s result, Johnson et al. demonstrated that dimethylsphingosine inhibited nociception and alteration of metabolites in the same model (Johnson et al., 2015). Another study demonstrated that pain increased the permeability of the blood brain barrier (Wisniewski et al., 1991). The reparation of damaged blood brain barrier may provoke alterations in phospholipid and protein metabolism.

**Conclusion**

Metabolomics is a useful approach to identify pathways altered by disease and to obtain a holistic view of the pathogenesis of the disease and metabolism. The effects of hyperalgesia on metabolism were investigated by UHPLC-Q-TOF/MS metabolic profiling. CFA-induced hyperalgesia led to pronounced perturbation of amino acid metabolism, lipid metabolism, and vitamin metabolism. UHPLC-Q-TOF/MS metabolic profiling identified 34 metabolites as potential biomarkers of hyperalgesia. The results provide a global metabolic network for us to have a further understanding of hyperalgesia.

**Acknowledgments**

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Table 1: Potential biomarkers of pain status in the hyperalgesia model.

<table>
<thead>
<tr>
<th>Metabolic pathway</th>
<th>m/z</th>
<th>RT (min)</th>
<th>Formula</th>
<th>Biochemical name</th>
<th>Ion</th>
<th>1 DPI/ BI</th>
<th>3 DPI/ BI</th>
<th>7 DPI/ BI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>174.041</td>
<td>1.02</td>
<td>C6H9NO5</td>
<td>N-acetylaspartate</td>
<td>[M-H]-</td>
<td>0.392</td>
<td>0.601</td>
<td>0.31</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>188.057</td>
<td>1.13</td>
<td>C7H11N05</td>
<td>N-acetyl-L-glutamic acid</td>
<td>[M-H]-</td>
<td>0.555</td>
<td>0.689</td>
<td>0.51</td>
<td>0.007</td>
</tr>
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<td></td>
<td>260.054</td>
<td>0.72</td>
<td>C6H14N08 P</td>
<td>D-glucosamine 6-phosphate</td>
<td>[M+H]+</td>
<td>0.742</td>
<td>0.866</td>
<td>0.64</td>
<td>0.012</td>
</tr>
<tr>
<td>Ascorbate and aldarate metabolism</td>
<td>175.024</td>
<td>1.02</td>
<td>C6H8O6</td>
<td>L-Ascorbic acid</td>
<td>[M-H]-</td>
<td>0.490</td>
<td>0.571</td>
<td>0.51</td>
<td>0.001</td>
</tr>
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<td></td>
<td>179.057</td>
<td>0.72</td>
<td>C6H12O6</td>
<td>D-Glucose</td>
<td>[M-H]-</td>
<td>0.524</td>
<td>0.759</td>
<td>0.52</td>
<td>0.020</td>
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<tr>
<td></td>
<td>199.021</td>
<td>7.99</td>
<td>C6H8O6</td>
<td>L-xylo-hexulonolactone</td>
<td>[M+Na]+</td>
<td>0.918</td>
<td>0.882</td>
<td>1.28</td>
<td>0.031</td>
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<td>Glycerolipid metabolism</td>
<td>226.107</td>
<td>3.32</td>
<td>C9H17NO4</td>
<td>Acetylcarnitine</td>
<td>[M+Na]+</td>
<td>0.189</td>
<td>0.540</td>
<td>0.32</td>
<td>0.007</td>
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<tr>
<td></td>
<td>310.202</td>
<td>12.90</td>
<td>C15H29NO4 4</td>
<td>L-octanoylcarnitine</td>
<td>[M+Na]+</td>
<td>0.893</td>
<td>0.937</td>
<td>0.91</td>
<td>0.043</td>
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<tr>
<td></td>
<td>504.229</td>
<td>1.15</td>
<td>C21H40NO9P</td>
<td>PS(15:1(9Z)/0:0)</td>
<td>[M+Na]+</td>
<td>0.618</td>
<td>0.892</td>
<td>0.48</td>
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<tr>
<td>Glycerophospholipid metabolism</td>
<td>524.371</td>
<td>12.96</td>
<td>C26H54NO7P</td>
<td>LysoPC(18:0)</td>
<td>[M+H]+</td>
<td>0.334</td>
<td>0.517</td>
<td>0.45</td>
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<td></td>
<td>761.417</td>
<td>13.42</td>
<td>C36H67O13P</td>
<td>PI(12:0/15:1(9Z))</td>
<td>[M+Na]+</td>
<td>0.625</td>
<td>0.588</td>
<td>0.61</td>
<td>0.041</td>
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<td>792.595</td>
<td>7.83</td>
<td>C46H82NO7P</td>
<td>PC(20:4(5Z,8Z,11Z,14Z)/P-18:1(11Z))</td>
<td>[M+H]+</td>
<td>0.686</td>
<td>0.799</td>
<td>0.78</td>
<td>0.002</td>
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<td>Histidine metabolism</td>
<td>127.051</td>
<td>0.76</td>
<td>C5H6N2O2</td>
<td>Imidazoleacetic acid</td>
<td>[M+H]+</td>
<td>0.607</td>
<td>0.785</td>
<td>0.56</td>
<td>0.031</td>
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<td>Phenylalanine</td>
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<td>7.76</td>
<td>C9H8O3</td>
<td>Phenylpyruvic acid</td>
<td>[M-H]-</td>
<td>0.260</td>
<td>0.584</td>
<td>0.31</td>
<td>0.003</td>
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<tr>
<td>Metabolism</td>
<td>m/z</td>
<td>Retention</td>
<td>Formula</td>
<td>Name</td>
<td>Charge</td>
<td>MRM Transition</td>
<td>Peaks</td>
<td>Precursor</td>
<td>Charge</td>
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<tr>
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<td></td>
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<tr>
<td>Sphingolipid metabolism</td>
<td></td>
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<tr>
<td>C9H11NO2</td>
<td>188.07</td>
<td>9.15</td>
<td>L-phenylalanine</td>
<td>[M+Na]</td>
<td>+</td>
<td>0.209</td>
<td>0.509</td>
<td>0.35</td>
<td>0.007</td>
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<tr>
<td>C13H16N2O4</td>
<td>299.078</td>
<td>5.00</td>
<td>Alpha-N-phenylacetyl-L-glutamine</td>
<td>[M-Cl]^-</td>
<td>-</td>
<td>0.252</td>
<td>0.711</td>
<td>0.53</td>
<td>0.036</td>
</tr>
<tr>
<td>C18H37NO2</td>
<td>334.252</td>
<td>12.29</td>
<td>Sphingosine</td>
<td>[M-Cl]^-</td>
<td>-</td>
<td>0.639</td>
<td>0.700</td>
<td>0.78</td>
<td>0.001</td>
</tr>
<tr>
<td>C20H40NO6P</td>
<td>420.251</td>
<td>11.09</td>
<td>Ceramide 1-phosphate</td>
<td>[M-H]^-</td>
<td>-</td>
<td>0.936</td>
<td>0.942</td>
<td>0.80</td>
<td>0.002</td>
</tr>
<tr>
<td>C9H11NO2</td>
<td>160.04</td>
<td>5.26</td>
<td>4,6-Dihydroxyquinoline</td>
<td>[M-H]^-</td>
<td>-</td>
<td>0.166</td>
<td>0.470</td>
<td>0.35</td>
<td>0.033</td>
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<tr>
<td>C8H7NO3</td>
<td>166.05</td>
<td>1.62</td>
<td>N-formylantranilic acid</td>
<td>[M+H]+</td>
<td>+</td>
<td>0.373</td>
<td>0.624</td>
<td>0.39</td>
<td>0.001</td>
</tr>
<tr>
<td>Tryptophan metabolism</td>
<td>176.033</td>
<td>1.36</td>
<td>3-Hydroxyantranilic acid</td>
<td>[M+Na] +</td>
<td>+</td>
<td>0.619</td>
<td>0.688</td>
<td>0.45</td>
<td>0.009</td>
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<tr>
<td>C11H9NO3</td>
<td>204.066</td>
<td>7.84</td>
<td>Indolepyruvate</td>
<td>[M+H]+</td>
<td>+</td>
<td>0.364</td>
<td>0.685</td>
<td>0.33</td>
<td>0.001</td>
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<tr>
<td>C10H9NO4</td>
<td>208.06</td>
<td>5.13</td>
<td>4-(2-aminophenyl)-2,4-dioxobutanoic acid</td>
<td>[M+H]+</td>
<td>+</td>
<td>0.381</td>
<td>0.591</td>
<td>0.30</td>
<td>0.000</td>
</tr>
<tr>
<td>C9H12O4</td>
<td>183.066</td>
<td>6.71</td>
<td>3-Methoxy-4-hydroxyphenylethylene glycol</td>
<td>[M-H]^-</td>
<td>-</td>
<td>0.126</td>
<td>0.504</td>
<td>0.22</td>
<td>0.004</td>
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<tr>
<td>C7H6O3</td>
<td>137.025</td>
<td>4.07</td>
<td>2,5-dihydroxybenzaldehyde</td>
<td>[M-H]^-</td>
<td>-</td>
<td>0.123</td>
<td>0.443</td>
<td>0.26</td>
<td>0.004</td>
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<tr>
<td>C8H5NO2</td>
<td>148.039</td>
<td>1.62</td>
<td>Indole-5,6-quinone</td>
<td>[M+H]+</td>
<td>+</td>
<td>0.403</td>
<td>0.619</td>
<td>0.42</td>
<td>0.002</td>
</tr>
<tr>
<td>C9H13NO</td>
<td>174.09</td>
<td>0.76</td>
<td>N-methyltyramine</td>
<td>[M+Na] +</td>
<td>+</td>
<td>0.515</td>
<td>0.717</td>
<td>0.50</td>
<td>0.006</td>
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<tr>
<td>C9H10O4</td>
<td>183.065</td>
<td>3.95</td>
<td>Homovanillic acid</td>
<td>[M+H]+</td>
<td>+</td>
<td>0.368</td>
<td>0.582</td>
<td>0.52</td>
<td>0.001</td>
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<tr>
<td>C9H9NO4</td>
<td>196.06</td>
<td>3.93</td>
<td>Dopaquinone</td>
<td>[M+H]+</td>
<td>+</td>
<td>0.172</td>
<td>0.397</td>
<td>0.32</td>
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<tr>
<td>C9H11NO4</td>
<td>198.077</td>
<td>4.41</td>
<td>L-Dopa</td>
<td>[M+H]+</td>
<td>+</td>
<td>0.438</td>
<td>0.662</td>
<td>0.42</td>
<td>0.000</td>
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</table>

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<table>
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<tr>
<th>Metabolic Pathway</th>
<th>M.W.</th>
<th>P.D.</th>
<th>Molecular Formula</th>
<th>Relevant Compound</th>
<th>Charge State</th>
<th>M/z</th>
<th>Theoretical M/z</th>
<th>E.R. (ppm)</th>
<th>M.P. (ppm)</th>
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<tbody>
<tr>
<td>Valine, leucine, and isoleucine biosynthesis</td>
<td>175.061</td>
<td>4.43</td>
<td>C7H12O5</td>
<td>(2S)-2-Isopropylmalate</td>
<td>[M-H]-</td>
<td>0.500</td>
<td>0.870</td>
<td>0.41 (6)</td>
<td>0.027</td>
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<tr>
<td></td>
<td>169.097</td>
<td>2.21</td>
<td>C8H12N2O2</td>
<td>Pyridoxamine</td>
<td>[M+H]</td>
<td>0.478</td>
<td>0.702</td>
<td>0.50 (2)</td>
<td>0.001</td>
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<tr>
<td>Vitamin B6 metabolism</td>
<td>184.06</td>
<td>1.62</td>
<td>C8H9NO4</td>
<td>4-pyridoxate</td>
<td>[M+H]</td>
<td>0.369</td>
<td>0.602</td>
<td>0.40 (0)</td>
<td>0.001</td>
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<tr>
<td></td>
<td>192.065</td>
<td>6.36</td>
<td>C8H11NO3</td>
<td>Pyridoxine</td>
<td>[M+Na]+</td>
<td>0.456</td>
<td>0.684</td>
<td>0.38 (1)</td>
<td>0.003</td>
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</tbody>
</table>
**Fig. 1.** Inflammatory pain and swelling of the lower limb in the rat induced by Complete Freund’s adjuvant (CFA). (A) Behavioral data in adult rats after the induction of inflammatory pain by an injection of CFA in the left hind paw, expressed as the withdrawal threshold (g). Swelling on the leg: before the injection (B) and day 1 (C), day 3 (D) and day 7 (E) postinjection. * p<0.05 compared with before the injection.
Fig. 2. Representative total ion current (TIC) chromatograms of the urine samples obtained in the electrospray ionization (ESI) positive ion mode before the injection (A) and on day 1 (B), day 3 (C) and day 7 (D) postinjection and in the ESI negative mode before the injection (E) and on day 1 (F) day 3 (G) and day 7 (H) postinjection based on UHPLC-Q-TOF/MS.
Fig. 3. Principal component analysis (PCA) score plots of four groups in electrospray ionization (ESI) positive mode (A) and negative mode (B). Plot of the partial least squares discriminant analysis (PLS-DA) scores of the four groups in ESI positive mode (C) and negative mode (D). The arrows indicate the variation in the trend between the groups. Figure 3E and 3F represent validation of PLS-DA model in positive mode and negative mode respectively. BI: before injection; 1DPC: 1 day postinjection; 3DPC: 3 days postinjection;
7DPC: 7 days post injection; QC: quality control.

**Fig. 4.** A heat map based on the relative levels of the potential biomarkers in the before injection, day 1, day 3, and day 7 postinjection groups.
Fig. 5. Box plots showing the levels of 34 potential biomarkers in the before injection, day 1, day 3, and day 7 post-injection groups.
**Fig. 6.** The integrated metabolic pathway network resulting from different metabolites among CFA-induced inflammatory pain. The potential biomarkers (i.e., levels of metabolites that increased/decreased after CFA induction) are labeled in red (up-regulated) and green (down-regulated). Metabolites that did not change significantly following CFA induction are labeled in black. The words and dotted lines in blue around the metabolites indicate the related pathway of metabolites in each center.
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