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Review

# Metabolism and metabolomics of opiates: A long way of forensic implications to unravel

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ABSTRACT

Opium poppy has important medical, socioeconomic, forensic and political implications. More than 80 benzylisoquinoline alkaloids have been described, many of them with relevant therapeutic properties such as morphine, codeine, papaverine and noscapine. Heroin, a semi-synthetic drug produced from morphine is a worldwide serious cause of morbidity and mortality. Heroin dependence is complex phenomenon with environmental and genetic influence, and several biomarkers of exposure have been proposed. This work aims to review the metabolism and metabolomics of opiates with particular interest on their relevance as potential clinical and forensic *antemortem* and *postmortem* biomarkers. It is known that the heroin is mainly a prodrug that is rapidly deacetylated in blood to its active metabolite, 6-acetylmorphine, which is then subsequently slowly deacetylated to morphine. Therefore, 6-acetylmorphine has been used as the main target metabolite to prove heroin abuse in clinical, but mostly in forensic routine. Nevertheless, its applicability is limited due to the reduced detection window. Therefore, morphine (and its metabolites morphine-3-glucuronide and morphine-6-glucuronide), codeine, codeine-6-glucuronide, 6-acetylcodeine, noscapine (and its metabolites meconine, desmethylmeconine, and cotarnine), papaverine (and its metabolites 6-desmethylpapaverine, hydroxypapaverine, dihydroxypapaverine, 6-desmethylpapaverine-glucuronide) and thebaine (and acetylthebaol and the non-acetylated analog thebaol) have been additionally recommended to obtain the most reliable results possible. More recently, the identification by metabolomics analysis of several endogenous compounds offered an alternative approach of significant importance to uncover toxic effects. Profound alterations in the neurotransmitters levels and energy and amino acid metabolism have been reported with *l*-tryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetate being suggested as potential non-specific biomarkers of long-term heroin addiction. These endogenous metabolic profiles and exogenous components that together comprise the exposome will certainly help to uncover metabolic disturbances and patterns that may be associate to addiction with relevant clinical and forensic implications.

## 1. Introduction

The rapid progress in analytical technology, namely of the "omics sciences" (*i.e.*, transcriptomics, proteomics, lipidomics and metabolomics) lead to the comprehensive analysis of endogenous molecules.[1–3] Metabolomics or metabonomics or metabolic profiling (*i.e.*, comprehensive analysis of the metabolome) is one of the most widely used "omics techniques" and its application has been expanded to various scientific fields namely to toxicology in attempts to elucidate the mechanism of toxicity of xenobiotics by offering "clues" to explain phenotype variation.[4] As it is well known, metabolites are produced during different biosynthetic and catabolic pathways that contribute to the complexity of the metabolome. Therefore, metabolomics aims to extract, separate and analyze the totality of small molecules present in any biological sample (*i.e.*, fluids or solid tissues).[5] The word "metabolome" (*i.e.*, the total set of endogenous metabolites in an organism) was first coined in 1998 and refers to low-molecular weight molecules (< 1500Da) that can be used as biomarkers, such as amino acids, organic acids, fatty acids, sugars and sugar phosphates, and it is positioned at the bottom of "omics cascade" which is the closest link to

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phenotype.[6] Due to its potential to map early biochemical changes in disease, metabolomics provides an opportunity to develop predictive biomarkers of the diagnosis of certain pathological conditions, physiological states and interactions with environmental aspects (*i.e.*, diet, lifestyle, gut microbial activity, and genetics), drug discovery, pharmacometabolomics and personalized medicine.[7–9]

The use of the term "biomarker" dates back to 1980.[10] In 1998, the National Institutes of Health Biomarkers Definitions Working Group described a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological courses, pathogenic progressions, or pharmacologic responses to a therapeutic intervention".[10,11] The World Health Organization, defines biomarker as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence or outcome of the disease". Typically, biomarkers can be classified into four types: i) diagnostic biomarkers for a specific disease; ii) prognostic biomarkers to evaluate the clinical outcome; iii) predictive biomarkers to evaluate the response to a particular treatment; and iv) predisposition biomarkers to uncover the inherent or acquired ability of the organism of developing a disease.[12,13]

Opiates are defined as the derivatives of opium alkaloids, the most relevant representatives being morphine, codeine and heroin. Heroin (*i.e.*, 3,6-diacetylmorphine, diamorphine) is semi-synthesized drug obtained by diacetylation of morphine and has extremely higher potency than morphine due to its higher lipophilicity, which can strengthen its permeability through the blood-brain barrier.[14] The drug was firstly synthetized by the chemist Charles Romley Alder Wright in 1874 while boiling morphine with acetic anhydride and then introduced into medicine by Bayer® laboratories in 1898 as a cough suppressant, possessing higher opioid activity comparatively to morphine.[15] It was commercialized as Heroin® and the name likely comes from the German word "heroisch" (*i.e.*, something extremely powerful). When its addictive potential was recognized, Bayer® ceased its production in 1913. Nowadays, heroin is the second most popular recreational drug (after *Cannabis*) and is arguably the world's most widely abused opioid and the most physiologically and psychologically problematic illicit psychoactive substance; indeed more abusers die each year from heroin abuse, and more are forced to seek treatment for addiction, than for any other illicit drug.[16] Heroin has also the highest dependence, tolerance and withdrawal score.[17] Moreover, there is a tenuous difference between recreational and fatal doses, and variations in street drug purity may cause overdoses. In addition, heroin is the drug most frequently associated with intravenous administrations and therefore there is an increased risk of bloodborne diseases transmission such as human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) and hepatitis B and C.[16,18] Heroin has a very short half-life (*i.e.*, approximately 2–4 min) since it is rapidly metabolized to 6-acetylmorphine (6-AM), morphine and other metabolites, meaning that heroin itself cannot be a useful target analyte to identify heroin abuse in practice. Although 6-AM has been claimed to be the most relevant biomarker of heroin abuse, it also has some limitations.[19–24] Therefore, alternative approaches such as metabolomics, by providing information of endogenous metabolic profiling in a global view, may help to reveal heroin abuse and better understand related mechanisms. In this review, metabolomics application to biological samples such as urine, blood or other tissues, focusing on the studies of chronic and acute administration of opiates will be presented together with metabolism and an outline of basic analytical techniques used in metabolomics. Limitations of metabolism and metabolomics studies and future perspectives will be also discussed.

## 2. Methodology

An exhaustive computer assisted search of the literature was carried out in PubMed (US National Library of Medicine, Bethesda, MD), Web of Science (Thomson Reuters, Philadelphia, PA), SciFinder (Chemical Abstracts Service, Columbus, OH), Scopus (Elsevier B.V., Amsterdam, the Netherlands), Google Scholar (Google Inc, Mountain View, CA) and books without a limiting period, concerning the metabolomics and metabolism of opiates, namely morphine, heroin (or diamorphine), codeine, papaverine and thebaine, and biomarkers of exposure. Furthermore, governmental reports of the United Nations Office on Drugs and Crime (UNODC, 2018) and European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2018) were also reviewed for possible additional publications related to this topic while the retrieved journal articles as well as books were reviewed for possible additional publications related to this topic not included in the databases.

## 3. Opioids *versus* opiates

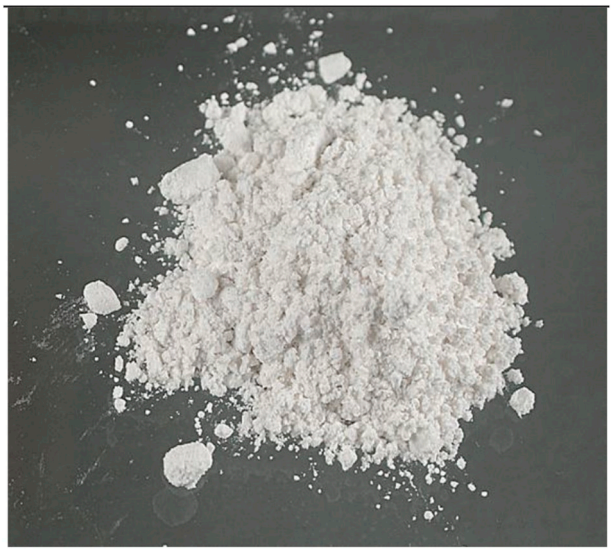
Opioids are substances that interact with opioid receptors.[25] Terms such as narcotics, hypnoanalgesics and narcoanalgesics are now considered inadequate since they includes several other xenobiotics that induce sleep. They have also been called opiates, firstly a generic name, but now restricted to naturally occurring alkaloids derived from the annual herbaceous opium poppy plant (*Papaver somniferum* and *Papaver album*), namely morphine (10%), codeine (or methylmorphine, 0.5%), papaverine (1%), thebaine (0.2%) and noscapine (8%), but also semi-synthetic compounds such as heroin.[26–28] *Papaver* is the Greek word for poppy and *somniferum* is the Latin word for sleep inducers.[25] Alkaloids are extracted from a milky exudate that flows when the green seed capsules are cut. When dry, the material is called raw opium and the percentage of each alkaloid is highly dependent on growth conditions and geographic region. The term opioid was proposed by Acheson to designate compounds acting similar to morphine, but with different chemical structures.[29] However, the opioid concept has evolved to include compounds related to opium, with similar effects although not limited to opioids. Therefore, the term opioid comprises compounds of natural, semisynthetic, synthetic and endogenous origin, which interact either as agonists or antagonists with opioid receptors and are completely antagonized by naloxone.[29] Morphine still remains the standard against which all other opioids are compared.

The opium poppy is legally cultivated worldwide for the pharmaceutical and food industries. The world's illicit crop of opium aiming heroin production originates predominantly from the 'Golden Triangle' (Myanmar, Lao People's Democratic Republic and Thailand) and Afghanistan. Three heroin presentations are typically available in the market[30–32] (Table 1). Pure heroin is a white powder, but street heroin presents in many different colors (*i.e.*, white, tan, brown, gray or black), depending the method of synthesis from morphine and accompanying "cutting" agents. No matter what its color or form, all heroin is either in salt or base form. For pharmaceutical industries, the plant is a source of opiates (*e.g.*, morphine, codeine and thebaine) that are extracted from poppy straw for the synthesis of morphine sulfate, codeine phosphate, hydrocodone, naloxone and buprenorphine.[33] Another by-product of the process of harvesting poppy straw is poppy seeds, which are legally used by the food industry in cakes, on bread products and as source of good quality cooking oil sold to supermarkets and specialist shops. Since they develop after the latex, it was originally claimed that the poppy seeds and related products would not contain any alkaloid compounds. Nevertheless, in the late 1970s, it was noted that poppy seeds actually contained alkaloids found in opium and the content varied greatly between lots, probably due to external contaminations and different harvesting technologies, poppy varieties and geographical origin.[34–36] Moreover, during food processing, the morphine content is considerably reduced up to 90%.[37] Over the last decades it has become increasingly apparent that the presence of alkaloids in the food chain is a problem and can potentially lead to serious forensic repercussions particularly in workplace and roadside drug testing.[38] Therefore, it is important to distinguish between poppy seed consumption and other sources of opiates in biological samples in case of positive analytical results.

**Table 1**
General characteristics of the three main forms of heroin available for abuse. Adapted from[30–32].

SALT/HYDROCHLORIDE POWDER

- Typically comes from the South East Asia
- White (the purest form) or slightly off-white to light tannish brown powdered
- Highly water soluble
- It is suitable for injection (intravenous – "mainlining" or subcutaneous – skin "popping") and snorting. It is not very good for smoking since easily decomposes when heated
- Easily mistaken for cocaine
- For injection heroin is placed in a "cooker" such as spoon or bottle cup, a small amount of water is added, and mixture is heated with a match or lighter until dissolved
- For intravenous administration, the vein can be distended by being tied with a tourniquet. Abuser might bring blood back into the hypodermic, where it can mix with the heroin, a process known as "booting"
- Injection carries many health risks (HIV/AIDS, HBV and HBC transmission) due to the use of "dirty" injection techniques and shared needles



BASE POWDER

- Typically comes from the South West Asian
- Poor water solubility but with a good heat stability
- It is usually a brown powder
- Suitable for smoking (*e.g.*, "chasing the dragon"). The "chasing" occurs as the abuser gingerly keeps the liquid moving in order to keep it from overheating and burning up too quickly by a flame placed below a heat conducting material such as aluminum foil. The moving smoke/pyrolysate is chased with a straw or a tube through which the user inhales
- By usually using a spoon, it can be converted into a water-soluble salt (usually using a spoon) by addition of acid (*e.g.*, citric acid, ascorbic acid) and heated, making it suitable for injection. Occasionally lemon juice or vinegar is used.

BLACK TAR

- Is a dark brownish to black solid, sticky and gumlike mass that is either gummy or rock hard
- Often produced in Latin America such as Mexico
- Its color and consistency results from the crude processing methods used to illicitly manufacture heroin that leaves behind impurities
- It is usually in the salt form
- Mostly smoked or injected
- Somewhat heat stable
- Leads to more frequent abscesses and more extensive vasculopathy
- It is a viscous, gummy and non-water-soluble, requiring additional handling and heating to prepare in an injectable solution
- Generally, less expensive than other forms of heroin

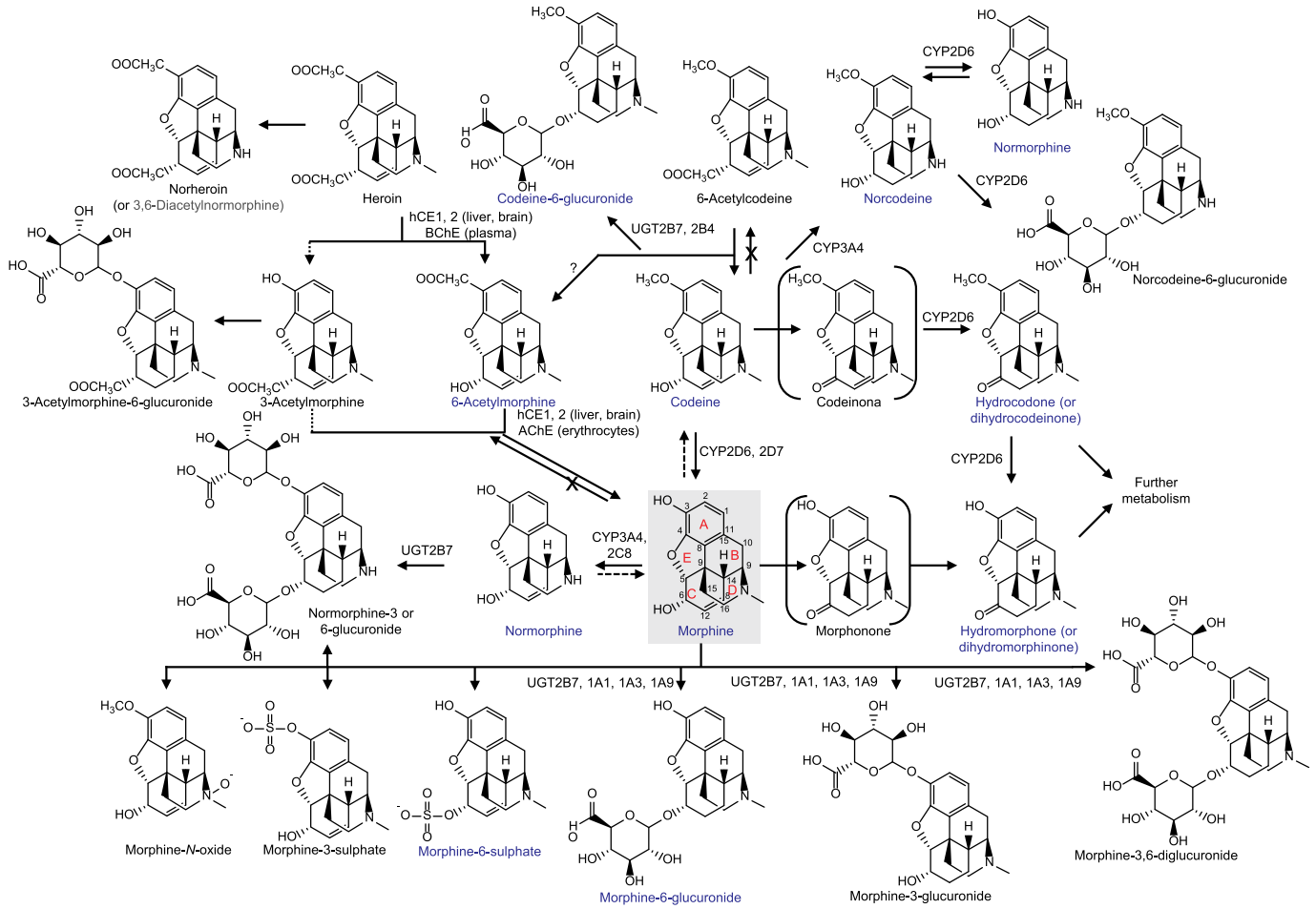



**Fig. 1.** Metabolic pathways of heroin, codeine and morphine. A – Aromatic ring; B – Cyclohexane ring; D – Piperidine ring; E – Tetrahydrofuran ring; C – Alcoholic ring. In blue are the main recognized active compounds. UGT - UDP-glucuronosyltransferase; CYP – Cytochrome P450; hCE – human carboxylesterase; BChE – butyrylcholinesterase; AChE – acetylcholinesterase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## 4. Metabolism of heroin, codeine and morphine

Fig. 1 presents the main metabolic routes of heroin, codeine and morphine. Heroin is rapidly either enzymatically or spontaneously metabolized by a sequential hydrolysis/deacetylation *in vivo* spontaneously to 6-AM and then to morphine.[39–42] Heroin has a very low affinity for μ-opioid receptors and functions mainly as a highly lipophilic prodrug of its active metabolites 6-AM, morphine and morphine-6-glucuronide.[14,26,43–45] The structural modifications of heroin (relative to morphine) allow it to more easily cross the blood-brain barrier and produce pharmacological activity in the central nervous system. Indeed, since lipophilicity is in the order heroin > 6-AM > morphine, the greater toxicity of heroin on peripheral administration is attributed to its enhanced ability to cross the blood-brain barrier and effectively deliver active metabolites to the brain. Moreover, since similar $LD_{50}$ values for heroin and 6-AM after peripheral administration were obtained, it was suggested that heroin is rapidly deacetylated to 6-AM, which then crosses the blood-brain barrier in order to exert its action.[46] Indeed, it was shown that 6-AM reached much higher concentrations than heroin or morphine in both blood and brain after parenteral administration of heroin to mice; moreover, only a small fraction of the heroin dose was able to reach the brain, while the high 6-AM concentrations in brain were reflecting transfer of 6-AM formed in blood.[47] Authors concluded that 6-AM might therefore mediate most of the effects observed shortly after heroin intake.[47] The primary enzymes responsible for the metabolism of heroin to 6-AM and then to the less potent morphine in humans are erythrocyte (and not neuronal) acetylcholinesterase (AChE) and plasmatic butyrylcholinesterase (BChE).[48,49] BChE has a higher catalytic activity for the hydrolysis of heroin to 6-AM and AChE has a higher catalytic activity for the hydrolysis of 6-AM to morphine.[48,49] Human carboxylesterases 1 and mostly the isoform 2 (hCE-1 and hCE-2) in liver and brain can also bioactivate heroin to 6-AM.[42,50]

Codeine has only an alcoholic hydroxyl at the 6 position available for glucuronidation leading to the formation of the active/analgesic codeine-6-glucuronide, which is the major metabolite representing 80% of the metabolism.[51] Of an oral dose, 0–15% is *O*-demethylated to morphine, namely by the polymorphic enzyme CYP2D6, and 10–15% is *N*-demethylated to norcodeine via CYP3A4. Norcodeine is in turn glucuronidated to norcodeine-6-glucuronide, and a minor part is *O*-demethylated to normorphine.[52–55] Therefore, codeine is considered a prodrug of codeine-6-glucuronide but also morphine.[51,56]

The primary site of morphine biotransformation is the free phenolic hydroxyl group at the 3 position which is converted to inactive morphine-3-glucuronide (57.3%), while only a small percent (10.4%) of the alcohol group at position 6 is converted to active morphine-6-glucuronide.[52,57,58] Both conjugations are catalyzed mainly by UDP-glucuronosyltransferase (UGT)2B7 but also UGT1A1, 1A3 and 1A9[59,60]. Morphine analgesic activity is now recognized to be mostly associated with its metabolite morphine-6-glucuronide, which is a strong μ-receptor agonist with higher affinity than morphine itself.[61–65] Indeed, it was shown that after intrathecal administration, morphine-6-

glucuronide was reported to be 650 times more potent than morphine[66] and therefore it has been extensively studied as a substitute for morphine.[67] In contrast, morphine-3-glucuronide has an up to 200 times lower μ-receptor binding compared with morphine.[66] However, compared to morphine-6-glucuronide, morphine causes significantly more respiratory depression.[68] Indeed, polymorphisms of UGT2B7 and UGT1A1 that lead to reduced glucuronidation and to a poor metabolizer phenotype, could cause an increase in the morphine to morphine-6-glucuronide ratio and increased risk of respiratory depression.[69,70]

Morphine-6-glucuronide and morphine-3-glucuronide are further hydrolyzed by β-glucuronidase, synthesized by both intestinal mucosal cells and gut bacteria, and subsequently reabsorbed as morphine.[71,72] *N*-demethylation to yield normorphine (catalyzed by the CYP3A4 and 2C8), which is then conjugated to normorphine-3 or 6-glucuronide, oxidation to form hydromorphone, morphine-3,6-diglucuronide, morphine-3- or -6-sulphate and morphine-*N*-oxide are also minor morphine metabolites; although *O*-methylation to codeine was claimed, this was not clearly demonstrated.[73–76] Morphine-6-sulphate is a highly potent analgesic, reported to have a similar effect to morphine-6-glucuronide.[73,74,77–79] Normorphine possesses analgesic properties and may contribute to morphine-induced toxicity.[80,81]

## 5. Interpretation pitfalls of the opiate toxicological analyses results

There are several factors that may complicate the interpretation of the results of the opiate toxicological analyses, namely: i) codeine contamination of heroin; ii) the presence of morphine and codeine in poppy seeds or medicines for the treatment of pain and cough suppression; iii) the metabolic conversion of codeine and heroin to morphine; iv) the genetic polymorphisms that can affect the metabolism; v) the different rates of urinary excretion of codeine and heroin metabolites; and vi) availability of a reliable and sensitive analytical methods.[23,82] Therefore, the simultaneous determination of biomarkers of heroin abuse such as 6-AM, morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, codeine-6-glucuronide, acetylcodeine, noscapine (and its metabolites meconine, desmethylmeconine, and cotarnine), papaverine (and its metabolites 6-desmethylpapaverine, hydroxypapaverine, dihydroxypapaverine and 6-desmethylpapaverine-glucuronide) and thebaine (and acetylthebaol and the non-acetylated analog thebaol) have been recommended to obtain the most reliable results possible and validity regarding the alleged heroin consumption.[83–87]

### 5.1. 6-Acetylmorphine

Since it is a specific metabolite, 6-AM has been used as the most relevant target to identify heroin abuse in practice.[22–24,88–90] Nevertheless, due to its short half-life (approximately 30 min) there is a short detection window (*i.e.*, no more than 8 h in urine and 2 h in blood) to document heroin consumption.[19–24] Moreover, since concentrations of 6-AM in urine are often very low compared with morphine or codeine, its detection is difficult. Indeed, less than 1% of the administered heroin dose was excreted in urine as 6-AM, whereas approximately 6% was excreted as free morphine and 57% as conjugated morphine (total morphine minus free morphine).[22,91] In addition, although 6-AM is not a constituent of opium, it may be produced as by-product of heroin synthesis due to incomplete acetylation of morphine. In *postmortem* cases, several other alternative samples besides blood can be used with 6-AM longer detection window.[92] Indeed, a longer time span between heroin intake and death is likely if 6-AM is detected in vitreous humor and urine but not in blood.[93,94] On other words, since humor vitreous lacks esterases that hydrolyse and therefore rapidly reduce the blood concentrations of 6-AM, its detection in blood matrix provides an indicator of very recent heroin exposure.[92,95]

### 5.2. Codeine and acetylcodeine

Codeine and especially acetylcodeine have been suggested as additional biomarkers of illicit heroin abuse but without higher success than 6-AM.[96–98] As previously mentioned, codeine is an alkaloid present in opium. During illicit heroin synthesis from opium, codeine is also concomitantly acetylated to acetylcodeine. This means that acetylcodeine is an impurity of the illicit heroin manufacture, and different amounts may be present (typically 2%–20% of the heroin concentration, but can be as high as 50%–80%), depending on the source and the extent to which morphine is purified from opium.[96,97] Similarly, to heroin and 6-AM, acetylcodeine was found to be more stable at acidic pHs.[96,97,99] Since acetylcodeine was only found in fewer samples and urine concentrations were lower than 6-AM, morphine, and codeine concentrations, it has been suggested that most of the acetylcodeine administered with heroin is hydrolyzed to codeine, and since acetylcodeine is stable in urine, the hydrolysis was occurring *in vivo* and not from spontaneous hydrolysis after storage.[96] Moreover, due to its short half-life (*i.e.*, approximately 4 h) in urine, it does not remain detectable for as long as morphine after heroin abuse.[98,100] Indeed, the average half-life for free morphine was found to be 3.6 h, but for total morphine (*i.e.*, the sum of free and conjugated morphine) was 7.9 h.[22] After heroin administration, morphine could be detected for 12 h and up to 24 h in blood and urine, respectively[101,102] and up to 53 h in urine after deglucuronidation.[103] As the pharmaceutical maintenance heroin is pure with less than 1% of acetylcodeine, the presence of codeine and especially of acetylcodeine or also of noscapine or papaverine in the urine of patients indicates that they may be complementing their prescribed heroin doses with illicit heroin.[87,104] This is particularly important since there is growing international interest in investigating the prescription of injectable diamorphine (*i.e.*, pharmaceutical heroin) in the treatment of heroin dependence.[105–107] Accordingly, although acetylcodeine may not be a suitable biomarker of illicit heroin use for forensic testing, its urinary acetylcodeine could be useful for urine addicts monitoring in opiates substitution programs.

### 5.3. Papaverine and metabolites

Papaverine [1-(3,4-dimethoxybenzy1)-6,7-dimethoxyisoquinoline] has a half-life of 0.8–1.5 h; it is metabolized to a large extent by demethylation, the mono- and didemethylated metabolites being excreted as glucuronides and sulfates conjugates.[108,109] About 50% of the metabolites of papaverine are excreted in the urine within 48 h, 6-desmethylpapaverine being the major metabolite in the urine.[104,110] 6-Desmethylpapaverine and its glucuronide conjugate were claimed to be potential biomarkers for illicit heroin abuse since they longer detection windows than 6-AM in urine samples.[111] Nevertheless, it should be remembered that papaverine can also be found in medicines for the treatment of erectile dysfunction[112] and in patients with histories of tracheal intubation for which neuromuscular relaxant atracurium was administered. Indeed, atracurium undergoes Hofmann elimination with laudanosine as one of the major metabolites, which is then partial dehydrogenated to papaverine.[113–115]

### 5.4. Thebaine and metabolites, thebaol and acetylthebaol

Thebaine or its *O*-demethylated metabolite oripavine are interesting biomarkers. Although it is also found in opium latex and poppy seeds, thebaine itself is not found in medicines and in illicit heroin because it is converted primarily to thebaol and acetylthebaol under the action of acetic anhydride.[85] Therefore thebaine is considered a specific marker for opium or poppy seed consumption, respectively, but the co-administration of street heroin cannot be excluded.[85] The acetylated-thebaine-4-metabolite glucuronide was recently suggested as potential biomarker, since it originates from the opium alkaloid thebaine during illicit heroin synthesis followed by metabolism and is not be detected in

urine samples after consumption of various poppy seed products.[116,117]

## 6. Metabolomics of morphine

Morphine treatment lead to profound alterations in the neurotransmitters levels and energy and amino acid metabolism.[118,119]

### 6.1. Neurotransmitters

The *l*-glutamate and *l*-aspartate, and *l*-cysteine and *l*-homocysteine are the major and minor excitatory amino acids of the central nervous system.[120] The neuronal membrane potential is also regulated by the inhibitory amino acids γ-aminobutyric acid (GABA), glycine, β-alanine and taurine. An adaptive response after morphine exposure was suggested since GABA significantly increased in *nucleus accumbens*, prefrontal cortex and striatum after morphine treatment probably due to increased conversion of glutamine to *l*-glutamate catalyzed by glutamic acid decarboxylase (GAD) in brain.[121] Indeed, acute morphine treatment of rats, increased GABA content and GAD activity at dorsal horn.[122] Moreover, previous studies have also demonstrated that activation of both $GABA_A$ and $GABA_B$ receptors may be effective in suppressing the reinforcing effects of morphine.[123,124] Glutamine also increased in *nucleus accumbens* and striatum but decreased in hippocampus and prefrontal cortex. *l*-Glutamate increased in hippocampus, *nucleus accumbens* and striatum but decreased in prefrontal cortex of morphine-dependent rats suggesting to be an important neurotransmitter involved in the mechanisms of morphine dependence,[125] Enhanced glutamine-glutamate cycle in *nucleus accumbens* and striatum might be the result of increased *de novo* synthesis via tricarboxylic acid cycle.[126] Interestingly, GABA returned normal levels only in *nucleus accumbens* and prefrontal cortex after clonidine ($\alpha_2$-noradrenergic agonist also effective in in opiate detoxification[127,128]) treatment, whereas it increased further in the three brain regions after methadone and in striatum by clonidine treatment. Authors suggested that findings may reflect a geographical different regulation of GABA, glutamine and *l*-glutamate in suppressing the reinforcing effects of morphine by methadone or clonidine.[129]

### 6.2. Oxidative metabolism

Regarding oxidative metabolism, it is known that brain energy supply relies almost entirely in the mitochondrial glucose oxidation and that the lactic acid will be produced by glycolytic processes when neuronal energy demands transiently exceed the rate of oxidative metabolism.[130,131] Increased lactic acid is related to the reduced use of pyruvate in the tricarboxylic acid cycle and the increase of anaerobic glycolysis; moreover, it may also result from the activated lactate dehydrogenase, which reflects the increased oxidative stress.[132] In addition, lactate is metabolized through the tricarboxylic acid cycle, and comparing to glucose, it is equivalent concerning its access to the tricarboxylic acid cycle in neurons.[133] Thus, increased lactic acid in *nucleus accumbens*, hippocampus, prefrontal cortex and striatum may indicate the deficiency of mitochondria function or upregulation of lactate dehydrogenase in morphine-dependent rats.[119] Lactic acid increased also in hippocampus and prefrontal cortex of morphine-dependent monkeys.[118] After clonidine administration (and not methadone), lactate reached the normal levels in hippocampus, *nucleus accumbens* and striatum, suggesting that this $\alpha_2$-noradrenergic agonist can help to recover neurochemical functions and energy metabolic alterations induced by chronic morphine treatment.[119]

### 6.3. Creatine, phosphocholine, taurine and proline

Hu et al.[119] also observed that creatine increased markedly in hippocampus and *nucleus accumbens* but decreased in prefrontal cortex and striatum of morphine-dependent rats. In opposition, phosphocholine and creatine increased in prefrontal cortex but decreased in hippocampus after chronic morphine exposure of rhesus monkeys.[118] Creatine is thought to facilitates the recycling of adenosine triphosphate (ATP), the energy currency of the cell, primarily in muscle and brain tissue and to exert direct antioxidant effects and to normalize mitochondrial mutagenesis.[134] Therefore, the increase of creatine levels may reflect a protective mechanism against energy exhaustion and oxidative stress in certain brain regions and the opposite is also true.

The authors[119] also found a significantly increase of taurine in the hippocampus, *nucleus accumbens* and striatum of morphine-dependent rats, while the opposite was verified in the hippocampus and prefrontal cortex of morphine-dependent monkeys.[118] Taurine, a sulfur-containing β-amino acid, is essential for cardiovascular function and development and function of skeletal muscle, the retina, and the central nervous system; neuroprotective effects,[135,136] namely modulation of intracellular calcium homeostasis, apoptosis inhibition and antioxidant properties have been found.[137–139] Moreover, it has been suggested to be a possible urinary biomarker for liver damage[140,141] and has also been implicated in the mechanism of cell shrinkage during apoptosis in several cell types including cerebellar granule neurons.[142–145] Notably, creatine and taurine in most of the brain regions have returned towards normal levels after methadone or clonidine treatment, indicating that antioxidation could be an effectively therapeutic option for chronic morphine.

Meng et al.[146] reported that proline, a non-essential amino acid, increased in the brain of the morphine-induced conditioned place preference model mice. Authors also registered loss of appetite and body weight in morphine-treated rats. Under nitrogen shortage, proline is produced via the *l*-glutamate pathway.[147] Elevation of free amino acids such as proline and valine by protein hydrolysis during anorexia, results a negative nitrogen balance state in the body.[148,149]

### 6.4. N-acetyl aspartate

*N*-acetyl-aspartate is synthesized in the mitochondria and is considered a marker for neuronal density.[150] *N*-acetyl aspartate increased in the hippocampus, *nucleus accumbens* and striatum.[119] Authors suggested that these findings may reflect the morphine-induced complementary increase of amino acids, as its metabolic function is to transfer amino nitrogen from mitochondrion to cytoplasm.[151,152] *N*-acetyl aspartate in the above three brain regions have clearly recovered after methadone or clonidine treatment, indicating the disturbance of neuronal activity can be modified by methadone or clonidine.[119] More recently, Hansen et al.[153] shown that the ratios of *l*-glutamate/creatine, *N*-acetyl-aspartate/creatine and myo-inositol/creatine decreased after morphine treatment during experimental pain in healthy volunteers.[153]

### 6.5. Myoinositol

Myoinositol, a significant intracellular osmolyte and a marker of astrocytic activity, is required by cells for the synthesis of membrane phosphoinositides and for the maintenance of intracellular free myoinositol.[154] Myoinositol was reduced in hippocampus, *nucleus accumbens* and pre frontal cortex of rats[119] and rhesus monkeys[118] chronically exposed to morphine, indicating alterations in brain tissue osmolarity.[155,156] On the other hand, Meng et al.[146] reported that myoinositol increased in the brain of the morphine-induced conditioned place preference model mice. Accordingly, astrocyte proliferation and changed morphology in the hippocampus was also observed.[157,158] Moreover, myoinositol phosphate, a derivative of myoinositol, was accumulated in morphine-treated mice brains. Indeed, it was demonstrated that the accumulation of inositol phosphates was induced by agonist activated opioid receptors.[159,160] In mouse spinal cord, opioid agonists binding μ- or δ-opioid receptors increase the formation of myoinositol phosphates in a concentration-dependent manner, and this activity seems to be mediated through the activation of opioid

receptors.[161] Using $^{1}$H nuclear magnetic resonance (NMR), Sharma et al.[162] have shown changes in *l*-glutamate and *l*-glutamate-linked metabolites (inositols) in the *locus coeruleus* and periaqueductal gray. These metabolites decreased with acute morphine treatment in the spinal cord and a compensatory increase was registered with chronic morphine treatment, and a dramatic increase, or overcompensation during naloxone-precipitated withdrawal.

### 6.6. Tricarboxylic acid cycle intermediates

Succinate, an intermediate of the tricarboxylic acid cycle, increased in the *nucleus accumbens* probably in attempt to maintain the ATP levels, but decrease in striatum of rats.[119] Other studies have found that succinate at subconvulsing doses causes significant oxidative damage and behavioral effects through *N*-methyl-D-aspartate (NMDA) receptor-mediated mechanisms.[163] Moreover, similar excitatory effects at the NMDA receptors were found in the presence of succinate dehydrogenase inhibitors.[164] Therefore, toxic effects are expected from secondary succinate accumulation while decreased succinate levels may reflect an adaptive response in brain. Accordingly, these energetic changes were also evident by altered levels of other tricarboxylic acid cycle intermediates such as the 2-ketoglutaric acid, fumaric acid, and malic acid in the urine of morphine-induced conditioned place preference rat model.[165]

Zaitsu et al.[165] also suggested disruption of the biotransformation of glutamic acid to 2-ketoglutaric acid since glutamic acid decreased significantly in urine. The concentration of *l*-tryptophan decreased significantly in the plasma of morphine-addicted rats and authors suggested that morphine addiction was related to uptake of *l*-tryptophan from the blood by the brain.[165] The 3-hydroxybutyric acid concentration also decreased significantly in the plasma of the morphine-addicted rats suggesting that morphine dependence may downregulate the β-oxidation pathway of fatty acids and/or ketone production (*e.g.*, 3-hydroxybutyric acid and acetone) from acetyl-CoA.[165] There were also changes in other urinary metabolites (*e.g.*, cystine and isoleucine) but the biological implications were not further explored.[165]

### 6.7. Nicotinamide

Nicotinamide was also found increased after morphine treatment in conditioned place preference model mice.[146] The preclinical findings suggest that oral nicotinamide may prevent cognitive deficits in a mouse model of Alzheimer's disease with mild to moderate pathology in a manner consistent with an inhibition of brain sirtuins and an effect on microtubule stability.[166] The neuroprotection provided by nicotinamide against the long-term effect of perinatal asphyxia, focusing on delayed cell death and mossy fibre sprouting in hippocampus, as well as on cognitive performance and anxiety, were also confirmed.[167]

## 7. Metabolomics of heroin

Previous metabolomics studies in rat's serum and urine samples exposed to heroin have shown metabolic alterations that returned to normal levels after deprivation of the drug.[168] Moreover, the reduced weight registered in heroin exposed animals is most probably related to the attempt to maintain energy/ATP levels by[168]: i) upregulation of tricarboxylic acid cycle as evidenced by the increase of citrate, a crucial intermediate, after heroin administration even for 4 days after withdrawal; and ii) free fatty acids (*e.g.*, 9-(z)-hexadecenoic acid and palmitic acid) increased metabolism evidenced by their decrease in serum concentrations.

Heroin administration also decreased *l*-tryptophan and 5-hydroxytryptamine levels in serum but increased urinary *l*-tryptophan and 5-hydroxyindoleacetate.[168] Withdrawal of heroin for 4 days efficiently restored all metabolites to baseline, except the increased serum levels of myoinositol phosphate, the decreased serum levels of threonate and 9-(z)-hexadecenoic, and the decreased levels of hydroxyproline in the urine. Phosphoinositide signaling regulates a series of important neuronal processes that are involved in mood disorders.[169] Since the myoinositol phosphate, a metabolite of the phosphoinositides, is one of the lipids involved in membrane receptor function, its increased serum levels may help to explain the underlying mechanisms of heroin in brain.

5-Hydroxytryptamine also plays an important role as a neurotransmitter in the circuits of mood and behavior. A previous study revealed that the level of 5-hydroxytryptamine is significantly increased in the putamen of heroin users, but its metabolite 5-hydroxyindoleacetic acid is reduced in the *corpus striatum*.[170] Accordingly, Zheng et al.[171] registered a decrease of the serum levels of 5-hydroxytryptamine in rats administered heroin, although this decline was not detected in the urine. Instead, heroin administration clearly elevated urinary 5-hydroxyindoleacetic acid and *l*-tryptophan levels. These peripheral metabolites were identified as potential surrogate markers characterizing the metabolic effect of heroin on central nervous system function.

Li et al.[172] identified in mice's brain samples, amino acids, tricarboxylic-acid cycle intermediates, neurotransmitters, nucleotides, melatonin and other compounds as potential biomarkers following chronic heroin exposure and withdrawal. A significant decrease of histidine and tyrosine, and a significant increase of phenylalanine and *l*-tryptophan were observed after heroin was withdrawn while the increased levels of catecholamines (*i.e.*, dopamine, adrenaline and noradrenaline) were restored to normal after the withdrawal of heroin for 2 days.[172] Particularly, it should be remembered that phenylalanine is converted to tyrosine by phenylalanine hydroxylase in the liver and both amino acids are implicated in the biosynthesis of catecholamines, and particularly dopamine is largely implicated in the mechanisms of psychoactive substance dependence.[173–178] Furthermore, heroin produced an elevation of histamine while a significant decrease in histidine was observed after heroin withdrawal. Indeed, codeine, morphine and other opiates increase the release of histamine from mast-cells and may be the cause of side effects such as flushing, warming of the skin, sweating, itching and postural hypotension.[26,179] Moreover, nucleotide monophosphates (*i.e.*, metabolites of nucleotide triphosphates) increased significantly in the brain following heroin treatment probably in attempt to restore energy.[180] Remarkably, while melatonin was significantly reduced during the sub-chronic heroin exposure, their precursors *l*-tryptophan and *N*-acetylserotonin was increased after the heroin withdrawal. It was shown that melatonin supplementation is associated with the syndrome elicited by heroin withdrawal.[181] Altogether, data support evidences that heroin disrupts energy metabolism (*e.g.*, consumption of more energy source materials), the biosynthesis of both catecholamines, nucleotides and melatonin and at least some of them could be potential biomarkers of heroin abuse and/or withdrawal but further studies are needed to clarify the effect on the levels of mRNA and protein expression of genes related to these pathways.[172] Similarly, it has been reported that loss of appetite and weight loss are symptoms of heroin addiction.[171,182,183]

A heroin self-administered rat model was employed, and analysis of the $^{1}$H NMR based metabolomics was performed to investigate the characteristic metabolome upon reintroduction to the drug after abstinence.[184] This model has been quoted with a high degree of validity in providing a direct correspondence with dependence behavior that occurs in the natural environment.[185] Sixteen metabolites in rat's serum, including phospholipids, intermediates in of the tricarboxylic acid cycle, keto bodies and neurotransmitter's precursors, underwent a significant change in the reinstatement stage compared with those in the control group. Particularly, the energy production was changed as evidenced by the significant serum increase of glucose and decrease of pyruvate (both intermediates of glycolysis) and of the intermediate of the tricarboxylic acid cycle fumarate. The levels of lactate in the serum also decreased significantly during the relapse stage. Since the levels of

the ketone bodies, 3-hydroxybutyrate and acetoacetate, increased significantly in serum, it may be suggested that energy production was activated from fatty acids, since these can serve as an energy source alternative to glucose for the brain.[186] The concentration of the neurotransmitter's precursors phenylalanine, glutamine and choline also increased during the reinstatement stage. Studies have shown that acute heroin treatment significantly increases the number of choline acetyltransferase-positive cells in the *nucleus accumbens* shell,[187] and another study demonstrated that cure-induced reinstatement was inhibited by physostigmine, an acetylcholinesterase inhibitor.[188]

In addition to studies using animal models, Mannelli et al.[189] reported the application of targeted metabolomics to plasma samples of opioid-dependent individuals diagnosed by the DSM-IV criteria. Their results suggest that some metabolites changes could be correlated to opioid dependence namely alterations of the: i) oxidation-reduction activity confirmed by the higher plasma levels of α- and γ-tocopherol and increase of the reduced glutathione/oxidized glutathione ratio; and ii) purine metabolism evidenced by the increase of guanine and xanthosine concentrations, and decrease of guanosine and hypoxanthine concentrations and hypoxanthine/xanthine and xanthine/xanthosine ratios.

## 8. Analytical techniques in metabolomics

Although various analytical techniques can be used for metabolomics, NMR spectroscopy and mass spectrometry (MS) are the most common categories.[168,190,191] NMR-based techniques have been used since the beginning of metabolomics history and have some advantages: i) requires minimal or no need for sample pretreatment before analysis; ii) it is useful for qualitative and quantitative analysis; iii) it is generally a nondestructive technique, allowing intact metabolites to be analyzed even in crude solution; iv) short analytical run times; and v) highly reproducible results.[192,193] However, the main limitations are the relatively lower sensitivity of NMR spectroscopy in comparison with that of MS, the low performance in lipid analysis and the results interpretation are generally problematic with NMR-based metabolomics.[194]

Compared to NMR-based metabolomics, MS-based metabolomics is faster and has higher sensitivity, selectivity, reproducibility, operability of instruments and separation ability of metabolites in combination with chromatography. Thus, metabolomic studies by using hyphenated separative techniques coupling MS detection of different ionization sources have become widely used, especially in biological fields. Two distinct research approaches (*i.e.*, untargeted and targeted metabolomics) have been followed, each with their own inherent advantages and disadvantages.[195,196] In targeted metabolomics a pre-defined group of metabolites (*e.g.*, amino acids, lipids, sugars, and fatty acids) are semi-quantified or quantified in order to clarify a specific metabolic pathway, without providing a global view.[195,196] In contrast, untargeted metabolomics analysis seems to represent an interesting strategy to provide a broader metabolite/biomarker coverage in a certain physiologic state to unveil biological alterations, where comprehensive metabolite identification is generally not the goal.[195,196] In the untargeted metabolomics, the coverage of the metabolome is only limited by the methodologies used in sample preparation and due to sensitivity and specificity of the analytical technique employed. Targeted metabolomics is generally executed using gas chromatograph (GC)-mass spectrometer (GC-MS), GC-tandem mass spectrometer (GC-MS/MS), liquid chromatograph-mass spectrometer (LC-MS/MS) and capillary electrophoresis (CE)/MS/MS, while untargeted metabolomics is carried out by using quadrupole-time of flight hybrid mass spectrometers (Q-TOFMS) or other high resolution instruments like Fourier transform mass spectrometers (FT-MS).[197]

## 9. Chemometric tools in metabolomics

In targeted metabolomics, by using internal standards, especially those isotopically labeled, semi-quantitative and quantitative analysis can be performed.[196] This approach can reduce the likelihood of analytical artifacts and the interference by high abundance species by applying specific metabolite extraction techniques.[196] However, since untargeted metabolomics provides information of endogenous metabolic profiling in a global view, the number of the detected metabolites ranges from at least hundreds to thousands, rendering difficult and complex the interpretation of the data obtained.[196] Therefore, untargeted metabolomics must be coupled to advanced chemometric techniques, such as univariate (UVA) and multivariate (MVA) statistical data treatment, in order to obtain a smaller set of manageable signals and efficiently analyze the metabolome.[198–200]

UVA are highly used statistical analysis in biomedicine to evaluate if the results for a specific variable differ or not significantly between groups of samples. Two UVA types are usually considered[194]: i) the parametric tests assume that the data come from a normal distribution (*e.g.*, *t*-test and one-way analysis of variance (ANOVA), if two or more groups are compared, respectively). If data do not follow a normal distribution and parametric test is to be used, a transformation (*i.e.*, log-transformation) can be applied in order to make data follow it; and ii) the non-parametric tests assume that data do not come from a normal distribution (*e.g.*, the Mann-Whitney U test and the Kruskal Wallis, if two or more groups are compared, respectively). For both UVA types, the *p*-value is obtained and the hypothesis is rejected when the *p*-value is smaller than a predetermined significance value.[201]

Typically, NMR coupled with statistical pattern recognition (PR) methods such as MVA (*i.e.*, takes into account more than one variable at the same time) are mandatory to interpret metabolome data (without bias) and display the maximum variance.[192,193] Of these statistical MVA, principal component analysis (PCA), partial least squares (or projection to latent structures) discriminant analysis (PLS-DA) and orthogonal partial least squares projection to latent structures discriminant analysis (OPLS-DA) are currently used.[202] PCA, an unsupervised PR technique, is a bilinear decomposition method used for overviewing “clusters” within multivariate data typically with score plots (S-plots) and is useful as an exploratory preliminary step that simplifies the multivariate data to a low-dimensional plot to identify the similarities and differences which cannot be directly observed within the datasets.[203] PLS, one of the most popular supervised PR methods, is described as the multiple linear regression extension of PCA that attempts to derive latent variables, analogous to PCA, which maximize the co-variation between the measured data ($X$) and the response variable ($Y$) regressed against. Once a PLS model has been calculated and validated, it can be used for the prediction of class membership of unknown samples.[203,204] Orthogonal signal correction (OSC), a data filtering method to effectively separating $Y$ -predictive variation from $Y$ -uncorrelated variation in $X$, can be used to optimize the separation by PLS (*i.e.*, OPLS-DA), thus improving the performance and the predictive power of the model in NMR-based metabonomic studies.[205] At the end of the whole process, a large and complex set of metabolites resulting statistically significant will be obtained, allowing the identification of the metabolic pathways altered as consequence of drug exposure. The $^{1}$H NMR chemical shifts and assignments of endogenous metabolites are typically identified using a web-based bioinformatic/cheminformatics databases and libraries of commercial or in-house origins, the last with limited or no public access.[195,196,206]

## 10. Concluding remarks

Metabolomics is a relatively new but also a rapidly growing technique that provides information of endogenous metabolic profiling in a global view. It may revolutionize knowledge about pathophysiology and diagnosis of diseases, namely uncovering the multiple metabolic changes that may be related to the toxic effects of psychoactive substances. Metabolomics is also a valuable early diagnostic technique to identify patients at increased risk of neuropsychiatric disorders prior

clinical symptoms development and for researching better pharmacological approaches for treatment of dependence.[207–210] Moreover, it was also suggested the possibility of predict the extent of dependence by using the PLS-regression model of the metabolic profiling since strong relationships between metabolome and the mechanism of action of each psychoactive substance on the brain reward circuitry was obtained.[165]

In this work, toxicological biomarkers related to the metabolism and metabolomics of opiates, was reviewed. The biomarkers, 6-AM, morphine (and its metabolites morphine-3-glucuronide and morphine-6-glucuronide), codeine, codeine-6-glucuronide, 6-acetylcodeine, noscapine (and its metabolites meconine, desmethylmeconine, and cotarnine), papaverine (and its metabolites 6-desmethylpapaverine, hydroxypapaverine, dihydroxypapaverine, 6-desmethylpapaverine-glucuronide) and thebaine (and acetylthebaol and the non-acetylated analog thebaol) have been applied and recommended to obtain the most reliable forensic results. While biomarkers related to the metabolism of opiates are substantially well described, metabolomics studies are scarce. Nevertheless, metabolomic profiling in combination with the use of MVA suggests that oxidative stress, neurotransmission, energetic and amino acids metabolisms are significantly changed after acute and chronic exposures to psychoactive substances and the mechanism of action being proposed.[6] Particularly interesting will be the application of metabolomics to uncover toxic effects of the opioid class of new psychoactive substances, since several new substances replacing other as soon as they are legally controlled.[211,212] Given the global impact and high potency fentanyl derivatives, special attention should be given to this subgroup of opioids.[212]

Although metabolomics has undoubtedly enhanced our understanding regarding the addiction mechanisms, it has some limitations and it is yet naive in providing robust biomarkers regarding toxicological mechanisms of psychoactive substances, since drug dependence is a highly complex disease integrating a rewarding effect, tolerance, withdrawal effect, multiple coadministered drugs and genetic variability. Firstly, by using only metabolomics, it is difficult to stablish a casal link between a specific psychoactive substance and effects not only for acute but also for chronic exposures. Therefore, the concept of multi-omics or trans-omics techniques, namely the integration of multiple omics layers, has been developed and applied to toxicology and will certainly increase in the near future.[213] Indeed, Yang et al.[214] revealed that specific proteins related to energy metabolism were significantly changed in the frontal lobe cortex of morphine-addicted rats. In addition, untargeted metabolomics techniques will be used more commonly, unraveling the currently-unknown biomarkers and hidden toxic mechanisms. Secondly, to perform a metabolomics study, the experimental conditions should be carefully controlled since the metabolome is easily influenced by different factors such as diet, sex, age, ethnicity, other administered drugs, stress, weight, pathologic states and gut microbiome.[210,215–218] Therefore, controlled animal models are equally important to investigate metabolome profiles since they can reveal potential metabolic biomarkers and provide data for exploring the underlying mechanisms of action especially in dose-dependent studies. Although it is difficult to extrapolate the results obtained from animal models to humans, metabolomics is by far the most promising among other omics sciences since the metabolome is common between species. In addition, after the clinical usefulness has been demonstrated, the biomarker test must get regulatory approval, be commercialized, and incorporated into clinical practice guidelines.[219] Finally, several studies perform metabolomic analysis in easily accessible and minimally invasive biological samples, such as urine and plasma or serum. These samples are also particularly relevant from a forensic perspective but several other biological alterative samples exists and can lead to different results, especially the metabolic highly competent liver tissue.[92] Nevertheless, it should be highlighted that urinary, but also the blood metabolic profiling could not document the real alterations in target organs such as the central nervous system or peripheral regions. Indeed, in comparison with other organs, brain entails greater metabolic and geographic complexity rendering metabolomics analysis of different areas very difficult and laborious. Moreover, brain it is relatively isolated compartment due to the presence of blood-brain barrier that limits de drug influx and the flux of endogenous metabolites between the brain and the rest of the body.[194] One of the possible solutions to this problem may be to apply metabolomics to certain brain areas or cerebrospinal fluid in future studies.[165] Finally, longitudinal studies are needed to approve and expand on these initial findings. Certainly, the joint effort of physicians, forensic experts, researchers, bioinformaticians, and biostatisticians, in academia and industry will certainly make progress towards the development of sensitive and specific diagnostic, prognostic, predictive and predisposition biomarkers.[1] Moreover, physicians should recognize the complexity of the metabolism of opiates and the determinant influence of pharmacogenomics in the pharmacokinetics and pharmacodynamics.

## Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties. No writing assistance was utilized in the production of this manuscript.

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