Urine Proteomics in Patients with Methamphetamine Addiction

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Abstract

Drug addiction is a severe chronic relapsing brain disease; methamphetamine addiction has a complex disease course, is difficult to treat, and poses a serious public health burden. This study comparatively analyzed the urinary proteome of methamphetamine-addicted patients in the acute withdrawal phase (discontinued use within 24 hours), methamphetamine withdrawal rehabilitation phase patients (discontinued use for more than 3 months), and healthy volunteers, revealing that the urinary proteome of methamphetamine-addicted patients (both acute withdrawal phase and withdrawal rehabilitation phase) was significantly different from that of healthy individuals; some differential proteins and their enriched biological functions were shown to be related to addiction or methamphetamine neurotoxicity and may be potential intervention targets for drug addiction; methamphetamine withdrawal rehabilitation phase patients still struggled to return to normal levels, which may be used to reveal the reasons for the high relapse rate of methamphetamine. This study established a method for studying addictive drugs from a urinary proteomics perspective, demonstrating that the urinary proteome can relatively systematically and comprehensively reflect the effects of methamphetamine abuse on the organism, providing clues for clinical research and practice on addictive diseases.

Full Text

Urine Proteomics in Methamphetamine-Addicted Patients

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Abstract

Drug addiction is a serious chronic relapsing brain disease. Methamphetamine dependence has a particularly complex course and is difficult to treat, creating a substantial public health burden. This study comparatively analyzed the urine proteomes of methamphetamine-addicted patients in acute withdrawal (within 24 hours of cessation), patients in recovery (abstinent for over 3 months), and healthy volunteers. The urine proteomes of methamphetamine-addicted patients (both acute withdrawal and recovery groups) differed significantly from those of healthy individuals. Several differential proteins and their enriched biological functions were associated with addiction or methamphetamine neurotoxicity, representing potential intervention targets for drug addiction. Notably, patients in the recovery group still failed to return to normal levels, which may help explain the high relapse rate in methamphetamine addiction. This study establishes a novel approach for investigating addictive drugs through urine proteomics and demonstrates that the urine proteome can systematically and comprehensively reflect the effects of methamphetamine abuse on the body, providing valuable clues for clinical research and practice in addiction medicine.

Keywords: urine; proteomics; methamphetamine; addiction; substance use disorder; drug addiction

1. Introduction

Drug addiction represents a global public health and social challenge of major significance. According to the 2021 World Drug Report, approximately 284 million people aged 15-64 used drugs worldwide in 2020, a 22% increase over the past decade. Beyond the severe physical and psychological harm to users, the growing population of addicted individuals imposes enormous burdens on society in terms of healthcare costs, economic impact, and public safety. The International Drug Control Conventions currently classify addictive substances into three categories: (1) narcotic drugs, including opioids, cocaine, and cannabis; (2) psychotropic substances, including sedative-hypnotics, central stimulants, and hallucinogens; and (3) others, including tobacco, alcohol, and volatile organic solvents [1].

Methamphetamine (METH), commonly known as "ice," is a highly addictive drug that has caused severe social and health problems worldwide. In China, there are nearly 1.5 million registered drug users, with nearly 800,000 abusing methamphetamine, which has replaced heroin as the most widely abused drug in the country [2]. Chronic methamphetamine abuse causes serious physical and psychological damage. Due to its high lipophilicity [3], methamphetamine more

readily enters the central nervous system (CNS) compared to other psychostimulants, distributes throughout the brain, and has longer-lasting effects, making it more addictive [4]. Methamphetamine induces long-term neurotoxicity, causing severe and persistent damage to the CNS [5]. Methamphetamine addicts frequently suffer from long-term psychiatric disorders and cognitive decline [6]. The neurochemical mechanisms underlying neurotoxicity and excitotoxicity are complex and remain unclear.

Research indicates that methamphetamine addiction involves alterations in multiple neurotransmitter systems and neural circuits. The chemical structure of methamphetamine resembles that of monoamines [7]. In the brain, methamphetamine acts on dopamine, serotonin, norepinephrine, and epinephrine neurotransmitter systems, altering neurotransmitter release and reuptake, thereby producing intense euphoria and addictive behavior [8]. The ultimate result of methamphetamine's action is excessive stimulation of monoaminergic pathways in both central and peripheral nervous systems, leading to severe dysfunction and even neuronal degeneration in multiple brain regions including the striatum, prefrontal cortex, and hippocampus, which further exacerbates the formation and maintenance of addictive behavior [7]. Methamphetamine dependence is characterized by a complex disease course that is difficult to treat, creating a serious public health burden [9].

Drug addiction is a severe chronic relapsing brain disease involving reward, motivation, learning, memory, and decision-making—high-level neural activities with extremely complex mechanisms involving multiple brain regions, circuits, and systems. The mechanisms of drug addiction are not yet fully understood, presenting significant challenges for prevention and treatment. Therefore, understanding the effects of drug addiction on the body and identifying relevant biomarkers are crucial for developing effective intervention and treatment strategies.

Drug addiction is a dynamic behavioral and physiological process. Metabolomics and proteomics technologies have been widely applied in addiction research. Proteomics is a global approach for studying protein expression and function in organisms, currently capable of analyzing protein expression changes during drug addiction. Proteomics has been extensively used to study the effects of various addictive substances on animal models and humans. For example, researchers have analyzed plasma from methamphetamine-addicted patients [10] or evaluated protein expression in brain tissue from animals and humans exposed to alcohol, amphetamine, methamphetamine, cocaine, cannabis, morphine, nicotine, and other addictive drugs [11], providing rich resources for further investigation of addiction-related biochemical pathways, genes, and proteins. However, few studies have employed urine proteomics to investigate drug addiction.

Urine proteins contain abundant information that can reflect subtle changes in different systems and organs throughout the body. Compared to other body fluid samples, urine is not subject to homeostatic mechanisms and can accumulate early changes in physiological status, offering high-sensitivity indications

that may assist in early disease diagnosis, treatment, and prognosis monitoring. Additionally, 得益于 non-invasive collection methods, urine can be collected continuously, in large volumes, and repeatedly, with convenient and stable storage and relatively simple composition for analysis. These advantages make urine an ideal sample for detecting disease biomarkers [12].

Numerous animal model and clinical sample studies have demonstrated that urine proteomics research can provide clues for early diagnosis and intervention in neurodegenerative diseases and other brain disorders [13]. Zhang et al. identified the urine proteome in Alzheimer's disease mouse models and found that Alzheimer's-related biomarkers appeared in urine before β-amyloid deposition became pathologically evident [14]. Song et al. discovered that urinary exosome proteins in 5XFAD mouse models showed Alzheimer's-related differences before amyloid plaque deposition could be detected [15]. The Matthias Mann research team combined proteomics technology, genetic screening, and machine learning to show that urine proteomics can distinguish individuals carrying different Parkinson's disease-related mutations with different disease manifestations, demonstrating its application in familial Parkinson's disease stratification [16].

Furthermore, urine proteomics research has addressed psychiatric and behavioral disorders. Meng et al. found that screened urinary protein biomarker panels could effectively differentiate healthy children from autistic children across different age groups, with potential for assisting early diagnosis and intervention of autism, using random grouping methods to validate result credibility [17]. Wang et al. found that autism patient proteomes displayed biological pathway changes related to disease mechanisms [18]. Huan et al. detected differentially expressed proteins in major depressive disorder patients with different responses to antidepressants, suggesting that urinary biomarkers could potentially predict effective therapeutic measures for major depressive disorder patients, providing clues and basis for precision treatment to improve quality of life [19].

To date, no urine proteomics studies on addictive drugs such as methamphetamine have been published. Given that urine proteomics can reflect changes caused by various brain diseases, this study aims to expand the application potential of urine proteomics to drug addiction—a complex and difficult-to-treat brain disorder.

This study investigates differences between the urine proteomes of methamphetamine-addicted patients and healthy individuals, conducting systematic analysis at different levels including biological pathways and population behavioral characteristics. Our objectives are to reveal the effects of methamphetamine addiction on physiological status, identify potential protein biomarkers related to methamphetamine addiction and candidate drug targets for addiction treatment, thereby providing new clues for addiction diagnosis and treatment while offering a novel perspective for addiction research.

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2. Materials and Methods

2.1 Experimental Materials

2.1.1 Consumables 1.5ml/2ml centrifuge tubes (Axygen, USA), 50ml/15ml centrifuge tubes (Corning, USA), 96-well cell culture plates (Corning, USA), 10kD filters (Pall, USA), Oasis HLB solid-phase extraction columns (Waters, USA), 1ml/200 l/20 l pipette tips (Axygen, USA), BCA assay kit (Thermo Fisher Scientific, USA), high pH reverse-phase peptide separation kit (Thermo Fisher Scientific, USA), iRT (indexed retention time, BioGnosis, UK).

2.1.2 Equipment High-speed refrigerated centrifuge (Thermo Fisher Scientific, USA), vacuum concentrator (Thermo Fisher Scientific, USA), DK-S22 electric constant temperature water bath (Shanghai Jinghong Experimental Equipment Co., Ltd.), full-wavelength multifunctional microplate reader (BMG Labtech, Germany), shaker (Thermo Fisher Scientific, USA), TS100 constant temperature mixer (Hangzhou Ruicheng Instrument Co., Ltd.), electronic balance (METTLER TOLEDO, Switzerland), -80°C ultra-low temperature freezer (Thermo Fisher Scientific, USA), EASY-nLC1200 ultra-high performance liquid chromatography (Thermo Fisher Scientific, USA), Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA).

2.1.3 Reagents Trypsin Golden (Promega, USA), dithiothreitol DTT (Sigma, Germany), iodoacetamide IAA (Sigma, Germany), purified water (Wahaha, China), MS-grade methanol (Thermo Fisher Scientific, USA), MS-grade acetonitrile (Thermo Fisher Scientific, USA), MS-grade pure water (Thermo Fisher Scientific, USA), Tris-Base (Promega, USA).

2.1.4 Analysis Software Proteome Discoverer (Version 2.1, Thermo Fisher Scientific, USA), Spectronaut Pulsar (Biognosys, UK), Ingenuity Pathway Analysis (Qiagen, Germany), R studio (Version 1.2.5001), Xftp 7, Xshell 7.

2.2 Experimental Methods

2.2.1 Subject Selection and Study Design Subjects were divided into three groups: the acute group (Group A), comprising patients in acute methamphetamine withdrawal within 24 hours of cessation who were hospitalized for treatment; the recovery group (Group R), comprising patients who had abstained from drug use for over 3 months and entered recovery treatment; and the control group (Group H), comprising healthy volunteers. In collaboration with hospitals and research institutes, urine samples were collected and stored at -80°C for later use. A total of 22 acute patient urine samples, 26 recovery patient samples, and 28 healthy volunteer samples were collected. Some samples were excluded due to low peptide content or detection of other diseases during physical examination. The final analysis included 19 acute patient urine samples, 22 recovery patient samples, and 25 healthy volunteer samples. This study was

approved by the Ethics Committee of Peking University Health Science Center, and all subjects provided informed consent.

[Figure 1: see original paper] Research methodology and technical workflow

2.2.2 Urine Sample Processing Two milliliters of urine sample were thawed and centrifuged at 12,000×g for 30 minutes at 4°C to remove cell debris. The supernatant was collected, and 40 l of 1M dithiothreitol (DTT, Sigma) stock solution was added to achieve a final DTT concentration of 20mM. After mixing, the sample was heated in a metal bath at 37°C for 60 minutes. After cooling to room temperature, 100 l of iodoacetamide (IAA, Sigma) stock solution was added to achieve the working concentration, mixed, and reacted in the dark at room temperature for 45 minutes. Following the reaction, the sample was transferred to a new centrifuge tube and mixed thoroughly with three volumes of pre-cooled absolute ethanol, then placed at -20°C for 24 hours to precipitate proteins. After precipitation, the sample was centrifuged at 10,000×g for 30 minutes at 4°C, the supernatant was discarded, and the protein precipitate was dried. The protein precipitate was resuspended in 200 l of 20mM Tris solution. The resuspended sample was centrifuged and the supernatant was retained. Protein concentration was determined using the Bradford method. Using the filter-aided sample preparation (FASP) method, the urinary protein extract was added to the membrane of a 10kD ultrafiltration tube (Pall, Port Washington, NY, USA), washed three times with 20mM Tris solution, and resuspended in 30mM Tris solution. Trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) was added to each sample at a ratio of 50:1 (urinary protein:trypsin) for digestion, followed by incubation at 37°C for 16 hours. The filtrate after enzymatic digestion was the peptide mixture. The collected peptide mixture was desalted using Oasis HLB solid-phase extraction columns, vacuum-dried, and stored at -80°C. The lyophilized peptide powder was resuspended in 30 l of 0.1% formic acid water, and peptide concentration was measured using a BCA assay kit. Peptide concentration was diluted to 0.5 g/ L, and 4 l from each sample was taken to create a mix.

2.2.3 LC-MS/MS Tandem Mass Spectrometry Analysis All identification samples were spiked with diluted 100× iRT standard solution at a sample:iRT volume ratio of 20:1 to unify retention time. All samples underwent data-independent acquisition (DIA), with each sample run in duplicate. One mix sample was inserted every 10 runs as a quality control. One microgram of sample was separated using EASY-nLC1200 liquid chromatography (elution time: 90 min, gradient: mobile phase A: 0.1% formic acid, mobile phase B: 80% acetonitrile). Eluted peptides were analyzed by Orbitrap Fusion Lumos Tribrid mass spectrometer to generate corresponding raw files.

2.2.4 Data Processing and Analysis Raw files acquired in DIA mode were imported into Spectronaut software for analysis. High-confidence proteins were

defined as those with peptide q-value < 0.01. Protein quantification was performed using peak area quantification of all fragment ion peaks from secondary peptides, with automatic normalization. Proteins containing two or more specific peptides were retained, missing values were replaced with 0, and protein content was calculated for each sample. Different samples were compared, and differential proteins were screened according to the criteria FC ≥ 1.5 or ≤ 0.67, P < 0.05.

Unsupervised cluster analysis (HCA), principal component analysis (PCA), and OPLS-DA analysis were performed using the Wukong platform (https://omicsolution.org/wkomics/main/). Functional enrichment analysis of differential proteins was conducted using the DAVID database (https://david.ncifcrf.gov/) to obtain results in three aspects: biological process, cellular localization, and molecular function. Differential proteins were also analyzed using Ingenuity Pathway Analysis (IPA). The PubMed database (https://pubmed.ncbi.nlm.nih.gov/) was used to search for differential proteins and related pathways. Protein-protein interaction network analysis was performed using the STRING database (https://cn.string-db.org/).

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3. Results

3.1 Sample Characteristics

Using label-free quantification technology in data-independent acquisition mode, we performed mass spectrometry identification on 19 acute patient samples, 22 recovery patient samples, and 25 healthy volunteer samples, identifying a total of 2,917 high-confidence urinary proteins (unique peptides ≥ 2, FDR < 1%).

Sample identification summary

Class	A group	R group	H group	Total
Number of samples	19	22	25	66
Age (Years)	32$±7|34±8|31±6|32±7||*Averageofproteingroups*|2,057±335|2,013±268|2,052±303|2,040±$3			

3.2 Acute Group vs. Healthy Group Comparison

3.2.1 Differential Protein Analysis In the comparative analysis between the healthy group and acute patient group, 143 differential proteins were identified using screening criteria of FC > 1.5 or < 0.67, P < 0.05.

Among these, six differential proteins had an FC of 0, showing a "presence-to-absence" change—detected as 0 in all methamphetamine-addicted patients' urine but present in several healthy individuals' urine.

[Figure 2: see original paper] Proteins with fold change (FC) of 0 in methamphetamine patients vs. healthy individuals ($p < 0.05$)

3.2.2 IPA Pathway Analysis of Differential Proteins Differential proteins were enriched in 38 IPA pathways, many of which are closely related to psychiatric disorders and drug addiction.

IPA pathways enriched by differential proteins between acute patients and healthy individuals

Ingenuity Canonical Pathways	-log(p-value)	Related to drug abuse
L-carnitine Biosynthesis	[23,22,21]	
Tryptophan Degradation X (Mammalian, via Tryptamine)	[25,24]	
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	[28,27,26]	
Role of JAK family kinases in IL-6-type Cytokine Signaling	[25,24]	
Xenobiotic Metabolism AHR Signaling Pathway	[30,29]	
Role Of Chondrocytes In Rheumatoid Arthritis Signaling Pathway	[25,24]	
Histamine Degradation	[32,31]	
Role Of Osteoclasts In Rheumatoid Arthritis Signaling Pathway	[34,33,32]	
LPS/IL-1 Mediated Inhibition of RXR Function	[23,8]	
Fatty Acid α-oxidation	[25,24]	
Putrescine Degradation III		
Neuroprotective Role of THOP1 in Alzheimer's Disease		
Ethanol Degradation IV		
Acute Phase Response Signaling		
LXR/RXR Activation		
PI3K/AKT Signaling		
Dopamine Degradation		
STAT3 Pathway		
Ethanol Degradation II		
Noradrenaline and Adrenaline Degradation		

Ingenuity Canonical Pathways	-log(p-value)	Related to drug abuse
Oxidative Ethanol Degradation III		
Aryl Hydrocarbon Receptor Signaling		
Ascorbate Recycling (Cytosolic)		
D-glucuronate Degradation I		
S-adenosyl-L-methionine Biosynthesis		
N-acetylglucosamine Degradation I		
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis		
Retinoate Biosynthesis I		
Retinoate Biosynthesis II		
Pentose Phosphate Pathway (Oxidative Branch)		
Catecholamine Biosynthesis		
Trans, trans-farnesyl Diphosphate Biosynthesis		
N-acetylglucosamine Degradation II		
Serotonin and Melatonin Biosynthesis		
Glutathione Redox Reactions II		
Rapoport-Luebering Glycolytic Shunt		
Hepatic Fibrosis / Hepatic Stellate Cell Activation		

The enriched IPA pathways include metabolic processes for many important neurotransmitters and their precursors, derivatives, and neuromodulators, such as L-carnitine biosynthesis, tryptophan degradation, histamine degradation, fatty acid α-oxidation, putrescine degradation, THOP1 neuroprotective role in Alzheimer's disease, dopamine degradation, ethanol degradation, noradrenaline and adrenaline degradation, aryl hydrocarbon receptor signaling, ascorbate recycling, S-adenosyl-L-methionine (SAM) biosynthesis, retinoate biosynthesis, catecholamine biosynthesis, serotonin and melatonin biosynthesis, and glutathione redox reactions.

L-carnitine biosynthesis was the most significant pathway (smallest p-value). L-carnitine is an amino acid-like compound that facilitates the conversion of

fat into energy. Chronic methamphetamine and opioid abuse leads to appetite suppression and weight loss, which we hypothesize may be related to L-carnitine metabolism. L-carnitine may exert neuroprotective effects against methamphetamine toxicity through actions at the level of dopamine release or mitochondrial function [20].

Three differential proteins were enriched in tryptophan degradation. Tryptophan metabolites are precursors for serotonin, melatonin, kynurenine, and quinolinic acid pathways, and their levels have been found to be affected by methamphetamine addiction [21]. The kynurenine pathway is the primary route of tryptophan degradation, and studies have found that modulating kynurenine metabolism at certain stages can reduce, prevent, or eliminate drug-seeking behaviors [22].

According to literature, methamphetamine's mechanism of action involves increased release of catecholamine neurotransmitters such as norepinephrine [23]. Dopamine and serotonin are neurotransmitters that play important roles in addiction. Addictive drugs induce changes in histamine levels in the hypothalamus, striatum, and other regions, with histamine playing a significant role in drug addiction [30,29].

Four differential proteins were enriched in the neuroprotective role of THOP1 in Alzheimer's disease, with a z-score of -1. THOP1 (thimet oligopeptidase 1) is an enzyme that primarily degrades neuropeptides and hormones such as oxytocin and angiotensin II. THOP1 plays important roles in neuromodulation in the central nervous system and regulation of the immune system, and is closely associated with the development and prognosis of many diseases including Alzheimer's disease. Methamphetamine abuse may lead to premature development of Alzheimer's disease and neurodegeneration [35].

L-ascorbate prevents methamphetamine-induced cortical cell neurotoxicity by inhibiting oxidative stress, autophagy, and apoptosis [38]. Cocaine significantly reduces the S-adenosyl-L-methionine (SAM)/S-adenosyl-L-homocysteine (SAH) ratio level in the dorsal striatum [39]. Long-term methamphetamine self-administration increases mesolimbic mitochondrial oxygen consumption and reduces striatal glutathione [41].

Several proteins were enriched in the role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis. Methamphetamine causes increased dopamine in the central nervous system. The dopaminergic system strongly influences rheumatoid arthritis progression [24]. Dopamine may be involved in bone formation, bone remodeling, and joint erosion in rheumatoid arthritis [25].

The enriched IPA pathways also include several signal transduction pathways, such as the role of JAK family kinases in IL-6-type cytokine signaling, xenobiotic metabolism AHR signaling pathway, LPS/IL-1 mediated inhibition of RXR function, acute phase response signaling, LXR/RXR activation, PI3K/AKT signaling, and STAT3 pathway.

Four differential proteins were enriched in the role of JAK family kinases in IL-6-type cytokine signaling, with a z-score of -2. Additionally, four differential proteins were enriched in the STAT3 pathway. Serum TNF-α, IL-6, and IL-18 levels are elevated in long-term methamphetamine users [27,26]. Studies have also shown that TNF-α and IL-6 have protective effects against methamphetamine-induced microglial death through IL-6 receptors, particularly by activating the JAK-STAT3 pathway, thereby altering pro-apoptotic and anti-apoptotic proteins [28].

Four differential proteins were enriched in xenobiotic metabolism AHR signaling pathway and aryl hydrocarbon receptor signaling. The aryl hydrocarbon receptor (AHR) can recognize some xenobiotic substances and natural compounds, such as tryptophan metabolites.

Six differential proteins were enriched in LPS/IL-1 mediated inhibition of RXR function. Studies have shown that IL-1β protein levels significantly increase in hippocampal tissue of mice exposed to methamphetamine [31]. Retinoid X receptor (RXR) is involved in amphetamine-induced locomotor activity [32].

Five differential proteins were enriched in PI3K/AKT signaling. Methamphetamine can mediate neuroprotection by activating the dopamine/PI3K/AKT signaling pathway [36].

Four differential proteins were enriched in hepatic fibrosis/hepatic stellate cell activation. Serum alanine aminotransferase and aspartate aminotransferase activity levels were significantly elevated in rats intraperitoneally injected with methamphetamine compared to controls, indicating obvious liver injury [42].

Glycolysis-related pathways and fatty acid oxidation were also enriched. Methamphetamine causes alterations in carbohydrate metabolism [40]. Methamphetamine inhibits glucose uptake in human neurons and astrocytes, which adaptively use fatty acid oxidation as an alternative energy source during glucose restriction [33]. Methamphetamine can cause structural changes in astrocytes through multiple targets, where cell structure, steroid biosynthesis, and fatty acid biosynthesis may play important roles in nerve injury [43]. Amphetamine exerts lipolytic effects through the release of endogenous catecholamines [34].

3.2.3 GO and KEGG Pathway Analysis of Differential Proteins Functional analysis of differential proteins using the DAVID database enriched 26 biological processes (BP), including regulation of gene silencing, regulation of gene expression (epigenetics), telomere organization, DNA replication-dependent nucleosome assembly, nucleosome assembly, negative regulation of endopeptidase activity, cell adhesion, regulation of presynapse assembly, bone mineralization, prostaglandin metabolic process, positive regulation of synapse assembly, negative regulation of neuron death, positive regulation of protein metabolic process, positive regulation of cell-matrix adhesion, memory, carnitine biosynthetic process, developmental process involved in reproduction, osteoclast proliferation,

proteolysis, viral entry into host cell, angiogenesis, regulation of protein localization to membrane, and neural crest cell migration.

Ten differential proteins were enriched in biological processes including regulation of gene silencing, regulation of gene expression (epigenetic), telomere organization, DNA replication-dependent nucleosome assembly, and nucleosome assembly. DNA methylation primarily adds methyl groups to cytosines in gene promoter regions, hindering transcription factor binding and thereby causing long-term gene expression suppression, which may be a key molecular mechanism underlying the persistence of addictive behaviors [44].

Three differential proteins were enriched in prostaglandin metabolic process. Studies indicate that prostaglandins are drugs that regulate brain disease processes in positive or negative ways. Prostaglandins (PGs) are formed through sequential oxidation of arachidonic acid under physiological and pathological conditions. For PG production, cyclooxygenase is an essential enzyme with two isoforms named cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [41]. Cyclooxygenase-2 is a necessary factor for methamphetamine-induced neurotoxicity [45]. Cannabis patients' serum metabolome was found to have higher prostaglandin F1a than controls [46].

Six differential proteins were enriched in angiogenesis. Methamphetamine administration can induce retinal hypoxia and angiogenesis [47].

Several differential proteins were enriched in biological processes related to synapse regulation, neuron death regulation, memory, and neural crest cell migration.

Biological processes (BP) enriched by differential proteins between acute patients and healthy individuals

Biological Process	Count	%	P-Value
regulation of gene silencing			3E-18
regulation of gene expression, epigenetic			1.5E-13
telomere organization			2.3E-13
DNA replication-dependent nucleosome assembly			1.3E-12
nucleosome assembly			2.2E-06
negative regulation of endopeptidase activity			
cell adhesion			
regulation of presynapse assembly			
heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules			
bone mineralization			

Biological Process	Count	%	P-Value
prostaglandin metabolic process			
positive regulation of synapse assembly			
negative regulation of neuron death			
positive regulation of protein metabolic process			
positive regulation of cell-matrix adhesion			
memory			
carnitine biosynthetic process			
cell-substrate junction assembly			
developmental process involved in reproduction			
osteoclast proliferation			
proteolysis			
homophilic cell adhesion via plasma membrane adhesion molecules			
viral entry into host cell			
angiogenesis			
regulation of protein localization to membrane			
neural crest cell migration			

Differential proteins were enriched in 22 molecular functions, including cadherin binding, structural constituent of chromatin, protein heterodimerization activity, protein binding, serine-type endopeptidase inhibitor activity, interleukin-1 receptor activity, aminobutyraldehyde dehydrogenase activity, metal aminopeptidase activity, aminopeptidase activity, viral receptor activity, fatty acid binding, muscle structural constituent, peptidase activity, actin binding, glyceraldehyde-3-phosphate dehydrogenase (NAD+) (non-phosphorylating) activity, serine-type peptidase activity, dipeptidase activity, vinculin binding, catalytic activity, titin binding, and oxidoreductase activity.

Differential proteins were enriched in 10 KEGG pathways, including Shigellosis, Systemic lupus erythematosus, Alcoholism, Transcriptional misregulation in cancer, Neutrophil extracellular trap formation, Metabolic pathways, beta-Alanine metabolism, Osteoclast differentiation, Cell adhesion molecules, and Cytokine-cytokine receptor interaction.

Eleven differential proteins were enriched in alcoholism. Studies show that abusive drugs such as alcohol and psychoactive substances have similar effects, such as reduced dopaminergic markers [37].

Three differential proteins were enriched in beta-alanine metabolism pathway.

Beta-alanine is a neuromodulatory substance. Four cyclohexene analogs of γ-aminobutyric acid (GABA) and beta-alanine were designed as conformationally rigid analogs of vigabatrin, an antiepileptic and anti-addiction drug, and as potential mechanism-based inactivators of γ-aminobutyric acid transaminase (GABA-AT) [48].

Four differential proteins were enriched in biological processes such as bone mineralization and osteoclast proliferation. Five differential proteins were enriched in the osteoclast differentiation KEGG pathway. Studies show that bone mineral loss is substantial in a high proportion of methamphetamine abusers [49].

Seven differential proteins were enriched in cytokine-cytokine receptor interaction. Changes in cytokine balance are associated with blood-brain barrier damage, leading to altered brain plasticity and persistent neurotoxicity [76].

KEGG pathways enriched by differential proteins between acute patients and healthy individuals

KEGG Pathway	Count	P-Value
Shigellosis		5.80E-06
Systemic lupus erythematosus		8.50E-06
Alcoholism		1.70E-05
Transcriptional misregulation in cancer		2.10E-05
Neutrophil extracellular trap formation		1.20E-04
Metabolic pathways		7.70E-03
beta-Alanine metabolism		3.80E-02
Osteoclast differentiation		4.00E-02
Cell adhesion molecules		7.30E-02
Cytokine-cytokine receptor interaction		7.80E-02

3.3 Recovery Group vs. Healthy Group Comparison

3.3.1 IPA Pathway Analysis of Differential Proteins In the comparative analysis between the healthy group and recovery patient group, 312 differential proteins were identified using screening criteria of FC > 1.5 or < 0.67, P < 0.05.

Differential proteins were enriched in 62 IPA pathways. The most significant pathways were FXR/RXR activation and LXR/RXR activation. The primary substrate for amphetamine (AMPH)-induced locomotor activity is related to dopaminergic forebrain circuits. Brain regions associated with AMPH-induced locomotor activity express high levels of retinoid receptors, and retinoid X receptors are involved in amphetamine-induced locomotor activity [32].

Synaptogenesis signaling pathway and axon guidance signaling were enriched. Methamphetamine can cause destruction of dopaminergic nerve terminals in the mammalian forebrain, affecting neuronal growth and synapse formation [8,75].

Differential proteins were also enriched in many immune-related pathways, such as coagulation, lymphocyte signaling, complement system, rheumatoid arthritis signaling, and systemic lupus erythematosus signaling.

Six differential proteins were enriched in coagulation, with a z-score of 0.8. The intrinsic prothrombin activation pathway was also enriched. Opioid addiction causes changes in the coagulation system [50]. Four differential proteins were enriched in the complement system, with a z-score of -1. The complement system is involved in methamphetamine addiction [51], and synthetic psychoactive drug abuse may trigger multiple complement system mutations [52].

Three differential proteins were enriched in sucrose degradation. Sucrose intake activates the mesocorticolimbic system in a manner similar to abused substances [53]. Several differential proteins were enriched in glycolysis and gluconeogenesis-related pathways. Four differential proteins were enriched in maturity-onset diabetes of the young (MODY) signaling. Six differential proteins were enriched in iron homeostasis signaling pathway, and five differential proteins were enriched in ferroptosis signaling pathway. Methamphetamine induces a toxic syndrome characterized by altered carbohydrate metabolism, dysregulated calcium and iron homeostasis, increased oxidative stress, and disrupted mitochondrial function [40].

Differential proteins were enriched in biosynthesis or degradation processes of several important amino acids, including alanine biosynthesis, alanine degradation, glycine biosynthesis, tryptophan degradation, methionine salvage, and tyrosine synthesis. The metabolism of these amino acids may be related to the addiction process.

Glycine transporter (GlyT)-1 plays a key role in maintaining glycine levels at glutamatergic synapses. Glycine is an allosteric agonist of N-methyl-D-aspartate (NMDA) receptors, and NMDA receptor activation is an important step in inducing methamphetamine dependence and psychosis [54]. Glycine can reduce methamphetamine-induced locomotor activity [55].

The plasma metabolome of methamphetamine-abstinent individuals shows significant changes in alanine, aspartate and glutamate metabolism pathways, and cysteine and methionine metabolism pathways [56].

Dopaminergic neurons are deeply involved in addiction. Tyrosine hydroxylase catalyzes the first and rate-limiting step in dopamine (DA) biosynthesis. Overexpression of tyrosine hydroxylase in dopaminergic neurons increases sensitivity to methamphetamine. Tyrosine hydroxylase may be a drug target for improving sensitivity to abused drugs [57].

The anandamide degradation pathway was enriched. Anandamide, also known as arachidonoylethanolamide, was the first endocannabinoid discovered. Drug abuse disrupts synaptic plasticity in brain circuits involved in addiction, and alterations in normal endocannabinoid activity play an important role. This promotes abnormal brain changes and the development of addictive behaviors

characteristic of substance use disorders [58]. Several studies indicate that anandamide has an overall modulatory effect on brain reward circuits. Some reports suggest it is involved in addictive behaviors to other abused drugs and can also act as a behavioral reinforcer in animal models of drug abuse. These effects of anandamide appear to be enhanced by pharmacological inhibition of its metabolic degradation; after treatment with fatty acid amide hydrolase inhibitors (the primary enzyme responsible for its degradation), elevated brain levels of anandamide seem to affect the reward and reinforcement of many abused drugs [59].

Many signal transduction-related pathways were enriched.

Sixteen differential proteins were enriched in p70S6K signaling. Mitogen-stimulated p70-S6 kinase (p70-S6K) is involved in the development of sensitization to methamphetamine-induced reward effects in rats [60]. p70S6K is now considered a new therapeutic target for drug development in diseases such as cancer.

Twelve differential proteins were enriched in IL-15 signaling, and seven differential proteins were enriched in IL-15 production. Methamphetamine (METH) addiction and withdrawal cause severe damage to the immune and nervous systems. Patients acutely withdrawing from methamphetamine show significantly reduced levels of interleukin (IL)-1β, IL-9, and IL-15 [61].

Five differential proteins were enriched in Ephrin B signaling, with a z-score of -1.3. Ephrins are axon guidance molecules that may be related to behaviors associated with opioid addiction [62]. Eph receptor A4 plays a role in demyelination and depression-related behaviors [63].

Ten differential proteins were enriched in clathrin-mediated endocytosis signaling. Endocytosis of dopamine receptors is regulated by many components, such as clathrin, β-arrestin, caveolin, and Rab family proteins. Dopamine receptors escape lysosomal digestion, and their recycling occurs rapidly, strengthening dopaminergic signal transduction [64].

Eight differential proteins were enriched in actin cytoskeleton signaling, with a z-score of -0.4. Literature shows that the actin cytoskeleton can serve as a therapeutic target for preventing methamphetamine relapse [65]. Methamphetamine reduces tight junction protein expression, rearranges the F-actin cytoskeleton, and increases blood-brain barrier permeability through RhoA/ROCK-dependent pathways [66].

Nine differential proteins were enriched in sperm motility. Methamphetamine affects intracellular calcium homeostasis through calcium signaling pathway-related proteins. Additionally, it may disrupt ion homeostasis in sperm through GABA A-$ $1 receptors and calcium-binding proteins, triggering changes in intracellular calcium and chloride ions related to sperm motility [67].

Five differential proteins were enriched in macropinocytosis signaling. During exposure to methamphetamine, overstimulation of macropinocytosis leads to

lysosomal dysfunction in SH-SY5Y human neuroblastoma cells [68].

Seventeen differential proteins were enriched in phospholipase C signaling. Studies show that the central renin-angiotensin system is related to neurological diseases. Inhibition of PLC$ 1$ $effectively alleviates methamphetamine-induced neurotoxicity and methamphetamine self-administration. Central blockade of PLC$ 1-related signaling pathways significantly reduces the reinforcing and incentive effects of methamphetamine [69].

Six differential proteins were enriched in Th1 and Th2 activation pathways. Cocaine use disorder is associated with changes in Th1/Th2/Th17 cytokines and lymphocyte subsets [70].

Seven differential proteins were enriched in RHOGDI signaling. Rho GDP-dissociation inhibitor (Rho GDI) is identified as a down-regulator of Rho family GTPases [71]. Rho kinase inhibitors can improve cognitive impairment in a male mouse model of methamphetamine-induced schizophrenia [72].

Fourteen differential proteins were enriched in the role of NFAT in regulation of the immune response. Methamphetamine induces calcineurin activation and nuclear translocation of NFAT [73], and calcineurin/NFAT-induced upregulation of the Fas ligand/Fas death pathway is involved in methamphetamine-induced neuronal apoptosis [74].

Furthermore, in the comparison between recovery patients and healthy individuals, many pathways overlapped with those enriched in the acute vs. healthy comparison. Overlapping pathways included LXR/RXR activation, acute phase response signaling, neuroprotective role of THOP1 in Alzheimer's disease, role of JAK family kinases in IL-6-type cytokine signaling, hepatic fibrosis/hepatic stellate cell activation, role of chondrocytes in rheumatoid arthritis signaling pathway, PI3K/AKT signaling, STAT3 pathway, tryptophan degradation, L-carnitine biosynthesis, LPS/IL-1 mediated inhibition of RXR function, and role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis. As shown in the table, "-" indicates no overlap and represents pathways unique to recovery patients. Many of these pathways may be relevant to methamphetamine and addiction.

THOP1 (thimet oligopeptidase 1) is an enzyme that primarily degrades neuropeptides and hormones such as oxytocin and angiotensin II. THOP1 plays important roles in neuromodulation in the central nervous system and regulation of the immune system, and is closely associated with the development and prognosis of many diseases including Alzheimer's disease. Methamphetamine abuse may lead to premature development of Alzheimer's disease and neurodegeneration [35].

Serum TNF-α, IL-6, and IL-18 levels are elevated in long-term methamphetamine users [27,26]. Studies have also shown that TNF-α and IL-6 have protective effects against methamphetamine-induced microglial death through IL-6 receptors, particularly by activating the JAK-STAT3 pathway, thereby

altering pro-apoptotic and anti-apoptotic proteins [28].

Methamphetamine can mediate neuroprotection by activating the dopamine/PI3K/AKT signaling pathway [36]. L-carnitine may exert neuroprotective effects against methamphetamine toxicity through actions at the level of dopamine release or mitochondrial function [20].

Tryptophan metabolites are precursors for serotonin, melatonin, kynurenine, and quinolinic acid pathways, and their levels have been found to be affected by methamphetamine addiction [21]. The kynurenine pathway is the primary route of tryptophan degradation, and studies have found that modulating kynurenine metabolism at certain stages can reduce, prevent, or eliminate drug-seeking behaviors [22].

Methamphetamine addiction is closely related to the dopaminergic system. The dopaminergic system strongly influences rheumatoid arthritis progression [24]. Dopamine may be involved in bone formation, bone remodeling, and joint erosion in rheumatoid arthritis [25].

We found that some differential proteins and pathways in individuals who have abstained from methamphetamine for over three months (recovery vs. healthy comparison) overlap with those in active users (acute vs. healthy comparison). These consistencies may reflect that even after more than three months of methamphetamine abstinence, patients still retain lasting effects that prevent them from returning to healthy levels. These persistent effects may also explain the high relapse rate among patients after methamphetamine withdrawal. However, after longer abstinence periods, methamphetamine-withdrawn patients may potentially recover to healthy levels. In addition to overlapping pathways, many other pathways also show relevance to addiction. These differential proteins and pathways may provide potential drug targets for addiction treatment and clues for investigating addiction mechanisms, warranting further exploration in future studies.

IPA pathways enriched by differential proteins between recovery patients and healthy individuals (RH indicates recovery vs. healthy comparison, AH indicates acute vs. healthy comparison)

Ingenuity Canonical Pathways	RH -log(p-value)	AH -log(p-value)	References
FXR/RXR Activation			
LXR/RXR Activation			
Acute Phase Response Signaling			
Neuroprotective Role of THOP1 in Alzheimer's Disease			
Coagulation System			

Ingenuity Canonical Pathways	RH -log(p-value)	AH -log(p-value)	References
p70S6K Signaling			
PI3K Signaling in B Lymphocytes			
B Cell Development			
Role of JAK family kinases in IL-6-type Cytokine Signaling			
Sucrose Degradation V (Mammalian)			
IL-15 Signaling			
FcγRIIB Signaling in B Lymphocytes			
IL-15 Production			
Hepatic Fibrosis / Hepatic Stellate Cell Activation			
Ephrin Receptor Signaling			
Clathrin-mediated Endocytosis Signaling			
Systemic Lupus Erythematosus In B Cell Signaling Pathway			
Complement System			
Synaptogenesis Signaling Pathway			
Axonal Guidance Signaling			
Intrinsic Prothrombin Activation Pathway			
B Cell Receptor Signaling			
Ephrin B Signaling			
Role Of Chondrocytes In Rheumatoid Arthritis Signaling Pathway			
Pathogen Induced Cytokine Storm Signaling Pathway			
Glycolysis I			
Macropinocytosis Signaling			
Gluconeogenesis I			
SPINK1 Pancreatic Cancer Pathway			

Ingenuity Canonical Pathways	RH -log(p-value)	AH -log(p-value)	References
Glycine Betaine Degradation			
PI3K/AKT Signaling			
Sperm Motility			
Iron homeostasis signaling pathway			
STAT3 Pathway			
Phospholipase C Signaling			
Communication between Innate and Adaptive Immune Cells			
Extrinsic Prothrombin Activation Pathway			
Osteoarthritis Pathway			
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis			
Actin Cytoskeleton Signaling			
VDR/RXR Activation			
Maturity Onset Diabetes of Young (MODY) Signaling			
Alanine Degradation III			
Alanine Biosynthesis II			
Glycine Biosynthesis I			
Tryptophan Degradation X (Mammalian, via Tryptamine)			
Systemic Lupus Erythematosus Signaling			
Th1 and Th2 Activation Pathway			
Ferroptosis Signaling Pathway			
IL-6 Signaling			
L-carnitine Biosynthesis			
Guanine and Guanosine Salvage I			
Methionine Salvage II (Mammalian)			
Anandamide Degradation			
Tyrosine Biosynthesis IV			

Ingenuity Canonical Pathways	RH -log(p-value)	AH -log(p-value)	References
RHOGDI Signaling			
Hepatic Fibrosis Signaling Pathway			
Granulocyte Adhesion and Diapedesis			
Role of NFAT in Regulation of the Immune Response			
LPS/IL-1 Mediated Inhibition of RXR Function			
Th2 Pathway			
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis			

3.3.2 GO and KEGG Pathway Analysis of Differential Proteins Differential proteins were enriched in 83 biological processes ($p < 0.05$), including negative regulation of endopeptidase activity, cell adhesion, axon guidance, multicellular organism development, positive regulation of cell migration, positive regulation of kinase activity, positive regulation of synapse assembly, carbohydrate metabolic process, proteolysis, ephrin receptor signaling pathway, complement activation (classical pathway), heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules, receptor-mediated endocytosis, transmembrane receptor protein tyrosine kinase signaling pathway, innate immune response, negative chemotaxis, peptidyl-tyrosine phosphorylation, blood coagulation, angiogenesis, negative regulation of fibrinolysis, immune response, regulation of extracellular matrix disassembly, axonal fasciculation, glycoside catabolic process, induction of bacterial agglutination, immunoglobulin production, epithelial cell differentiation, retina homeostasis, viral entry into host cell, inflammatory response, neural crest cell migration, negative regulation of plasminogen activation, cell-matrix adhesion, antimicrobial humoral immune response mediated by antimicrobial peptide, fructose metabolic process, negative regulation of cysteine-type endopeptidase activity, regulation of blood coagulation, nucleotide metabolic process, plasminogen activation, adaptive immune response, cellular iron ion homeostasis, glycosaminoglycan catabolic process, defense response to Gram-positive bacterium, thyroid hormone transport, defense response to fungus, negative regulation of blood coagulation, dendritic spine development, transforming growth factor beta receptor signaling pathway, positive regulation of viral entry into host cell, cell-cell junction assembly, positive regulation of interleukin-6 production, protein catabolic process, response to hypoxia,

acute-phase response, positive regulation of tumor necrosis factor production, phospholipid homeostasis, cell migration, calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules, cell-cell signaling, smooth muscle cell-matrix adhesion, glycolipid catabolic process, iron ion homeostasis, positive regulation of substrate adhesion-dependent cell spreading, retinal ganglion cell axon guidance, cytokine-mediated signaling pathway, iron ion transport, neutrophil chemotaxis, positive regulation of phagocytosis, positive regulation of angiogenesis, oviduct epithelium development, complement-dependent cytotoxicity, cytoplasmic actin-based contraction involved in cell motility, zymogen activation, myelination, and lysosome organization.

The biological processes enriched in differential proteins include many neuroregulation-related processes, such as axon guidance, synapse assembly, neural crest cell migration, neuron development, dendritic spine development, and myelination. They also include many immune-related processes, such as complement activation, innate immune response, immune response, immunoglobulin production, and inflammatory response. Methamphetamine has the ability to modulate immune cells [76] and has major impacts on the brain, immune system, and digestion [77].

In the comparison between recovery patients and healthy individuals, many biological processes overlapped with those enriched in the acute vs. healthy comparison. Overlapping biological processes included negative regulation of endopeptidase activity, cell adhesion, positive regulation of synapse assembly, proteolysis, heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules, homophilic cell-cell adhesion via plasma membrane adhesion molecules, angiogenesis, viral entry into host cell, and neural crest cell migration. Many of these biological processes may be related to addiction.

Seventeen differential proteins were enriched in regulation of endopeptidase activity. Many endopeptidases can regulate the degradation of neuropeptides and enkephalins, which are involved in the regulation of mood, anxiety, reward, euphoria, and pain [78]. Among the differential proteins, carboxypeptidase E (CPE) (FC = 0.55, P = 0.02) regulates dopamine transporter activity [79].

Thirteen differential proteins were enriched in angiogenesis. Biological processes such as retina homeostasis and retinal ganglion cell axon guidance were also enriched. Methamphetamine administration can induce retinal hypoxia and angiogenesis [47].

Seven differential proteins were enriched in ephrin receptor signaling pathway. Many axon guidance molecules, such as integrins, semaphorins, and ephrins, may promote oxycodone-induced neural adaptations by altering axon-target connections and synaptogenesis, which may be related to behaviors associated with opioid addiction [62]. Eph receptor A4 plays a role in demyelination and depression-related behaviors [63].

Ten differential proteins were enriched in transmembrane receptor protein tyrosine kinase signaling pathway. Receptor tyrosine kinases (RTKs) are a large

family of proteins that can regulate behavior in the nervous system by modulating neuronal and glial functions, and are therefore associated with neurodegenerative diseases and psychiatric disorders such as depression and addiction. Multiple receptor tyrosine kinases regulate alcohol consumption and other behaviors related to alcohol addiction. Receptor tyrosine kinases are drug targets for developing treatments for alcohol use disorder (AUD) [80].

Biological processes (BP) enriched by differential proteins between recovery patients and healthy individuals

Biological Process	Count	%	P-Value
negative regulation of endopeptidase activity			1.40E-09
cell adhesion			5.10E-09
axon guidance			2.40E-07
multicellular organism development			3.90E-07
positive regulation of cell migration			1.10E-06
positive regulation of kinase activity			2.20E-06
positive regulation of synapse assembly			5.40E-05
carbohydrate metabolic process			9.60E-05
proteolysis			1.30E-04
ephrin receptor signaling pathway			1.50E-04
complement activation, classical pathway			1.60E-04
heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules			1.70E-04
receptor-mediated endocytosis			2.30E-04
transmembrane receptor protein tyrosine kinase signaling pathway			2.70E-04
innate immune response			2.70E-04
negative chemotaxis			3.60E-04
homophilic cell-cell adhesion via plasma membrane adhesion molecules			4.30E-04
peptidyl-tyrosine phosphorylation			4.60E-04
blood coagulation			4.90E-04
angiogenesis			7.40E-04
positive regulation of peptidyl-tyrosine phosphorylation			7.80E-04
negative regulation of fibrinolysis			1.00E-03
negative regulation of cell adhesion			1.40E-03
immune response			2.30E-03

Biological Process	Count	%	P-Value
regulation of extracellular matrix disassembly			2.40E-03
axonal fasciculation			3.20E-03
fibrinolysis			3.20E-03
glycoside catabolic process			3.60E-03
induction of bacterial agglutination			3.60E-03
immunoglobulin production			3.80E-03
epithelial cell differentiation			3.90E-03
retina homeostasis			5.70E-03
viral entry into host cell			6.40E-03
inflammatory response			6.40E-03
neural crest cell migration			6.60E-03
opsonization			6.60E-03
negative regulation of plasminogen activation			7.10E-03
cell-matrix adhesion			7.80E-03
antimicrobial humoral immune response mediated by antimicrobial peptide			8.40E-03
ovulation cycle			1.00E-02
fructose metabolic process			1.00E-02
negative regulation of cysteine-type endopeptidase activity			1.20E-02
regulation of blood coagulation			1.20E-02
nucleotide metabolic process			1.20E-02
plasminogen activation			1.20E-02
central nervous system projection neuron axonogenesis			1.30E-02
adaptive immune response			1.30E-02
cellular iron ion homeostasis			1.50E-02
glycosaminoglycan catabolic process			1.70E-02
defense response to Gram-positive bacterium			1.70E-02
thyroid hormone transport			1.80E-02
defense response to fungus			2.00E-02
negative regulation of blood coagulation			2.00E-02
dendritic spine development			2.20E-02
transforming growth factor beta receptor signaling pathway			2.30E-02
positive regulation of viral entry into host cell			2.40E-02
cell-cell junction assembly			2.50E-02

Biological Process	Count	%	P-Value
positive regulation of interleukin-6 production			2.50E-02
protein catabolic process			2.70E-02
response to hypoxia			2.90E-02
acute-phase response			2.90E-02
positive regulation of tumor necrosis factor production			2.90E-02
phospholipid homeostasis			2.90E-02
cell migration			3.00E-02
calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules			3.10E-02
cell-cell signaling			3.10E-02
smooth muscle cell-matrix adhesion			3.10E-02
glycolipid catabolic process			3.20E-02
iron ion homeostasis			3.20E-02
positive regulation of substrate adhesion-dependent cell spreading			3.30E-02
retinal ganglion cell axon guidance			3.60E-02
cytokine-mediated signaling pathway			3.80E-02
iron ion transport			3.90E-02
neutrophil chemotaxis			4.10E-02
positive regulation of phagocytosis			4.50E-02
positive regulation of angiogenesis			4.70E-02
oviduct epithelium development			4.70E-02
complement-dependent cytotoxicity			4.70E-02
cytoplasmic actin-based contraction involved in cell motility			4.70E-02
zymogen activation			4.70E-02
myelination			4.90E-02
lysosome organization			

Differential proteins were enriched in 11 KEGG pathways, including complement and coagulation cascades, pertussis, lysosome, salivary secretion, axon guidance, hematopoietic cell lineage, virion - herpesvirus, cytokine-cytokine receptor interaction, Staphylococcus aureus infection, glycolysis/gluconeogenesis, and other glycan degradation.

Twelve differential proteins were enriched in complement and coagulation cascades. The complement system is involved in methamphetamine addiction; complement factor H (CFH) is upregulated in serum of ice addicts and rats, and in certain brain regions of rats [51]. Synthetic psychoactive drug abuse may trigger multiple complement system mutations [52]. Opioid addiction causes changes

in the coagulation system [50].

Thirteen differential proteins were enriched in cytokine-cytokine receptor interaction. Changes in cytokine balance are associated with blood-brain barrier damage, leading to altered brain plasticity and persistent neurotoxicity [76]. Additionally, 11 differential proteins were enriched in axon guidance.

KEGG pathways enriched by differential proteins between recovery patients and healthy individuals

KEGG Pathway	Count	P-Value
Complement and coagulation cascades		8.70E-07
Pertussis		1.20E-04
Lysosome		2.70E-04
Salivary secretion		2.40E-03
Axon guidance		3.20E-03
Hematopoietic cell lineage		3.40E-03
Virion - Herpesvirus		1.30E-02
Cytokine-cytokine receptor interaction		1.40E-02
Staphylococcus aureus infection		4.10E-02
Glycolysis / Gluconeogenesis		4.30E-02
Other glycan degradation		4.80E-02

3.4 Acute Group vs. Recovery Group Comparison

In the comparative analysis between acute and recovery patient groups, 200 differential proteins were identified using screening criteria of FC > 1.5 or < 0.67, $P < 0.05$.

Functional analysis of differential proteins using the DAVID database enriched 57 biological processes (BP), including immunoglobulin production, immune response, cell-cell adhesion, cell adhesion, non-canonical Wnt signaling pathway, adaptive immune response, synaptic membrane adhesion, cellular response to amino acid stimulus, macromolecular complex assembly, regulation of presynapse assembly, cell migration, receptor-mediated endocytosis, regulation of cell-matrix adhesion, regulation of cell growth, ossification, positive regulation of myoblast fusion, blood coagulation, negative regulation of endopeptidase activity, positive regulation of apoptotic process, leukotriene D4 catabolic process, substrate adhesion-dependent cell spreading, lipoprotein metabolic process, positive regulation of interleukin-6 production, barbed-end actin filament capping, protein localization to plasma membrane, proteolysis, extracellular matrix organization, endodermal cell differentiation, regulation of fibrinolysis, sequestering of TGFβ in extracellular matrix, positive regulation of keratinocyte apoptotic process, neuron projection development, negative regulation of cell proliferation, signal transduction, tissue development, skeletal system development, animal organ morphogenesis, inflammatory response, nervous system development, endocytosis, actin filament polymerization, acute-phase response,

multicellular organism development, high-density lipoprotein particle clearance, cytokine-mediated signaling pathway, canonical Wnt signaling pathway, positive regulation of phagocytosis, negative regulation of Wnt signaling pathway, antimicrobial humoral immune response mediated by antimicrobial peptide, and regulation of gastrulation.

Differential proteins were enriched in 27 molecular functions (MF), including calcium ion binding, extracellular matrix structural constituent, signaling receptor activity, NAD+ nucleotidase (cyclic ADP-ribose generating), NAD(P)+ nucleotidase activity, carbohydrate binding, enzyme binding, interleukin-1 receptor activity, lipid transporter activity, antigen binding, Wnt protein binding, NF- B binding, complement component C3b binding, extracellular matrix structural constituent conferring tensile strength, transmembrane receptor protein tyrosine phosphatase activity, coreceptor activity, biotinidase activity, core promoter proximal region sequence-specific DNA binding, hormone activity, mannose binding, protein binding involved in cell-cell adhesion, cell adhesion molecule binding, heparin binding, translation elongator activity, transmembrane signaling receptor activity, and viral receptor activity.

Differential proteins were enriched in 6 KEGG pathways, including complement and coagulation cascades, cytokine-cytokine receptor interaction, cell adhesion molecules, ECM-receptor interaction, hematopoietic cell lineage, and amoebiasis.

Differential proteins were enriched in 56 IPA pathways, including hepatic fibrosis/hepatic stellate cell activation, LXR/RXR activation, B cell development, acute phase response signaling, PI3K signaling in B lymphocytes, pathogen-induced cytokine storm signaling pathway, role of osteoclasts in rheumatoid arthritis signaling pathway, FXR/RXR activation, IL-15 signaling, p70S6K signaling, role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis, FcγRIIB signaling in B lymphocytes, coagulation system, role of chondrocytes in rheumatoid arthritis signaling pathway, osteoarthritis pathway, systemic lupus erythematosus signaling in B cell signaling pathway, complement system, communication between innate and adaptive immune cells, altered T cell and B cell signaling in rheumatoid arthritis, WNT/β-catenin signaling, role of JAK family kinases in IL-6-type cytokine signaling, GP6 signaling pathway, leukotriene biosynthesis, role of NFAT in regulation of the immune response, idiopathic pulmonary fibrosis signaling pathway, PPAR signaling, role of osteoblasts in rheumatoid arthritis signaling pathway, granulocyte adhesion and diapedesis, myelination signaling pathway, atherosclerosis signaling, IL-6 signaling, VDR/RXR activation, maturity onset diabetes of young (MODY) signaling, STAT3 pathway, pulmonary healing signaling pathway, pyrimidine ribonucleotides interconversion, and phospholipase C signaling.

3.5 Comprehensive Analysis and Discussion

Beyond group comparison analysis, we also employed a one-to-many analysis method, comparing each acute patient sample with a group of age- and sex-matched healthy individuals to identify common differential proteins across all acute patients, aiming to find addiction-related biomarkers and pathway information. Screening criteria were FC > 1.5 or < 0.67, P < 0.05.

The differential protein common to all acute patients was Amphoterin-induced gene and ORF 1 (AMIGO-1). AMIGO-1 is expressed in various brain cell types, may regulate dendritic growth and neuronal survival, and may play a role in regeneration and neural plasticity in the adult nervous system [81], which is closely related to the addiction process.

In pairwise comparisons among the three sample groups, AMIGO-1 was also identified as a differential protein. The identification of AMIGO-1 across the three groups was as follows: acute group all 0, recovery group average 149, healthy group average 1,255. We hypothesize that urinary proteome AMIGO-1 levels may be negatively correlated with methamphetamine use; methamphetamine use may reduce urinary AMIGO-1 levels, which increase again after withdrawal and recovery. AMIGO-1 may have potential as a biomarker for methamphetamine use.

Notably, we found that some differential proteins and pathways in individuals who have abstained from methamphetamine for over three months (recovery vs. healthy comparison) overlap with those in active users (acute vs. healthy comparison). These consistencies may reflect that even after more than three months of methamphetamine abstinence, patients still retain lasting effects that prevent them from returning to healthy levels. These persistent effects may also explain the high relapse rate among patients after methamphetamine withdrawal. In addition to overlapping pathways, many other pathways also show relevance to addiction. These differential proteins and pathways may provide potential drug targets for addiction treatment and clues for investigating addiction mechanisms, warranting further exploration in future studies.

Furthermore, during analysis, the recovery group showed more differential proteins and pathways compared to healthy individuals than the acute group, with more significant differences. Possible reasons include: methamphetamine produces profound effects on the body in a short time, physiological indicators fluctuate dramatically over time, and responses may vary under different drug doses, usage durations, and metabolic states. Urine serves as a window that is highly sensitive to short-term physiological changes. Additionally, human susceptibility to addiction varies greatly [82]. Therefore, samples from acute patients within 24 hours of cessation have relatively high heterogeneity. Our results may help refine future experimental designs by creating more detailed sample groupings to ensure small within-group variation, facilitating better identification of commonalities.

In summary, this study comparatively analyzed the urine proteomes of methamphetamine-using patients (within 24 hours of cessation), methamphetamine-abstinent patients (abstinent for over 3 months in recovery treatment), and healthy individuals. The urine proteomes of methamphetamine-abusing patients differed significantly from healthy individuals, with some differential proteins and their enriched biological functions showing relevance to addiction or methamphetamine neurotoxicity. This study innovatively establishes a method for studying addictive drugs through urine proteomics, demonstrating that urine proteome can systematically and comprehensively reflect the effects of methamphetamine abuse on the body, with potential to provide clues for clinical addiction disease research and practice.

This study holds important significance for understanding the mechanisms and biological basis of methamphetamine addiction. By analyzing changes in the urine proteome of methamphetamine-addicted patients, we can identify protein biomarkers related to addiction, providing new clues and methods for early diagnosis and treatment of methamphetamine addiction. Additionally, through in-depth investigation of methamphetamine addiction's effects on the body, we can reveal important details of addiction mechanisms, contributing to the development of more effective prevention and treatment strategies.

Meanwhile, this study provides a novel urine proteomics perspective and effective research methods and analytical strategies for addictive drug research. Urine offers advantages such as high sensitivity, non-invasive collection, and sample stability, making it a promising tool for studying addictive substances.

Although this study has important significance and potential, there is room for improvement. Future research can combine our results with clinical data to further validate the clinical application value of the urine proteome in methamphetamine-addicted patients. Through large-sample clinical studies, we can evaluate the sensitivity, specificity, and predictive value of these protein markers, providing reliable biomarkers for early diagnosis and treatment of methamphetamine addiction.

Finally, our results can also guide the development of intervention and treatment strategies for methamphetamine addiction. By deeply understanding methamphetamine addiction's effects on the body, we can design targeted interventions to reduce addictive behaviors and alleviate addiction-related physical and psychological problems. Further research can explore the effectiveness of different intervention methods and identify more effective and personalized treatment options.

In conclusion, this study is significant for revealing methamphetamine addiction mechanisms, identifying biomarkers, and guiding clinical diagnosis and treatment. Additionally, this study provides inspiration for subsequent experiments and clinical research, promoting deeper understanding and effective management of methamphetamine addiction.

Note: Figure translations are in progress. See original paper for figures.