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REVIEWS

Special Issue: Biomarkers of Substance Abuse
Opinion

Using Metabolomics to Investigate Biomarkers of Drug Addiction

Reza Ghanbari[1,2] and Susan Sumner[1,*]

Drug addiction has been associated with an increased risk for cancer, psychological complications, heart, liver, and lung disease, as well as infection. While genes have been identified that can mark individuals at risk for substance abuse, the initiation step of addiction is attributed to persistent metabolic disruptions occurring following the first instance of narcotic drug use. Advances in analytical technologies can enable the detection of thousands of signals in body fluids and excreta that can be used to define biochemical profiles of addiction. Today, these approaches hold promise for determining how exposure to drugs, in the absence or presence of other environmentally relevant factors, can impact human metabolism. We posit that these can lead to candidate biomarkers of drug dependence, treatment, withdrawal, or relapse.

Highlights

With modern technology, it is feasible to determine the exposome of an individual and to assess how multiple types of exposure (e.g., environmentally relevant chemicals, tobacco products, or clinically relevant drugs) contribute to addiction, withdrawal, and adverse health outcomes associated with substances of abuse.

The use of metabolomics in well-designed studies of opiate addiction may enable the identification of targets for pharmacological and/or nutritional intervention.

Metabolomics is an ideal approach to study human individuality in response to both drug exposure and treatment.

Metabolomics and the Dole–Nyswander Theory

Drug addiction is a chronic disease and an ongoing social problem world-wide. A treatment for narcotic addiction evolved during the 1960s, when Marie Nyswander and Vincent Dole conducted clinical trials that led to the development of the **Methadone Maintenance Program** (MMP) (see Glossary). During this period, these researchers became convinced that there was no evidence to support the theory that addicts have sociopathic tendencies or addictive personalities [1]. However, they noticed that methadone prevented withdrawal and reduced cravings in opioid addicts, enabling them to return to normal life activities [1]. It appeared that methadone, similar to insulin for a diabetic, restored normal homeostasis [1]. Together, these observations led to the theory that addiction was initiated through a disruption in metabolism resulting in a persistent neurochemical disturbance and, furthermore, that this imbalance could lead to the types of psychological disturbance (such as underhanded tactics or deviant behaviors) reported for addicts (reviewed in [1]).

Metabolomics has been used in clinical and laboratory medicine for the discovery of potential diagnostic, prognostic, and therapeutic biomarkers [2], and may provide a means to reveal underlying metabolic perturbations associated with drug addiction, withdrawal, and relapse [3]. Metabolomics technologies enable the analysis of the low-molecular-weight (LMW) complement of cells, tissues, or biological fluids. Given that body fluids and tissues are rich in LMW endogenous metabolites, **untargeted metabolomics** provides a means to profile the biochemistry of an individual, or a cellular or organ system [4]. Indeed, many metabolites are involved in multiple biochemical pathways, and their quality and quantity in biospecimens represent a comprehensive metabolic status [4].

Through the study of perturbations in metabolic status, mechanisms underlying diseases or specific phenotypes of an organism can be revealed [5]. For example, by comparing metabolomics profiles between the phenotypes of current drug abusers, withdrawal period, duration of abstinence, relapse, or no drug use, biomarkers can be identified that correlate with the

[1]Department of Nutrition, Nutrition Research Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[2]Digestive Oncology Research Center, Digestive Diseases Research Institute, Tehran University of Medical Sciences, Tehran, Iran

*Correspondence: susan_sumner@unc.edu (S. Sumner).

phase of addiction [6]. These metabolic profiles can be used to determine how addiction might contribute to metabolic disturbances and patterns associated with the adverse health effects of addiction. While the use of metabolomics in studies of addiction is in its infancy, there is a wealth of literature demonstrating that metabolomics signatures (metabotypes) can correlate with gender, race, age, ethnicity, drug use, chemical exposure, alcohol use, tobacco product use, stress, weight, blood pressure, disease states, mental health, behavior, and nutrition, as well as changes in the gut microbiome; in their aggregate, these endogenous and exogenous components comprise the **exposome** [7–9]. In this opinion, we outline the rationale for using metabolomics in substance abuse research, discuss study design considerations, highlight the newest advances in the field, and present the hypothesis that metabolomics may be useful in biomarker discovery for substance abuse disorders (SUDs).

Why Use Metabolomics in Substance Abuse Research?

The identification of endogenous metabolites that correlate with addiction profiles can provide clinically relevant biomarkers. Mapping metabolic perturbations to pathways can improve our knowledge of the underlying etiology of the drug addiction profile. The Dole–Nyswander theory indicated a role for metabolism in addiction, relapse, and withdrawal, in part because it was noted that these individuals developed devious behaviors following drug use, while they did not exhibit these behaviors before the first occurrence of consumption [1]. In addition, the methadone treatment trials demonstrated that it was possible to titrate individual methadone doses based on metabolic rates [1].

Opiates bind to receptors localized in various regions of the mammalian brain, spinal cord digestive tract, and immune cells [10]. Indeed, mammals express endogenous opiate-like compounds (e.g., endorphins and enkephalins) in both GABA neurons and astrocytes, which have been implicated in the modulation of pain, mood, and immune responses, as well as cravings for food or other substances [11]. Opium is the dried resin obtained from the opium poppy and contains many chemical constituents, primarily alkaloids and opioids, where morphine is the active ingredient used medically for pain relief, and recreationally, for euphoria. Given that morphine binds to the same receptors (mu, delta, and kappa opioid receptors) as endogenous opioids, it also interacts with **GABA-inhibitory mechanisms**, inhibiting the production of cAMP in nerve terminals; consequently, it is expected that exposure to opium and morphine will have a significant impact on endogenous biochemical pathways that lead to the excitation of neurons, modulating responses to pain, cravings, and euphoria [8].

From another angle, there is growing concern about the link between opium exposure and cancer endpoints [12]. For example, a study of the Golestan Cohort Study (comprising approximately 50 000 subjects from a rural region in Iran) provided epidemiological evidence of a link between self-reported opium use and an increase in esophageal cancer [13], pancreatic cancer [14], bladder cancer [15], and gastrointestinal cancer [16]. By using metabolomics in these studies, metabotypes might be revealed, potentially indicating an association with a variety of factors, including the duration and cessation of opium use, and links to health-related phenotypes, as well as links to confounding factors, such as tobacco use and alcohol use. Understanding the perturbations in the metabotypes that correlate with these factors could help identify biomarkers for diagnosis, monitor intervention, or determine targets for drug development or nutritional intervention.

Study Design Considerations and Metabolomics Approaches

Metabolomics studies have been conducted using samples derived from human, animal, and plant models. Given that metabolic profiles are perturbed by many factors, such as genetics,

Glossary

Ambient pressure ion mobility mass spectrometry: analytical technique that uses a carrier buffer gas to separate ions for identification.
Chemometric approaches: statistical and multivariate approaches used to determine patterns of signals in complex data sets that correspond with specific phenotypes.
Clonidine: a prescription drug used for the treatment of hypertension, attention deficit hyperactivity disorder, and anxiety, and to ease the symptoms of substance abuse withdrawal (narcotics, nicotine, alcohol, etc.).
***Datura stramonium*:** one of the well-known folklore medicinal herbs belonging to the Solanaceae family with hallucinogenic effects.
Exposome: the totality of exposures from conception onwards.
GABA-inhibitory mechanisms: one of the inhibitory neurotransmitters that is used by the endogenous analgesia system.
Gas chromatography (GC): a method for separation of compounds in the gas phase.
Gas chromatography mass spectrometry (GC-MS): analytical method that combines GC for the separation of analytes, with MS detection.
Liquid chromatography mass spectrometry (LC-MS): analytical method that combines LC for the separation of analytes, with MS detection
Metabolomics: a powerful tool for analysis of metabolites in biological fluids, excreta, cells, and organ tissues; used to determine biomarkers and explore mechanisms and metabolic pathways related to the impact of exposures on health outcomes.
Metabotype: the metabolomics signature of an individual or system.
Methadone maintenance program (MMT): a comprehensive treatment program for opioid dependencies that includes long-term methadone treatment.
Nuclear magnetic resonance spectroscopy (NMR): a spectroscopic method that uses radio frequency pulses, and magnetic properties of certain atomic

lifestyles, microbial populations, and exposures, these parameters should be taken into consideration in the design of investigations in humans aimed at the discovery of putative metabolite biomarkers [17]. In addition, the collection and storage of samples should be consistent throughout clinical or epidemiological investigations, ensuring that factors, such as types of anticoagulant used, collection vessels, clotting times, stabilizers, or storage temperatures, do not introduce artifacts into the analyses [4].

Moreover, studies using *in vivo* model systems should be designed with dose and time to response (e.g., addiction, withdrawal, or relapse) to enable the identification of early or predictive markers of an addiction profile. Designs using both biological fluids and organ tissues should provide a means to establish noninvasive (e.g., blood, feces, or urine) corollary markers of target organ (e.g., brain) effects [18]. Furthermore, because metabolites are generally readily conserved across species, studies conducted in model systems (such as rats, mice, or nonhuman primates) might be translatable for validation in investigations in humans [19]. In addition, cell-based assays can be designed to determine how **xenobiotic** exposure (e.g., opiates) perturbs the cellular metabotype in the absence and presence of treatment; alternatively, it may be possible to compare cell metabotypes derived from substance-abuse subjects who are responders or nonresponders to treatments [20]. Such study designs might reveal markers and pathways important to opioid addiction treatment responses, and could result in the identification of druggable targets, or targets that could be considered for nutritional intervention.

Comprehensive analysis of LMW components of cells, tissues, and biological fluids can be achieved using a variety of methods [4,21]. The most-common analytical methods for metabolomics include **gas chromatography and liquid chromatography mass spectrometry** (GC-MS and LC-MS), and **nuclear magnetic resonance spectroscopy** (NMR). **Targeted metabolomics** approaches are often used when the research hypothesis is focused on specific analytes or pathways. Accordingly, specific methods are established to detect, identify, and quantitate exogenous and endogenous metabolites [22,23].

By contrast, broad-spectrum metabolomics (or **untargeted metabolomics**) are often used in hypothesis-generation research; in this scenario, techniques such as **high-resolution orbitrap** or **time-of-flight (TOF) mass spectroscopy** are used to capture signals for thousands of metabolites, and statistical and **chemometric approaches** are then used to reveal the signals that are important for defining the phenotypes of interest [24]. The specific methods selected for a metabolomics investigation will rely on factors such as: (i) the metabolites and pathways of interest; (ii) the sensitivity and resolution needed for low-abundance or difficult-to-identify analytes (e.g. neurotransmitters or endocannabinoids); (iii) the need for nondestructive sample analysis for retention of biospecimens; and (iv) the need for high-throughput methods for large-scale epidemiological studies [25].

Recent Findings in Drug Addiction Research

Metabolomics studies have been conducted using samples from both human and animal models to find putative metabolite biomarkers related to opioid addiction and lifestyle choices, such as the use of tobacco products and alcohol, which can influence addiction-related outcomes. In an early study, NMR metabolomics were used to analyze brain tissue from morphine-treated versus saline-treated monkeys; the authors reported perturbations in the concentrations of myoinositol, taurine, lactic acid, phosphocholine, creatinine, *N*-acetyl aspartate, γ-aminobutyric acid, glutamate, glutathione, methionine, and homocysteic acid in brain hippocampus and prefrontal cortex (PFC) in the morphine-treated monkeys relative to controls

nuclei, to characterize the structure of molecules.
Orbitrap Mass Spectrometry: MS with an ion trap mass analyzer.
Serotonergic syndrome: a potentially life-threatening syndrome associated with increased serotonergic activity in the central nervous system.
Targeted metabolomics: assessment of defined groups of chemically characterized metabolites.
Time-of-flight mass spectroscopy (TOF MS): a MS method that uses an electric field to accelerate ions through a tube, and determines the mass-to-charge ratio of the ion via a time measurement.
Ultra-performance liquid chromatography (UPLC): a method for the separation of compounds in liquid phase.
Untargeted metabolomics: the simultaneous assessment of thousands of signals for known and unknown metabolites.
Xenobiotic exposure: exposure to a drug or chemical that may found within an organism but that is not naturally produced.

[26]. Moreover, following morphine treatment, the administration of methadone or **clonidine** reversed metabolic perturbations, demonstrating the potential for using metabolomics to assess molecular mechanisms of opiate withdrawal and intervention [26]. Accordingly, the reversal of some metabolic perturbations by methadone supported the previously posited Dole–Nyswander theory.

Heroin consumption has been shown to significantly stimulate specific metabolic pathways. Recently, GC-MS was used to measure urine and serum concentrations of metabolites in Sprague–Dawley rats that were exposed to heroin twice a day to increasing doses administered intraperitoneally for 10 days, withdrawn for 4 days, and then readministered for 4 days [27]. The study reported several metabolites, such as myoinositol-1-phosphate and serotonin, as being implicated in underlying mechanisms of heroin reward [27]. Furthermore, in heroin-treated rats, disordered feeding behavior, accelerated energy metabolism (due to enhanced activity of the tricarboxylic acid cycle) as well as escalation of free fatty acid metabolism, were documented; these effects returned to baseline when heroin was withdrawn [27]. Again, these findings were consistent with the theory of metabolic influence of exposure in addiction and withdrawal that was alluded to above.

In another study from these authors, following intraperitoneal injection of escalating doses of methamphetamine in male Sprague–Dawley rats for 5 days and then withdrawal for 2 days, GC-MS metabolomics analysis of serum and urine samples showed disturbed energy metabolism during methamphetamine administration, including increased fatty acid beta oxidation, accelerated tricarboxylic acid activity, as well as a noticeable reduction in branched-chain amino acids, most of which resolved during the withdrawal period [28].

Moreover, in two separate studies, metabolomics was used to analyze the blood of cigarette smokers and menthol smokers, via **ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry (UHPLC-Q-TOF MS)** [29]. In menthol smokers, significant changes in 42 metabolites correlated with levels of menthol-glucuronide following the postsmoking boost relative to traditional non-menthol smokers. Metabolic profile changes were associated with processes that included cellular motility, as well as cell death (versus survival), including the cancer-related molecules (ABCB4, C3, CASR, CCK, IDO1, L-tryptophan, and UMOD) [29]. In non-menthol cigarette smokers, definitive changes in 31 smoking-related metabolites were reported; menthol-glucuronide was reduced relative to controls, along with 12 cancer-related biomarkers, glutamate, oleamide, and 13 glycerophospholipids [30].

A novel metabolomics approach, **nontargeted flow injection time-of-flight mass spectrometry**, has also been used in rat models of alcoholism (alcohol dependence induced by chronic intermittent alcohol vapor exposure) [31]. The authors reported global effects on specific neurometabolic profiles linked to alcohol consumption, which could be used to distinguish consumption history and identify a metabolic fingerprint associated with excessive alcohol consumption; indeed, alterations in metabolites related to energy metabolism in the rat accumbens shell were reported as potential pathophysiological mechanisms of alcohol dependency [31]. In addition, a prospective, case control study analyzed serum from patients with acute alcoholic hepatitis and found several altered metabolites associated with glutathione metabolism and energy homeostasis relative to controls; these 15 metabolites correlated with a 6-month survival in the patients [32].

Recently, using an NMR-based metabolomics approach, the metabolites tartrate, ethyl glucuronide, 2,3-butanediol, mannitol, and ethanol, and an endogenous response metabolite,

3-methyl-2-oxovalerate, were identified in the urine of wine consumers [33]. Of relevance, statistical analysis suggested that the measurement of combined tartrate and ethyl glucuronide biomarkers would be more valuable than single metabolite analysis in assessing alcohol consumption [33].

In a GC-MS metabolomics study using male Sprague–Dawley rats, the relationship between metabolites and ***Datura stramonium*** spectra demonstrated that administration of the hallucinogen *D. stramonium* resulted in increased or decreased levels of metabolites; 15 urinary (such as malonic acid, glycine, and galactonic acid) and ten plasma metabolites (such as alanine, butanedioic acid, and L-methionine) correlated with changes in amino, lipid. and energy metabolism [34].

Another metabolomics study used MS analysis to separately assess metabolic profiles of urine and plasma samples derived from morphine-, methamphetamine-, and cocaine-addicted male Sprague–Dawley rats; the levels of metabolites (such as 3-hydroxybutyric acid, L-tryptophan, cystine, lactose, spermidine, and stearic acid) were altered depending on the type of samples and drugs [35]. These differences might help explain, at least in part, the different actions of specific drugs on the brain reward circuitry [35]. This type of study helps demonstrate that metabolomics may be useful in predicting the extent or mechanism of drug addiction [35].

Moreover, using UPLC-HR-TOFMS analysis, changes in levels of certain endogenous metabolites, such as acylcarnitines, adenosine, inosine, AMP, and *S*-adenosyl-L-homocysteine, were recently identified in blood samples of illicit 3,4-methylenedioxymethamphetamine (MDMA) drug users relative to non-users [36]. The authors suggested that these metabolites are linked to greater energy utilization, drug-induced neurotoxicity, and **serotonergic syndrome** [36].

To assess the effects of opioids in different rodent addiction models, a LC-MS-based metabolomic analysis was performed in rat and mouse urine samples to compare cocaine metabolism [37]. After treating the rodents with the same dose of cocaine, the authors determined that, although benzoylecgonine levels were similar, metabolites from the oxidative metabolism of cocaine, such as *N*-hydroxybenzoylnorecgonine and hydroxybenzoylecgonine, were significantly higher in rats compared with mice [37]. These results were interesting because they indicated species-specific changes in cocaine metabolism; these types of finding would be relevant when attempting to translate animal studies to humans [37].

Additionally, in a neuronal metabolomics study in rats**, ambient pressure ion mobility mass spectrometry** was used to evaluate metabolic perturbations following cocaine exposure [38]; the results demonstrated cocaine effects on glucose and amine metabolites in different anatomical areas of rat striatum, PFC, and nucleus accumbens. Indeed, the metabolome diversity that was observed in treated rats was specific to the brain region, revealing that cocaine administration had the greatest effect on glycolysis metabolism in the thalamus [38].

It is also evident that choline is important in the generation of acetylcholine, a precursor to neurotransmitters, as well as in mammalian brain development [39]. The increased cancer risks associated with drug use [12,40], specifically with opium use [41], in countries that do not mandate folic acid enrichment of foods [42] may be related to perturbations in the folic acid pathway, with subsequent impact on choline-related metabolites (Figure 1). Furthermore, these

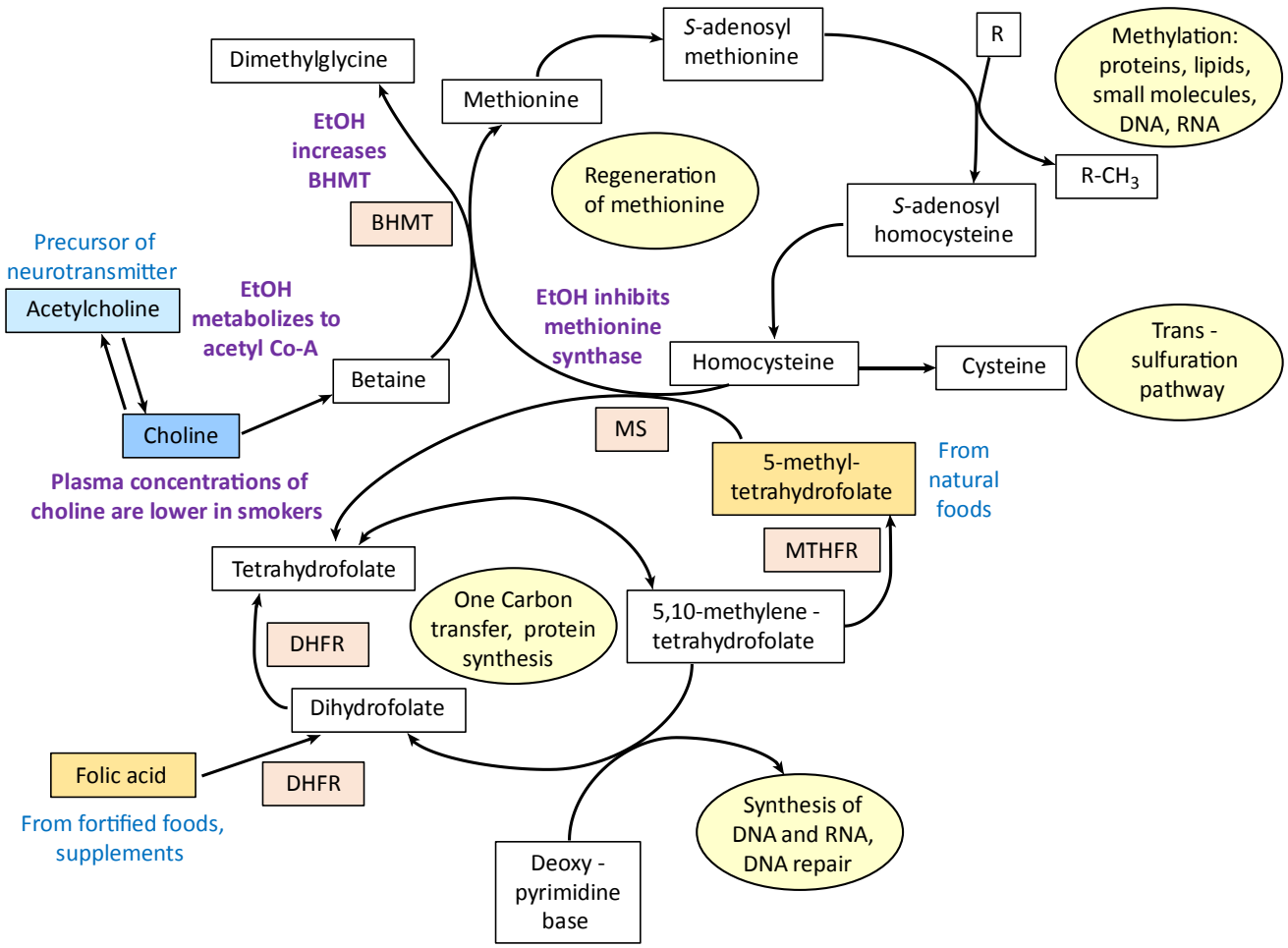

Figure 1. Effects of Human Alcohol Consumption and Smoking on Folate and Choline Metabolism. The use of tobacco products may reduce serum concentrations of choline, and ethanol use can inhibit methionine synthase and regeneration of methionine, as well as divert acetylcholine to acetyl Co-A, which impairs choline utilization, perturbing the conversion of betaine to dimethylglycine [43–47]. Methionine is essential for the methylation of proteins, lipids, and other small molecules, and for the regulation of DNA and RNA expression. Folate is an essential nutrient for the one-carbon transfer pathway, protein synthesis, DNA repair, and the synthesis of new DNA and RNA. In countries that do not fortify foods with folic acid, disturbances to folate metabolism and choline-related metabolites may contribute to increased cancer risks associated with drug use, although this remains to be further investigated. Abbreviations: BHMT, betaine-homocysteine *S*-methyltransferase; DHFR, dihydrofolate reductase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase.

perturbations might be enhanced by alcohol use, because ethanol is known to perturb the conversion of betaine to dimethylglycine [43], to inhibit methionine synthase (MetE) [43,44], and to metabolize acetylcholine to acetyl CoA [45]. Moreover, statistical methods have been used to show that lower concentrations of choline are associated with increased tobacco product use in humans [46,47]. Methyl donors (such as choline and folic acid) have been shown to impact responses to cocaine and opioids [48,49]. Thus, the impact of tobacco, to lower choline, might significantly contribute to an imbalance in the choline and folic acid pathways, and the addiction profile of an individual.

Box 1. Clinician's Corner

Addiction is a physiological response of the body to substance abuse. The individual gradually becomes dependent both physically and psychologically on drugs and, in some cases, increases the amount of drug intake.

Addiction can be defined as a noncommunicable disease that impacts community health and wellness. The biochemical changes in the brain related to drug use may be long-term, and associate with adverse response.

Some individuals who are physically and psychologically dependent on illicit drugs are genetically predisposed, and have a family history of drug addiction. In addition, an individual's response to treatment for addiction can also be related to genetics.

Similar to other diseases, the degree of vulnerability to substance abuse among individuals and subpopulations is different. The biological and metabolic capacity of an individual can be critical in terms of being susceptible to addiction, or to having a positive or negative response to treatment.

Using metabolomics to identify perturbations in biochemical process may provide a deeper understanding of the biological and molecular pathways of exposure, addiction, and withdrawal, and help reveal potential biomarkers for diagnosis or to monitor treatment.

Concluding Remarks

Metabolomics constitutes a powerful approach for revealing the impact of exposure on the overall biochemistry of an individual or system. Establishing the concise relationship between the phenotype and specific drug use is confounded by many types of exposure that an individual can simultaneously experience (e.g., drugs, chemical, nutrition, or stress), and by the impact of these exposures over a lifetime. Thus, studies in animal models and cell systems are critical for controlling experimental conditions and demonstrating links for validation in human cohorts. Important areas for such controlled dose- and time-to-response studies in animal models include using metabolomics to: (i) investigate peripheral metabolites that correlate with biochemical perturbations in the brain caused by opiate receptor binding; (ii) determine the impact of *in utero* exposure and neonatal exposure to opiates on biochemical processes later in life; (iii) investigate sex differences in response to opiate exposure; (iv) determine the impact of specific nutrient intake concurrent with opiate exposure on addiction-related responses; and (v) reveal the role of opiate receptor binding and metabolism on the gut microbiota (see Outstanding Questions and Box 1) [50,51]. We propose that, if addiction constitutes an overall metabolic disturbance in the organism, interventions that regulate metabolism might be used to treat and/or prevent SUDs. For instance, we recognize that optimum levels of folic acid are important in contributing to healthy pregnancies [52] and combatting the development of cancers, and that both low levels and high levels of folic acid have been associated with increased cancer risks [53,54]. Together, these insights have led us to hypothesize that opiate addiction, and related cancer risks, could be reduced through nutritional interventions, and we propose that extensive studies are warranted to further explore this hypothesis. We anticipate that using metabolomics for the analysis of human biospecimens, from a variety of studies, including the Golestan Cohort, will facilitate the discovery of diagnostic biomarkers and nutritional targets that may aid in intervention strategies to treat substance abuse, addiction, and withdrawal.

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Outstanding Questions

How can we use metabolomics in dose- and time-to-response studies with animal models to investigate the impact of *in utero*, lactational, and early in life exposure to opiates? How will this exposure impact the biochemistry of the developing offspring at different life stages, as well as in multiple generations?

What metabolic perturbations result from exposure to individual drugs of abuse compared with mixtures of drugs?

How does concurrent administration of opiates with tobacco, alcohol, stress, or other lifestyle factors associated with drug addiction and withdrawal perturb the biochemistry of a system compared with opiate exposure alone?

How can metabolomics be used to define nutritional targets that can be tested in model systems before translation to humans?

How does opiate exposure influence microbial populations? What are the biochemical relationships, and how are these populations related to disease outcomes?

Can metabolomics studies with cell-based assays answer questions related to the uptake and utilization of endogenous compounds (e.g., amino acids or sugars) in cells derived from subjects with different risks factors for addiction, and enable an informed approach to individualize nutrition?

How can metabolomics be used in human research studies and clinical trials to help address questions of compliance and adherence, and monitor biochemical processes that change during a successful versus a nonsuccessful program?

How can studies be designed to help unravel perturbations in our biochemistry that are attributed to the specific metabolism of the drug, adverse effects of the drug, confounding exposures, lifestyle factors, and genetics?

Can metabolomics be used to reveal how polymorphisms of susceptibility to addiction are related to pathways that are important in the response to treatment?

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