UNIVERSITY OF VERONA

Department of
Diagnostics and Public Health

Doctoral School of
Natural and Engineering Sciences

Doctoral Program in
Nanoscience and Advanced Technologies -
Technologies and nanotechnology in forensic sciences

XXXVI° Cycle (2020-2023)

POST-MORTEM INVESTIGATION OF CHRONIC USE OF FENTANYL AND RELATED ANALOGS BY HAIR TESTING

S.S.D. MED/43

Coordinator: Prof. Adolfo Speghini

Tutor: Prof. Federica Bortolotti

Ph.D. student: Dr. Nicola Pigaiani

YEAR 2024

ABSTRACT

Fentanyl and its derivatives, including both pharmaceutical and non-pharmaceutical fentanyls, represent the largest group among synthetic opioids. Fentanyls-related deaths have been reported in recreational use/abuse and therapeutic practice. Furthermore, fatalities from tampering with pharmaceutical products so that they can be smoked, snorted, injected, or taken orally have been reported.

In the United States, from 2013 to 2019, the age-adjusted rate of deaths involving synthetic opioids increased by 1,040%. Of them, about 50% was caused by fentanyl or derivatives alone or in association with other drugs. Moreover, in 2021, there were more than 80,000 opioid overdose deaths in the U.S. Most of those deaths were mainly driven by synthetic opioids (primarily fentanyls).

In addition, since the mid-2010s, an increasing number of unintentional overdose deaths involving opiates and/or fentanyl showed the presence as adulterant of xylazine, a veterinary drug able to worsen hypotension, central nervous system, and respiratory depression caused by opiates.

On the basis of the above evidence, it looks necessary to monitor the diffusion of fentanyl and adulterants among the population to understand their role in the overdose-risk environment and possibly prevent fentanyl overdose fatalities.

Hair testing can provide essential information regarding previous intake/exposure to xenobiotics in this frame. The use of hair analysis has gained attention over the years, especially for the retrospective investigation of chronic drug abuse and the unique ability of this matrix to serve as a long-term storage site for xenobiotics.

The present study has been carried out in collaboration with the Division of Forensics (Dept of Pathology, University of Alabama at Birmingham (UAB), AL, U.S.) and discusses the forensic toxicological value of the results of fentanyl, fentanyl analogs, and xylazine determination in hair from 250 post-mortem cases with different causes and manners of deaths also to verify the usefulness of extensive hair analysis in forensic pathology and epidemiological field.

The analytical procedure of hair testing was developed and validated at the Laboratory of Forensic Medicine of the Department of Diagnostics and Public Health of the University of Verona according to international guidelines for the quantitative analysis in forensic toxicology in terms of interferences (selectivity),

linearity, sensitivity (limit of detection [LOD] and limit of quantification [LOQ]), intra- and inter-day precision, accuracy (bias), matrix effect (M.E.) and extraction recovery (RE), carryover, and processed sample stability.

In 129 out of 250 cases (51,6%), hair samples tested positive for fentanyl, fentanyl analogs, and adulterants, counting 92 males (71.31%) and 37 females (28.69%), with a mean age of 41.72. The manner of death in the subpopulation with positive hair testing was stated as accident n = 92, homicide n = 14, natural n = 10, suicide n = 7, and undetermined n = 6. Regarding the means of death of the subjects who tested positive on hair, the most represented were drug-opioid (n = 76), gun (n = 19), and drug (n = 13).

Fentanyl was detected in almost all cases (125/129 cases), usually with analogs (101/125 cases). The analogs included: norfentanyl (n = 83, 66.4 %), acetylfentanyl (n = 16, 12.8 %), ß-hydroxy fentanyl (n = 42, 33.6 %), despropionyl para-fluorofentanyl (n = 26, 20.8%), 4-ANPP (n = 83, 66.4 %). In 51 out of 125 cases, the adulterant xylazine was identified (n = 51, 40.8 %). The hair fentanyls concentrations reported in the literature, ranging from tens to thousands of picograms per milligram, are similar to those determined in our study, and, to the best of our knowledge, this is the first time that xylazine has been detected in hair.

The concentration ratio maps of Jefferson County showed several areas of elevated hair-positive subjects. In addition, the maps showing the concentration ratio of xylazine-hair-positive subjects demonstrate a high incidence in a specific neighborhood of Birmingham.

Data from this study confirm the usefulness of systematic hair testing in post-mortem investigation, providing a qualitative representation of drug spread in near real-time in a specific area and evidence consistent with chronic organs and tissue pathologic alterations.

In addition, several risk factors for positive fentanyls hair testing, such as age and means of death, have been identified. Lastly, data provided evidence that recurrent consumption/exposure to fentanyl could lead to tolerance in subjects who would, therefore, require higher blood concentrations of fentanyl to cause death.

Given the constantly increasing diffusion of new synthetic opioids, particularly in North America, and the related social cost of the emergency, applying hair testing on a large non-pre-selected population confirms the usefulness of hair testing for forensic pathology, public health and epidemiological purposes.

SUMMARY

1. INTRODUCTION

"*Of all the remedies it has pleased almighty God to give man to relieve his suffering, none is so universal and so efficacious as opium.*" – Thomas Sydenham

"*Opium! Dread agent of unimaginable pleasure and pain! I had heard of it as I had of manna or of ambrosia, but no further. How unmeaning a sound was it at that time: what solemn chords does it now strike upon my heart! What heart-quaking vibrations of sad and happy remembrances!*" – Thomas de Quincey, Confessions of an English Opium Eater

Opium has always been part of human history. Since its discovery, it has played a fundamental role in the society in its dual use of medical and recreational drug. Moreover, its history is also closely linked to the major wars around the globe.
The use of opium throughout the centuries is characterized by the recurrence of "*opium epidemics*" that are provoked by the conditions of tolerance and withdrawal syndromes induced by this compound.
Dramatically, the last two "*opium epidemics*" occurred in times very close to us. The former occurred from 1850 to 1915, while the latter started in the mid-1980s and is still ongoing.
Over the last 20 years, several waves of opioid overdose deaths related to prescription and nonprescription opioids occurred in North America. Furthermore, in the last 10 years, the rise of fentanyl and other synthetic opioids in the U.S. on the drug market resulted in a constant increase in deaths. In 2021, nearly 71,000 drug overdose deaths involving synthetic opioids occurred in the United States.
In 2023, I had the unique opportunity to attend as a Research Scholar the Division of Forensics - Department of Pathology at the University of Alabama at Birmingham (UAB) / Jefferson County Coroner/Medical Examiner's Office, where about 1300 post-mortem cases are investigated every year.
The idea for this research project was born from this experience in consideration of high incidence of synthetic opioid-related deaths in the area and the scarce literature data on post-mortem hair analysis.

The project was focused on the application of hair testing to post-mortem cases to investigate the exposure to fentanyls of the population of the area under the jurisdiction of Jefferson County Medical Examiner.

The present PhD dissertation shows and critically discussed the results of the research project.

In particular, in the first part (4 chapters), after a general introduction on the use and abuse of opiates and opioids throughout history, with particular attention on the last century, the thesis paper faces the physiological and toxicological aspects of hair testing.

The last chapter is instead dedicated to the experimental part, in which the long-lasting analytical and toxicological experience of the Laboratory of Forensic Toxicology of the Department of Diagnostic and Public Health of the University of Verona in hair testing has been applied to the analysis of a significant number of post-mortem cases of Jefferson County Coroner/Medical Examiner's Office.

The results of hair testing were evaluated *per se* and in reference to the epidemiological, pathological and toxicological data of the cases in order to obtain a picture as complete as possible of the phenomenon.

2. PHARMACOLOGICAL ASPECTS OF OPIOIDS

2.1. OPIOIDS AND OPIATES

The term “opiate” refers to substances found naturally in poppy, such as morphine, or directly synthesized from a poppy source, such as heroin. The term “opioid” usually refers to any semisynthetic or synthetic derivative with a morphine-like activity that acts as an agonist or antagonist at an opioid receptor, including endogenous compounds (1).

Opium (or poppy tears) is dried latex obtained from the seed capsules of the opium poppy *Papaver somniferum*. Fresh raw opium is brownish with a characteristic odor, which becomes darker, hard, and brittle with aging.

The alkaloid content of opium is approximately 25% – 30% by weight. Five alkaloids, three phenanthrenes (morphine, codeine, thebaine), and two benzylisoquinolines (noscapine and papaverine) constitute the primary alkaloid content.

Morphine is a potent analgesic and psychoactive drug, codeine has minimal analgesic effect, and thebaine has no analgesic activity. Thebaine is the precursor of oxycodone and oxymorphone, both more potent than morphine. Noscapine has no analgesic activity, whereas papaverine has weak analgesic properties (2).

In contrast, the chemical classification of opioids falls into four major categories: phenanthrenes, benzomorphans, piperidines, and diphenylheptanes.

- The phenanthrene class of opioids has five rings, with the skeleton of three fused benzene rings (e.g., Buprenorphine).
- Benzomorphan derivatives have a three-ring structure and are selective for kappa opioid receptors (e.g., Pentazocine).
- Piperidine groups have two rings: a phenolic ring and a piperidine ring as essential components (e.g., Fentanyl).
- Diphenylheptanes have no heterocyclic ring and bear little resemblance to morphine but have a basic phenolic ring and a hydrophobic domain (e.g., Methadone) (3). (Table 1).

The family of synthetic opioids has expanded over the past 40 years, and many of the formulations have been synthesized since 1978 (Figure 1).

Opioid class	*Examples*
Phenanthrenes	Buprenorphine, etorphine, hydrocodone, hydromorphone, nalorphine, naloxone, naltrexone, oripavine, oxycodone, oxymorphone
Benzomorphans	Pentazocine, ketocyclazocine, bremazocine
Piperidines	Alfentanil, carfentanil, fentanyl, meperidine, remifentanil, sufentanil
Diphenylheptanes	Methadone, propoxyphene

Table 1*. Chemical class of opioids with examples.*

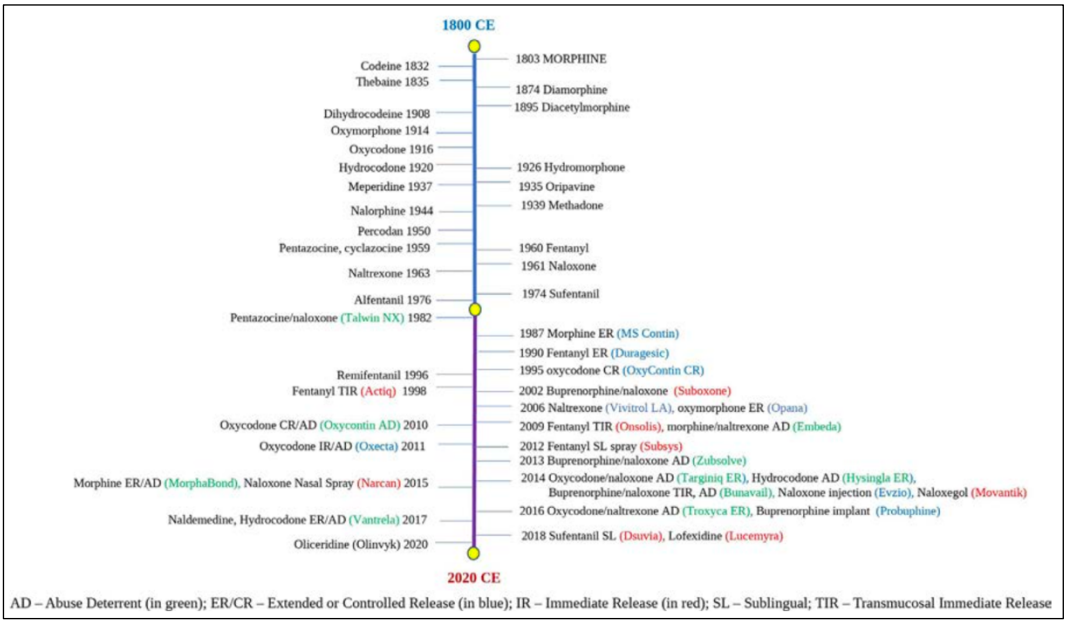


Figure 1. *Timeline of opioid development* (4).

2.2. PHARMACOKINETICS AND PHARMACODYNAMICS

At the level of organs and tissues are opioid receptors capable of binding endogenous compounds with morphine-like structures called enkephalins and endorphins. Exogenous opioids, as well as endogenous compounds, bind opioid receptors in the same way, and depending on the receptor bound, the result may be analgesia, dysphoria, or respiratory depression, among others.

Most opioids are absorbed either by oral administration or parenterally, but there are marked differences related to hepatic first-pass effects and the absorption capacity of the compounds. Lipid solubility, protein binding, ionization state, molecular size, and membrane physio-chemical properties significantly influence the absorption of opioids (5).

Drug-protein binding is high for opioids, and distribution to all tissues is remarkably rapid. Blood-brain barrier passage is privileged for liposoluble molecules such as fentanyl, but even heroin, which is hydrophilic, rapidly crosses the barrier and diffuses to the brain.

Opioid metabolism occurs primarily in the liver and consists of two phases. Phase I reactions may involve oxidation, hydrolysis, reduction, or hydration. The cytochrome P-450 enzyme system produces most metabolites during this phase. Phase II consists of a conjugation reaction that covalently attaches a small polar endogenous molecule, such as glucuronic acid, sulfate, or glycine, in order to reach an easier excretion. Many opioid metabolites produced in these phases are inactive, whereas some compounds are more potent than the parent drugs (e.g., morphine → morphine-6- glucuronide).

Opioids and their metabolites are excreted mainly with urine (almost 90% eliminated in the urine), and small amounts are also excreted with bile (6).

It is believed that at least five types of opioid receptors exist, although to date, only three have been recognized: **μ (mu), κ (kappa), and δ (delta).** These three receptors share 70% of the amino acid sequence and differ mainly at the N- and C-terminal levels. The receptor on the cell membrane consists of a transmembrane chain (7 transmembrane domains) linked to G-proteins coupled to the cyclic adenosine monophosphate (cAMP) second messenger system. Once an opioid compound (no matter whether morphine, heroin, or fentanyl) binds the receptor, the unit becomes activated. The second messenger pathway (signal cascade) begins, and intracellular effector proteins are activated, modifying the pre- and post-synaptic ion flux, achieving the effect only as long as the opioid compound is linked to the receptor (7).

Receptors		***Actions***
Mu (μ)	• *Brain* - Cortex - Thalamus - Striosomes - Periaqueductal gray • *Spinal cord* - Substantia gelatinosa • *Intestinal tract*	Analgesia, reinforcement euphoria, cough and appetite suppression, decreased respirations, decreased gastrointestinal motility, sedation, hormone changes, dopamine and acetylcholine release

Kappa (κ)	• *Brain* - Hypothalamus - Periaqueductal gray - Claustrum • *Spinal cord* - Substantia gelatinosa	Dysphoria, decreased gastrointestinal motility, decreased appetite, decreased respiration, psychotic symptoms, sedation, diuresis, and analgesia
Delta (δ)	• *Brain* - Pontine nuclei - Amygdala - Olfactory bulbs - Deep cortex	Analgesia, euphoria, physical dependence, hormone changes, appetite suppression, and dopamine release

Table 2. *Locations and Actions of Opioid Receptors*

The action of many opioids is related to the specific neuroanatomical region stimulated. Opioid receptors have been identified in the Ventral Tegmental Area and the Nucleus Accumbens, which are responsible for the euphoric and addiction effects. Other areas with high concentrations of receptors are the Periaqueductal Gray Region, the Superficial Dorsal Horn of the spinal cord (responsible for analgesia), and the Locus Coeruleus, responsible for respiratory depression. In particular, stimulation of μ receptors located in the pons (i.e., Locus Coeruleus), making the respiratory center less sensitive to carbon dioxide, leads to lethal respiratory depression, responsible for the mechanism of death in cases of opioid overdose (8).

Opioid tolerance and withdrawal are chronic effects related to the second messenger intracellular cascade. Opioid tolerance is the reduction of the response to an opioid agonist, and its manifestation is the need to increase doses to achieve the desired effect. In contrast, withdrawal refers to an intense and very uncomfortable, but usually not life-threatening syndrome that arises after abrupt cessation of chronic opioid use.

The molecular mechanisms that lead to tolerance and withdrawal are strictly linked. Cellular tolerance and withdrawal in opioid-sensitive neurons are due to multiple adaptations, as reported in Figure 2.

Firstly, receptor tolerance is related to loss in the coupling of opioid receptors to the G-protein-regulated potassium channel and perhaps the reduction in opioid receptor surface expression. In addition, hypertrophy of cAMP signaling, feedback circuit

adaptations, and synaptic plasticity contribute to this complex molecular mechanism (9).

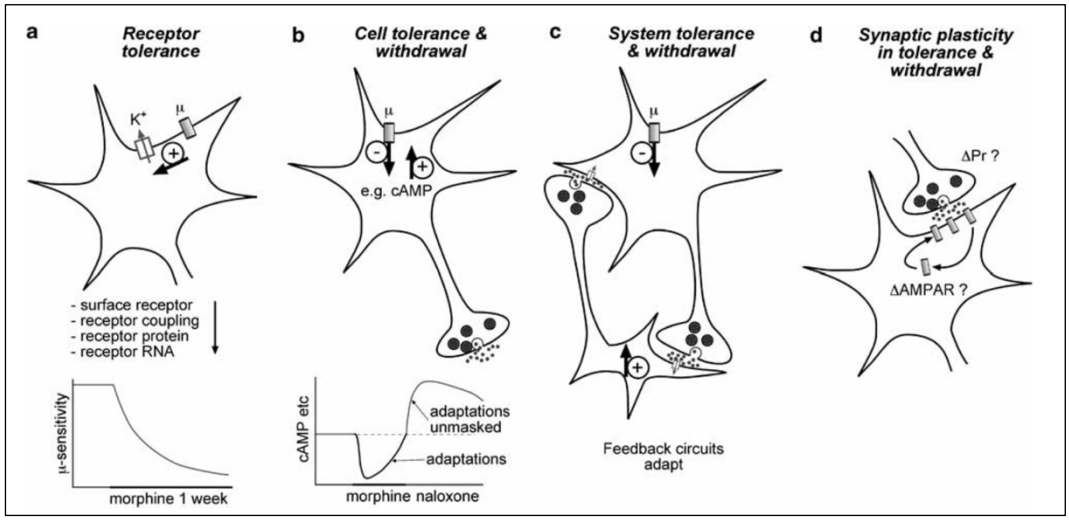


Figure 2. *Organization of opioid adaptations in the nervous system* (9)

From the clinical point of view, opioid withdrawal syndrome onset after a variable period from the last opioid intake. Symptoms usually begin after 8-12 hours from last usage, peak within 36-72 hours, and last for 7-10 days. Methadone withdrawal syndrome starts within 30 hours of last exposure, peaks at 72-96 hours, and lasts for ≥14 days. The types of symptoms reported vary with the withdrawal timeline. Early symptoms include drug craving, agitation, anxiety, muscle aches, stomach cramps, increased tearing, insomnia, runny nose, sweating, pupillary dilation, and yawning. In contrast, tachycardia, hypertension, dilated pupils, goosebumps and chills, anorexia, nausea, diarrhea, and vomiting are reported later (10).

2.3. FENTANYL AND ANALOGS

Starting from the early 2010s, an emerging number of new psychoactive substances (NPS) has been described in many countries. The emerging NPS are synthesized to mimic the effects of psychoactive compounds, which are often drugs of abuse. Novel synthetic opioids are a new trend in this context and represent an alarming warning to public health worldwide.

In this group are included high-potency fentanyl analogs (11).

Fentanyl is a highly potent synthetic μ opioid receptor agonist (but can also bind

to δ- and κ-receptor subtypes), synthesized for the first time in Belgium in December 1960 by Dr. Paul Janssen and the Janssen Company Beerse. Following the synthesis of fentanyl, some derivates were synthesized, such as sufentanil, alfentanil, and remifentanil, approved for human pharmaceutical use, and carfentanil and thiofentanil approved for veterinary purposes (12).

Nowadays, fentanyl is used as an anesthetic agent and pain reliever in the form of injection or transdermal patch, as well as in the management of persistent moderate to severe chronic pain in cancer patients who require continuous opioid administration for an extended time (13).

Fentanyl has 100 times the potency of morphine and 40 times the potency of heroin, being one of the most potent medications known to exist. Fentanyl's pharmacologic effects mime other opioid effects, including analgesia, anxiolysis, euphoria, drowsiness, feelings of relaxation, constipation, miosis, nausea, and cough suppression. However, excessive stimulation of μ receptor may lead to respiratory depression, causing death. Given its high lipophilic properties, enabling rapid diffusion through membranes like the blood-brain barrier, fentanyl can produce analgesia and unconsciousness within minutes (14).

Fentanyl was approved by FDA for medical use in the United States 1968. In the same year it was introduced into Controlled Substances Act (schedule II).

Fentanyl was initially abused by healthcare personnel in the 80s, having easy access to controlled substances and occupational exposure.

Later, a growing increase in illicit fentanyl use by opioid abusers, including subjects on opioid maintenance treatment, was reported.

Fentanyl is commonly sold as a powder to dissolve and inject, smoke, or inhale, as nasal sprays, liquids, or in tablet forms. The clandestine drug is often mixed up with heroin, increasing its potency at a small cost, and, in some cases, with cocaine.

Starting from the first documented large-scale use of fentanyl-laced heroin (so-called "China White") in California in the 1980s, fentanyl overdose started to rise worldwide. In Europe, the first report of fentanyl-related deaths was published in 1997 in Sweden, and later, east European countries such as Estonia became the region where fentanyl was the most used substance in injecting drug abusers (15).

The illicit production of fentanyl in clandestine laboratories is the primary source in the "recreational" market. In the case of European countries, the production of illegal fentanyl usually takes place in areas close to the east border of the European

Union, such as Russia, Belarus, and Ukraine. The vast majority of fentanyl and precursors for its synthesis aimed at the North American market comes from Asian laboratories, principally in China. Illicit fentanyl is also manufactured in Mexico and trafficked by the Cartels into the United States and Canada (16).

More recently, illicit laboratories started to synthesize **fentanyl analogs with a chemical structure similar to fentanyl, mainly to avoid toxicological positivity and to bypass laws banning psychotropic illicit substances, ensuring, at the same time, similar potency of the parent drug**. Fentanyl analogs are usually obtained by modification or replacement of fentanyl's propionyl chain or replacement of the ethylphenyl fraction, as reported in Figure 3 (17).

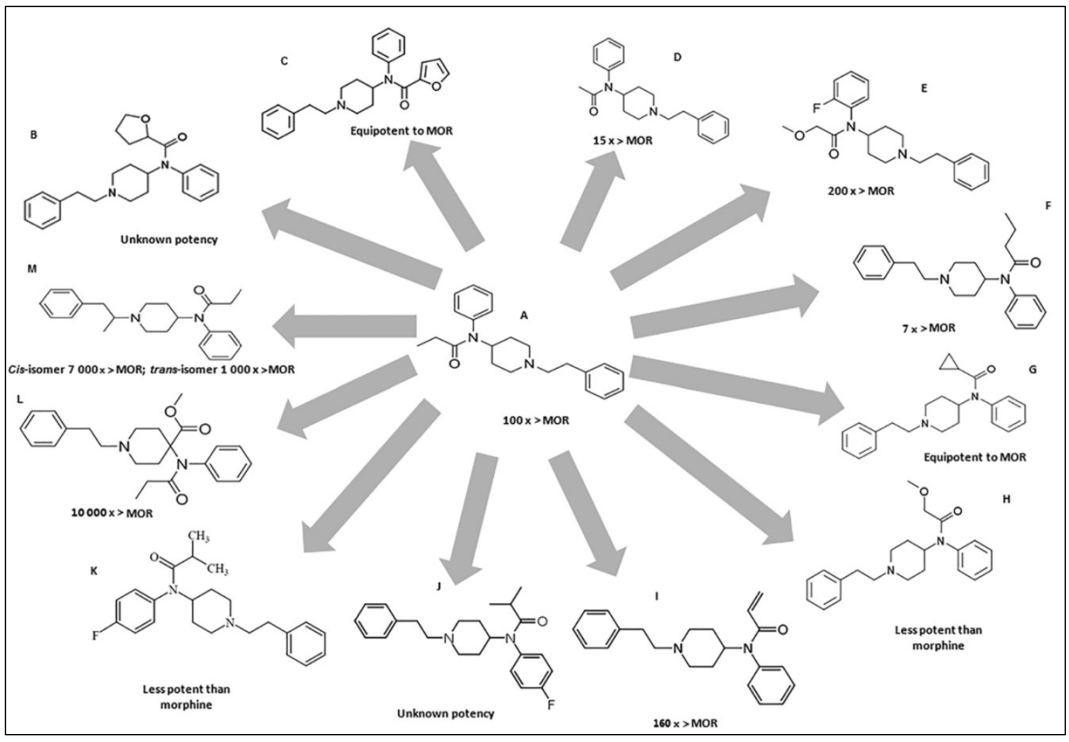


Figure 3. *Chemical structure of fentanyl and their analogues: (A) fentanyl; (B) tetrahydrofuranylfentanyl (THFF); (C) furanylfentanyl; (D) acetylfentanyl; (E) ocfentanil; (F) butyrfentanyl; (G) cyclopropylfentanyl; (H) methoxyacetylfentanyl; (I) acrylfentanyl; (J) para-fluoroisobutyrfentanyl; (K) para-fluoroisobutyrylfentanyl; (L) carfentanil; (M) a-methylfentanyl. The compounds' potency has been compared to that of morphine (MOR)* (11)

3. OLD AND NEW TRENDS OF OPIOIDS ABUSE

Illustrations of opium poppies antedate any written mentions in the Greek literature by more than 1000 years (18).

Writings from the classical period frequently discussed the medicinal qualities of poppies. In Greece, the poppy was called *opion*, a term derived from the word for "juice" (*opos*). When translated into Latin, *opion* becomes *opium*. For the ancients, the poppy symbolized sleep, occasionally everlasting. A well-known example of opiate use in the classical period was the cup given to Socrates, which contained the standard solution used at that time for suicide or euthanasia: a mixture of hemlock and opium. Opium was primarily known in Europe but used scarcely during the Middle Ages, probably because medieval physicians seemed to have been largely indifferent to the distress of their patients (19).

Afterward, opium advanced from being a sleep aid in ancient history to being prescribed for a variety of disorders during the Late Antiquity and Islamic Renaissance (20).

The popularity of opium in modern history is attributed to multiple factors, including the influence of the nonconformist physician Paracelsus on opium therapeutic use. In addition, the recreational use of various formulations of opium spread, driven by the romanticization of their use by so-called "*maudits poets*" (21).

Scientific advances with the identification of opium alkaloids and unscrupulous commercialization and prescription led to the **first opioid epidemic** in the modern era (1850-1915): indeed, opioid use increased during and after major wars of the 19th and 20th centuries [American Civil War (1861–1865), Franco-Prussian War (1870–1871), World War I (1914–1918), World War II (1939–1945), Korean War (1950–1953), and Vietnam War (1975–1995)] (22).

In the 1990s, a reappearance of Paracelsian philosophy on opium resulted in the second opioid outbreak. In particular, the promotion of pain as the "fifth vital sign", uncontrolled pharmaceutical marketing for OxyContin, and the use of opioids for chronic pain, lead to this **second epidemic** (23).

During the 2010s, the surge of prescription opioid abuse, especially in North America and Europe, and the illicit market for fentanyl-related substances resulted in the current opioid wave, leading to an increase in the number of overdose deaths worldwide(11,24).

3.1. PREHISTORIC ERA

The origin and spread of poppy worldwide during the prehistoric era are fascinating inquiries. Palaeobotanical and archaeological evidence and recent genomic analysis interpretation provide helpful data for reconstructing the relationship between poppy and humans.

Poppy is considered native to Asia Minor, in particular the areas of the Middle East overlooking the Mediterranean Sea (25). The cultivation of poppies by humans can find an interesting explanation from the theory proposed by Edgar Anderson, according to which the accumulation of rubbish near villages favored the vigorous growth of plants; from observing this phenomenon, humans cultivated specific plants useful for their needs (26).

Palaeobotanical data indicate that opium poppies were cultivated in Asia Minor and then spread to Europe as far as the Iberian Peninsula. Opium poppy seeds were found in the submerged village of La Marmotta in Lake Bracciano (Italy). The site was considered a farming community and dates back to around 5700 BC. The seeds were found in bowls together with other cereals, indicating possible cultivation. This is the earliest association in history between humans and poppies. Furthermore, poppy seeds have been found in Central Europe and Switzerland in Middle and Late Neolithic burial sites (4200-3000 BC) (27).

The oldest archaeological evidence of poppy-related artifacts includes necklaces with capsule pendants dating back to the Bronze Age. In addition, paintings of poppy gardens have been found in the tombs of pharaohs in the Valley of the Kings, as well as gold poppy-shaped earrings were found in the tombs of queens (1600-1400 BC). These findings suggest that the poppy was widespread in the aristocratic circles of Ancient Egypt and was possibly used for its psychedelic effects (28).

In the Aegean Sea, archaeological evidence of poppy capsules has been found starting from 2100 BC with paintings of Gods holding them in their hands. Numerous artifacts from the Mycenaean period (1500-1200 BC) have been found in Crete and Cyprus, such as ivory smoking pipes, jars, and clay jugs shaped like opium poppy (29).

The first evidence of the presence of opium, represented by its active components, morphine, codeine, and noscapine, was found in a juglet from ancient Egypt (1500 BC). Opium alkaloids such as thebaine and papaverine were also confirmed in a

juglet from Cyprus dated to the same period. This evidence suggests that during this Era, opium was extracted and consumed probably for its medicinal and recreational value (30).

Regarding genetics, recent studies confirmed two patterns of the spread of agriculture (including poppy) into Europe during the Neolithic period. One spread in southern Europe called "*Cardial Pottery Culture*" through the Mediterranean coastline into Iberia, and another called "*Linear Pottery Ceramic Culture*" following the Danube River into Central Europe (Figure 4). Opium poppy seeds were found with wheat, barley, chickpeas, and flax in many of these farming sites belonging to both those agriculture patterns (31).

All botanical, archaeological, and genetic evidence reported above provides solid data supporting that opium poppies, starting from the Neolithic Age, were cultivated and consumed by humans following the spread of agriculture into Europe.

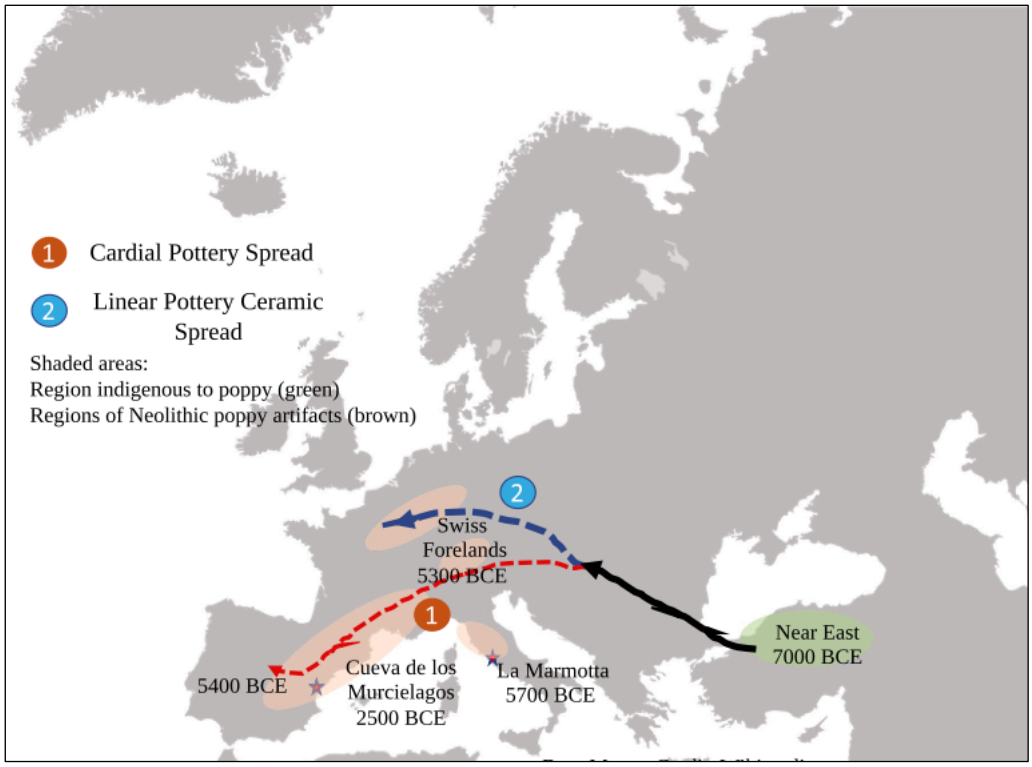


Figure 4. *Opium poppy and agriculture spread into Europe during the Neolithic Age* (4).

3.2. ANCIENT ERA

Some historical scholars initially considered that the Sumerians used poppies long before the Egyptians. The belief stemmed from interpreting some words presented on Sumerian tablets that could mean "poppy". In addition, some bas-reliefs showed people holding plants with capsular heads. It was later defined that the words assumed to be "*poppy*" were actually "*cucumber*" or "*melon*", and the bas-reliefs represented pomegranate. In a review, Krikorian concluded that there was no evidence that Sumerians, Babylonians, or Assyrians cultivated opium poppy (32).

Regarding ancient Egypt, there is evidence of poppy cultivation and use dating back to about 1,500 BC. Poppy was cultivated in Thebes, inspiring the name "thebaine" to one of the major alkaloid compounds in the poppy. In addition, thanks to the Rosetta Stone, a vocabulary for translating hieroglyphics, it was possible to discover that the Ebers Papyrus was a medical compendium containing prescriptions, a list of diseases, and some plant remedies, including the poppy. This papyrus enclosed the oldest written evidence of the use of opium poppy, particularly against headaches, to stop crying babies, and for abdominal discomfort. This finding testifies that poppy's soporific and analgesic effect was recognized during the Pharaohs (33).

Pharmacopeia of ancient Egypt was zealously studied by Classical Greek physicians and formed the basis of several therapeutics in Greek medicine. References to poppy in the literature have been found in Hesiod and Homer (Theogony, Iliad, and Odyssey) (18).

Around the fifth century BC, the writings of Hippocrates, Aristotle, and Theophrastus amplified the interest in the medical use of opium poppy.

Hippocrates of Cos (c. 460 BC - 377 BC), known as the "*Father of Clinical Medicine*", reported the use of poppy for the relief of pain in several passages of his Hippocratic Corpus (34). Physicians in later years knew and prescribed opium for a wide variety of medical conditions. In addition, flourishing was the written production relating to the properties, uses, and even side effects of poppies, popularizing the use of poppies. During this era, opium was well known for producing sleepiness, pinpoint pupils, progressing to stupor with muscular relaxation, slow respiration, and ultimately death from respiratory failure. When herbal medicine was at its peak, root-cutters were accepted as sources for plant products. At the same time, hawkers sold various herbal products from Egypt,

Arabia, and the Far East with no verifiable sources (35). These products were stored (*apothecae*), and the storeman (*apothecarius*) cataloged these products. Poppy was probably obtained from root-cutters as a poison to enable suicide and homicide in ancient times (36).

During the Roman Era, encyclopedists such as Celsus and Pliny the Elder translated Greek medical texts popularizing the use of poppy and "poppy tear" (opium) to induce sleep, calm tempers, and pain in general (37).

Further thrust for the use of opium came from the works of Claudius Galenus, a Hellenic physician in the Roman Empire. Galenus formulated the so-called "*Triaca magna*", a universal antidote (panacea). He also advocated Triaca for several illnesses, including malarial fever, and described the concept of tolerance. Although he used opium in many prescriptions, he cautioned to avoid it unless absolutely needed (38)

3.3. POST-CLASSICAL ERA

During the Dark Ages, great medical glossators translated many early Greek, Persian, and Sanskrit works into Arabic, thriving Arabic medicine. Furthermore, Arabic glossators added knowledge and experiences into their works.

During those years, scholars described diseases and cures. They advocated poppy to induce sleep and relieve pain from many conditions such as gallstones, toothaches, and headaches (39). In addition, some physicians recognized the importance of opium dosage (strengths) based on the disease treated and the physical condition of the patient and started using different preparations of opium resulting in different concentrations of morphine, a unique advancement for those times. In particular, morphine dosage in different preparations was approximately 27, 18, and 9mg (40).

Unfortunately, many historical scholars reported opium addiction to rise among several groups during this period, recognizing this condition as a sociocultural phenomenon. During the tenth century AD, the first writings appeared regarding the tolerance developed by the medical use of opium, denouncing the habit of taking opium daily until death caused by excess intake (41).

The peak of the Islamic Renaissance was reached with the advent of a great physician called Avicenna (980– 1037 AD). Unlike previous glossators, he advocated breakthrough concepts and guidelines for the use of opium, especially in

painful conditions. Avicenna recommended the primary use of opium as a sedative to soothe all types of pain, gout, earache, headache, conjunctivitis, persistent cough, and diarrhea, but cautioned it as a poison if the dose exceeded 1.2 g or 100 mg morphine at 10% content. He also discussed constipation from opium use, recommended regular use of castoreum, and advocated the use of warm honey with rose oil for opium addiction. Incidentally, Avicenna was the first physician to write an entire chapter on pain (in contrast to Galen, he recognized 15 different types of pain), including theory, nature, causes, and analgesics (42).

3.4. MODERN ERA

The Renaissance was a period of great cultural awakening in Europe with the outgrowth of sciences in general, including medicine. During this period, thanks to Paracelsus, a German-Swiss physician of many controversies, interest in the use of opium was revitalized. Paracelsus, whose real name was Philip von Hohenheim (1493 – 1541 AD), proposed opium as pain relief without investigating the cause of pain, an antithesis of Galenic logic (43).

Thomas Sydenham (1624 – 1789 AD) made a significant contribution to medicine in the early modern age, who attributed laudanum as a remedy for many conditions. The "*laudanum of Sydenham*" consisted of opium, sherry wine, saffron, and cinnamon powder indicated for many symptoms including pain, sleep, cough, alvine colic, diarrhea, dysentery, cholera, gout, kidney stones, lues venerea, childbirth, lochia, and in the later stages of fever and smallpox (44).

During the 17th century, animal experiments using intravenous opium started, coining the term "*rarefaction theory*" due to the observation that opium caused stagnation of blood circulation. During those experiments, major effects of opium were observed, and a comparison of doses on various preparations was carried out (45).

Opium continued to be commonly used for the treatment of the three major conditions, pain, sleeplessness, and diarrhea, till the advent of the "*maudits poets"*. Opium romantics, starting with Samuel Taylor Coleridge and Thomas De Quincey, followed by French Charles Baudelaire, Americans Fitz Hugh Ludlow, and Edgar Rice Burroughs, popularized opium through the description of self-experiences of loss of self-control and fantasy with Gothic gore (horror of addiction) and Faustian suggestions (devil as the master, dominance of Western over Eastern cultures) (21).

Regarding the Far East, opium was cultivated in all Chinese provinces for medical and recreational use during the Ming Dynasty (1368 – 1644 AD). Opium was used as an aphrodisiac in the "*art of commerce with women*" as well as a recreational substance: during this period, the average amount of opium smoked per person in China was estimated to be 3–6 g/day (46). This social and cultural trend persisted until the 19th century. Opium prospered along with sex industries in the city as well as the countryside in the so-called "opium dens". Smoking opium was even offered in public places such as restaurants. Only the political and economic reforms that started after 1949 were able to eradicate public opium dens (47).

In India, opium use in traditional medicine was limited to inducing sleep and controlling diarrhea and pain. Recreational use among locals increased during British rule. Concentrated preparations of opium called "*madak*" and "*chandu*" were available for smoking, but the incidence of opium addiction was estimated to be lower than the global average of the era (48).

At the turn of the 18th and 19th centuries, there were important discoveries and developments that forever changed the history of opioid use. Francois Magendie published a short article in 1818 describing the use of the alkaloid morphine for pain, proposing its use along with codeine for numerous painful conditions (49).

Intravenous injections of compounds had been described since the 1600s, but just in 1855, Alexander Wood, a Scottish physician, used a small glass syringe with a hollow, large-caliber steel needle to administer morphine under the skin. Wood called the procedure "subcutaneous," and morphine was locally injected in the area of pain. However, the needle was difficult to insert, and Charles Hunter improvised Wood's needle by adding a lateral opening to the point. Hunter proposed that morphine could be injected elsewhere, providing relief by systemic effects, and coining the term "*hypodermic*".

The use of a hypodermic syringe for administration changed the course of opium history. Morphine started to be used extensively in obstetrics, surgery, and anesthesia (50).

In the 19th century, a global opioid crisis manifested. Opium smoking was popular in China, and the habit spread along trade routes in the rest of the world. Smoking opium became trendy in the United States and Europe, a habit learned from Chinese immigrants.

Physicians, seeing that opium helped diseases most common in the era before public health and preventive medicine took hold, such as diarrhea and cough, used opium and opioids as the treatment of choice (51).

Morphine was not a controlled substance at that time. Entrepreneurs promoted its use for many conditions ranging from social phobia to morning sickness and menstrual pain (52).

Furthermore, morphine was used extensively during the American Civil War (1861–1865), which resulted in addiction among those returning from the war (53). The uncontrolled spread use of morphine was followed by numerous reports of addiction, which put victims in a "degrading slavery" to the drug (54).

In 1895, diacetylmorphine (Heroin) rose to the forefront, being proposed as a remedy for morphine addiction by Bayer pharmaceutical company; within a few years, heroin addiction also rose. Addiction to opium by smoking, oral, and intravenous use hurried in society, resulting in public health concerns, and centers for the treatment of addiction appeared in America and Europe (55).

A global opioid crisis called the "First Opioid Epidemic" occurred between 1850 and 1915. This crisis was addressed by the promulgation of many national and supra-national regulations (*i.e., 1868 England Pharmacy Act, 1920 England Dangerous Drugs Act, 1914 U.S. Harrison's Narcotic Act, 1901 American Episcopal Bishop Charles Brent (Brent Commission), 1909 International Opium Commission, 1912 Hague Opium Commission*).

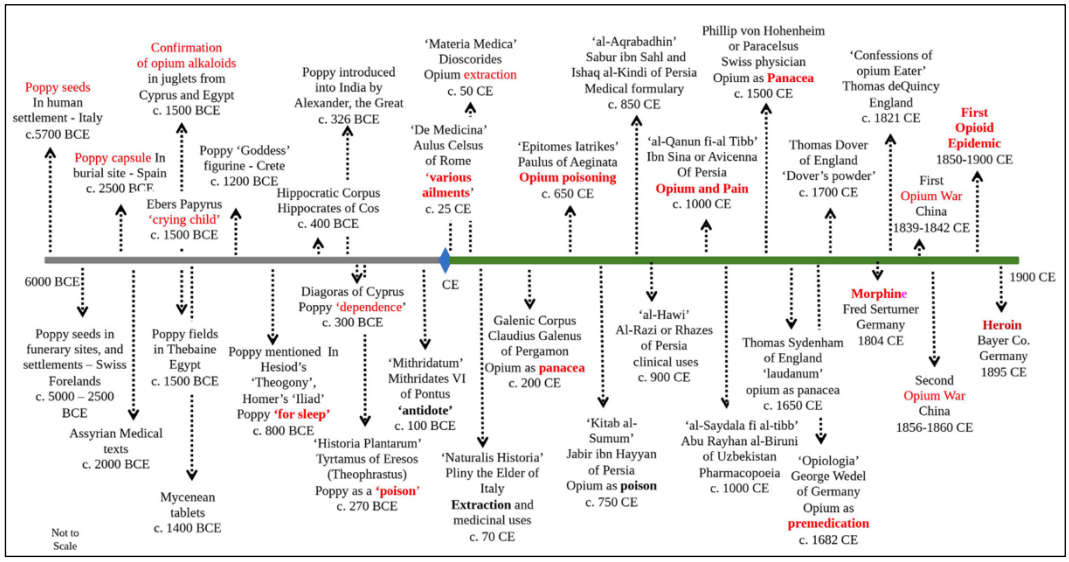


Figure 5. *Timeline of opioid use from prehistory to the beginning of the contemporary Era* (4).

3.5. CONTEMPORARY ERA

The contemporary history of opioids is interlaced with the history of wars, anesthesia technic development, cancer, and noncancer chronic pain (56).

Oral and intravenous use of morphine intensified during major wars of the last two centuries, starting with the American Civil War and arriving at the Vietnam War in the second half of the 20th century. Opium and its derivatives were used as major analgesics both in and outside the battlefield, increasing addiction among those returning from these wars (57).

The strong development of surgical procedures and anesthetic techniques resulted in the extensive medical use of morphine and opioids in anesthesia and postoperative pain. In 1959, “neuroleptanalgesia” was catching on using a combination of short-acting opioids with neuroleptic medications (58). For cardiovascular surgery purposes, in 1969, Lowenstein introduced a high-dose morphine technique for anesthetic induction by continuous infusion (59).

In 1979, spinal epidural injection of morphine was first tried for acute and chronic pain, being found to produce superior analgesia with lower doses in postoperative and obstetric analgesia, laying the foundations for continuous infusion via an epidural catheter from an implantable pump for chronic pain (60).

During the second half of the 20th century, the clinical use of opioids was limited to anesthesia and cancer-related pain. The limited use of opioids in clinical practice was reviewed in the 1970s and 1980s. A medical opinion brief that appeared in the New England Journal of Medicine reported that opioid habituation was rare in treated patients (61). The brief report was cited more than 600 times over the next 40 years to justify large-scale opioid administration (62).

The World Health Organization developed guidelines in 1986 to regulate cancer pain management. The guidelines were developed in 3 steps: non-opioid analgesics in step 1, weak opioids in step 2, and strong opioids in step 3 (63).

In the same year, however, an article based on the observation of a minor patient group extended the use of opioids to non-cancer chronic pain, often cited as evidence for the use of opioids in chronic noncancer pain (64). **This was the basis for a new epidemic wave of opioids since the emergence of heroin**.

In the following years, experienced pain researchers, as well as scientific societies, supported the use of opioids, so Paracelsus' philosophy of opioids as a panacea was resurrected.

In the 1990s, the aggressive marketing strategy of some pharmaceutical firms, such as Purdue Pharma (which introduced Oxycontin, controlled-release oxycodone), influenced physicians and medical societies, playing a pivotal role in the development of this crisis (65). Later, numerous adverse effects, including higher tolerance, accidents, emergency room (ER) visits, respiratory failures, cardiac risk, neonatal abstinence syndrome, addiction, and unintentional overdose, were reported. However, they were initially underestimated (56).

Subsequently, several prescription guidelines have been introduced to help healthcare providers evaluate risk and monitor opioid therapy. While abuse and deaths from prescription opioids decreased, chronic users deviated to other street drugs, like heroin, starting in 2010, and illicit synthetic opioids, such as fentanyl, since 2013 (66).

Data from the Centers for Disease Control and Prevention show that the number of people who died in the US from a drug overdose in 2021 was over six times the number in 1999. **The number of drug overdose deaths increased by more than 16% from 2020 to 2021.** Over 75% of the nearly 107,000 drug overdose deaths in 2021 involved an opioid. From 2020 to 2021, opioid-involved death rates increased by over 15%, prescription opioid-involved death rates remained the same, heroin-involved death rates decreased by nearly 32%, and synthetic opioid-involved death rates (excluding methadone) increased by over 22% (Figures 6 and 7) (67).

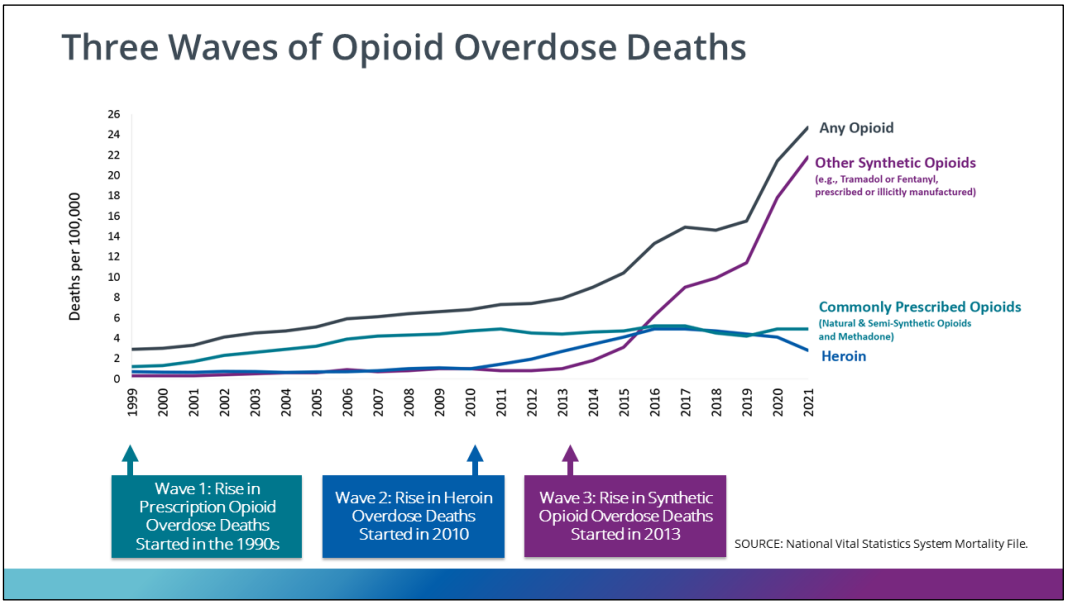


Figure 6. *Waves of opioid overdose deaths in U.S.* (67).

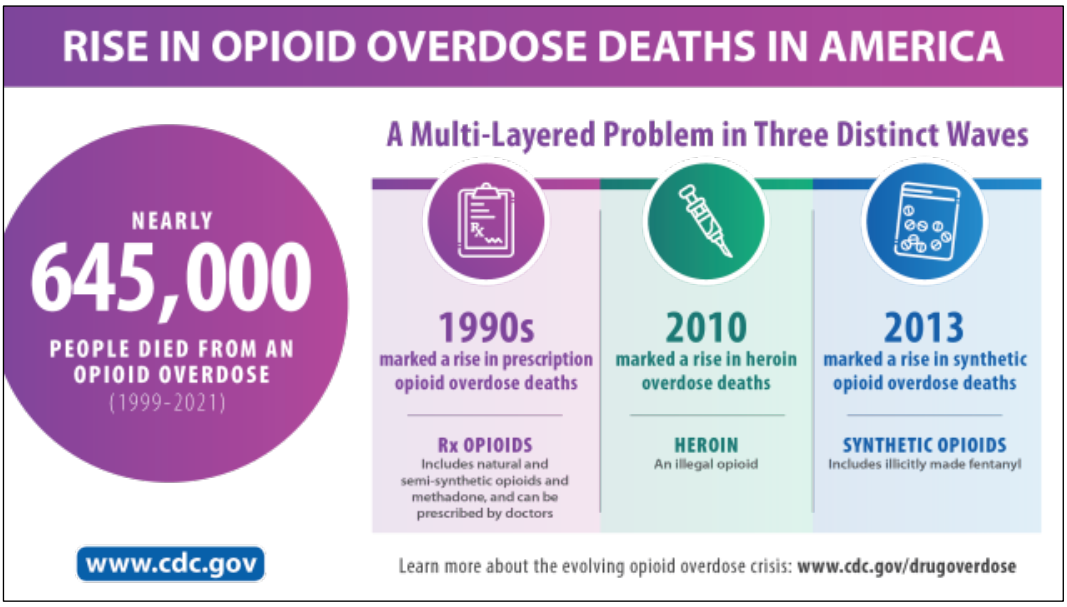


Figure 7. *The three waves of opioid overdose deaths in the U.S.* (67).

This rise in opioid overdose deaths can be outlined in three distinct waves.

The first wave began with increased prescribing of opioids in the 1990s, with overdose deaths involving prescription opioids (natural and semi-synthetic opioids and methadone) rising since at least 1999 (68).

The second wave began in 2010, with rapid increases in overdose deaths involving heroin (69).

The third wave began in 2013, with significant increases in overdose deaths involving synthetic opioids, particularly those involving illicitly manufactured

fentanyl (70–72). The market for illicitly manufactured fentanyl continues to change, and it can be found in combination with heroin, counterfeit pills, and cocaine(73).

Regarding synthetic opioids in 2021, nearly 71,000 drug overdose deaths involving synthetic opioids (other than methadone) occurred in the United States. Synthetic opioid-involved death rates increased by over 22% from 2020 to 2021 and accounted for nearly 88% of all opioid-involved deaths in 2021 (74).

The increases in synthetic opioid-related deaths are being driven by increases in fentanyl-related overdose deaths, and the source of the fentanyl is more likely to be illegally made than pharmaceutical (75).

Fentanyl analogs are also present across illegal markets, such as acetyl fentanyl, furanyl fentanyl, and carfentanil, which are similar in chemical structure to fentanyl and even much more potent than fentanyl. Carfentanil, the most potent fentanyl analog detected in the U.S. so far, for example, is estimated to be 10,000 times more potent than morphine (76).

4. HAIR TESTING AS OBJECTIVE TOOL TO INVESTIGATE RECURRENT DRUGS CONSUMPTION/EXPOSURE

4.1. BIOLOGICAL MATRICES IN POSTMORTEM FORENSIC TOXICOLOGY

While antemortem samples employed for toxicological analysis include blood, urine, sweat, oral fluid, and hair, in postmortem forensic toxicology, the samples most used are blood, urine, and vitreous humor. However, any fluid or tissue may be virtually available during autopsy.

4.1.1. Blood

Interpretations based on the analytical results obtained from blood samples are more significant than those of other samples. Therefore, when available, blood is the most used sample in postmortem forensic toxicology. However, "*cadaveric blood*" collected during autopsy is usually clotted or hemolyzed to a varying extent, not to mention the degree of dehydration to which the corpses are subjected.

The use of blood in screening analytical procedures has become more diffused with the availability of advanced methods of analysis, such as LC-MS/MS, which facilitate the detection of many analytes and their metabolites, carrying out a single analysis. The main disadvantage of using blood in screening analysis is that it may require several preanalytical operations, such as extractions. On the contrary, other samples, such as urine, may not require any such preparation. In addition, some analytes, such as cocaine and their metabolites, are measurable for short periods of time in blood than in urine and hair. For these reasons, urine or hair is often a better choice for screening than blood (77).

The main advantage of postmortem blood analysis is the presence of extensive databases of drug concentrations collected in postmortem cases correlated with the effects of those concentrations, generally categorized as therapeutic, toxic, and lethal, making the basis of forensic interpretations (78)

Another point of discussion is the collection site. This is due to the possible wide range of analytes differences in concentration between different collection points. This dissimilar distribution of analytes in blood samples collected from different sites may be related to either the lack of distribution equilibrium before death or

postmortem redistribution. Nevertheless, it is widely accepted by the forensic community that blood collected on the peripheral site is the most reliable indicator of antemortem analyte concentration (79).

Sometimes, forensic pathologists collect intracranial, subdural, or epidural hematomas. This procedure may be valuable if the subject survives several hours after the traumatic incident because drug concentrations often decrease at a slower rate than blood in general circulation (80).

4.1.2. Urine

Urine samples have several advantages in postmortem forensic toxicology, such as making them the most widely used analytical samples in forensic toxicology laboratories (81).

The advantages of urine samples include: i. numerous drugs and their metabolites are excreted and detectable in urine; ii. the urine concentrations of drugs and their metabolites are frequently greater than in blood; iii. the analytes may be detected in a window ranging from hours and days after the last use. In addition, given that urine is composed primarily of water, generally, no pre-analytical treatment is needed, and simple analysis methods may be employed, such as color tests and immunoassay.

However, urine can be obtained in only approximately 50% of postmortem cases (82). Some authors have suggested the use of bladder washing in those cases, given the low detection limits of analytical methods (83).

The main problem of urine analysis is that the urine concentration of analytes cannot be correlated with blood concentration and/or biological effects of drugs detected.

4.1.3 Vitreous humor

Vitreous humor, also known as the vitreous body or vitreous, is an inert, transparent, colorless, viscoelastic, hydrophilic gel contained between the crystalline lens and the retina. The anterior and posterior chambers of the eye contain aqueous fluids, aqueous humor, and vitreous humor, respectively. Its volume is approximately 4–5 mL, about two-thirds the volume of the entire eyeball. The vitreous humor matrix is almost entirely represented by water (99 %); the rest consists of collagen fibers,

hyaluronic acid (the main contributor to the viscosity), hyalocytes, inorganic salts, and organic compounds.

In forensic toxicology, vitreous humor is a useful alternative matrix because it shows a detection window similar to that of blood. In addition, it is relatively isolated from other body compartments/fluids due to its limited vascularization and is less affected by putrefaction, being easy to sample (84).

Most compounds of forensic interest are detected from vitreous humor, and those compounds within the ocular globe tend to be stable if certain storage conditions are warranted. Regarding preanalytical treatment, no more preparation is required than this necessary to ensure "cleanliness" (85).

Similar to urine, vitreous humor concentrations do not correlate with those of blood for tested analytes, making any quantitative interpretation impossible but giving importance to this matrix in the screening field.

4.1.4. Bile

Bile has gained interest in postmortem forensic toxicology since drug concentrations are often higher than in blood. Bile has both lipophilic and hydrophilic features, being surfactant with an aqueous component. As such, it is an excretion pathway for both neutral and ionized substances (86).

Bile has, therefore, assumed an essential role in screening investigations since the wide distribution of drugs in this matrix allows for identification even of substances absent in blood (87).

The disadvantage of using bile is the pre-analytical extraction, which is complicated due to the presence of fatty and bile acids.

4.1.6 Gastric content

Gastric contents are valuable samples for detecting recently ingested drugs because the concentrations of analytes may be in milligrams or grams in comparison to the microgram or nanogram concentrations found in blood and urine.

This is especially important in cases where there is evidence of intoxication by oral intake, in the case of body packers, or in the case of drug ingestion to avoid arrest (82).

4.1.7 Liver

The liver is a site of interest in forensic toxicology since it is the main organ of drug metabolism. As a result, many drugs and metabolites are found in the liver at higher concentrations than blood and urine. The criticalness of the liver sampling is that drugs distribute heterogeneously, given that the liver is susceptible to postmortem redistribution from the stomach, located nearby (88).

4.1.8 Brain

The brain has never been a preferred site for forensic toxicology investigations. This is due to the high concentration of fat that requires more problematic preanalytical procedures than other samples. In addition, analytical interpretations cannot be made outside of qualitative evaluation. An advantage of brain is that some compounds, such as 6-MAM, are less prone to degradation than blood, probably because of lower esterase activity (89).

4.1.9 Lung

The lungs are a repository for many drugs being richly vascularized. For this, lipophilic drugs can readily diffuse, and ionized drugs can rely on specialized transporters. Concentrations are often higher than those in the blood, making the samples useful for screening investigation. Lung tissue should be sampled at the apical lobes in order to avoid postmortem diffusion phenomena from the stomach to the lower lobes (90).

4.1.10 Adipose tissue

Adipose tissue is not the first-choice site for sampling in forensic toxicology, even if it is a repository for lipophilic drugs (THC, propofol, thiopental). Unfortunately, to date, there are no guides regarding the sampling site, although the abdominal layer seems to be the site of choice. Adipose tissue should be analyzed to investigate the injection site, given the high concentration of xenobiotics in the surrounding area (91).

4.1.11. Skeletal muscle

Skeletal muscle shows several advantages as a postmortem sample for forensic toxicology purposes, such as its availability in large quantities, its presence even in cases of putrefaction, trauma, and burning, its feature to be less prone to decomposition phenomena, and its presence in sites away from drugs reservoirs in liver, lung, or gastric content. One limitation of skeletal muscle is that its homogenization, in the preanalytical phase, is complex (92).

Even if, in most cases, drugs detected in blood are also detected in muscle tissue, sporadically, xenobiotics detected in blood are absent in muscle (93).

4.1.12. Bone and bone marrow

Bone may be a helpful specimen in all cases of extensive decomposition, burnt, or body fragmentation, where only a few tissues may be available. However, its usefulness should be considered only for the indication of exposure (77).

The intra- and inter-bone distribution of xenobiotics has been described to be variable mainly due to the different composition of the tissue (cortical bone, dense and compact *vs* cancellous bone, spongy and porous) (94).

Bone marrow can be divided into two types: red marrow, implied in hematopoiesis and located in the lower skull, vertebrae, shoulder, pelvis, ribs and sternum, and yellow marrow, which consists of fat cells located mainly in long bones.

A vast difference in the concentration of xenobiotics detected from different types of bone marrow has been described, probably due to the subject's age, postmortem redistribution, and postmortem degradation or synthesis (95).

4.1.13 Nails

In recent years, keratinized tissue such as nails and hair (described in the following chapters) have received significant attention, given that they have a wider detection window than blood and urine and could provide some interesting retrospective information.

Many drugs, such as opioids, cannabinoids, cocaine and its metabolites, benzodiazepines, and methadone, have been detected in nails since the first detection of amphetamines in 1984 (96).

Fingernails and toenails grow at a predictable rate of approximately 3-5 mm/month and 1 mm/month, respectively, possibly allowing for an estimation of the chronology of drug use by analyzing the longitudinal distribution of xenobiotics (90).

In conclusion, in postmortem forensic toxicologic analysis, no sample is "ideal", given that each sample has advantages and disadvantages associated with its use, as summarized in Table 3.

Sample	Advantages	Disadvantages
Blood	- Large database dose-effect correlation - Multiple collection sites - Multiple analysis methods	- Concentrations subject to postmortem alteration - Concentration influence of collection site - Wide interpersonal concentration-effect correlation - Influence of clotting and hemolysis
Urine	- Drugs detected in high concentrations - Wide detection window - Ease of analysis	- No correlation with blood concentrations - Often not available
Vitreous humor	- Less influenced by putrefaction - Ease of analysis	- Correlation with blood concentration only after establishment of equilibrium
Liver	- Drugs detected in high concentrations	- Poor correlation with drugs effect - Extensiveness of sample preparation
Brain	- Target of many drugs	- Extensiveness of sample preparation - Difficult analysis - Poor correlation with drugs effect

Table 3. *Summary of advantages and disadvantages of some of the different samples in forensic toxicology.*

4.2 HAIR TESTING

4.2.1. Anatomy and physiology of Hair

Hair covers almost the entire surface of the human body except for the outer surface of the lips, palms of the hands, soles of the feet, and some parts of the external genitalia (97). The primary function of body hair is to protect the skin surface from injury and help regulate the body temperature.

Body hair consists of two elements: the root and the shaft.

The root is located within the hair follicle, which is an introflexion of the epidermis located 3-4 mm below the skin surface, shaped like a sac with a deep dilatation called bulb. The follicle, hair, and sebaceous gland are fused anatomically and functionally into the so-called pilosebaceous complex.

The hair follicle includes three structures:

- *The outer root sheath*, composed of a single row of keratinocytes, creates a bulge area at the base of the erector pili muscle and is considered to be the source of the stem cells;
- *The inner root sheath*, present only in the deep portion, can be further divided into three layers, which, from the outermost to the innermost (relative to the hair), are:
    - Henle's layer: a single layer of cubical cells with clear flattened nuclei;
    - Huxley's layer: one or two layers of horny, flattened, nucleated cells;
    - The cuticle of the internal sheath which is formed by keratin material.

    The inner root sheath produces binding material and directs hair growth upward.
- *The root bulb* includes a more profound, mitotically active region and a superficial region, the keratinization center.

The dermal papilla, located at the base of the root bulb, provides blood supply.

Apocrine and eccrine glands secrete directly into the follicle. While apocrine glands are located only in the axilla and pubic region and secrete into the follicle, eccrine sweat glands secrete near the exit of the follicle (98,99).

The hair shaft, the part of the hair above the skin's surface, includes an external part called *cuticle*, an underlying area called *cortex*, and an internal area called *medulla*.

The cuticle consists of overlapping layers of keratin cells (6 to 10, depending on the thickness of the hair). Various factors can damage the cuticle, including exposure

to light or heat, chemical treatments such as bleaching and perming, and physical damage. The internal structure of the stem, the cortical area, contains protein chains (keratin) constituting the major component of the stem, along with cells in which melanin, the primary hair pigment, is located. The innermost region of the hair is the medulla, which can be continuous along the entire length of the hair, discontinuous or completely absent (100).

The microscopic structure of the hair is illustrated in Figure 8.

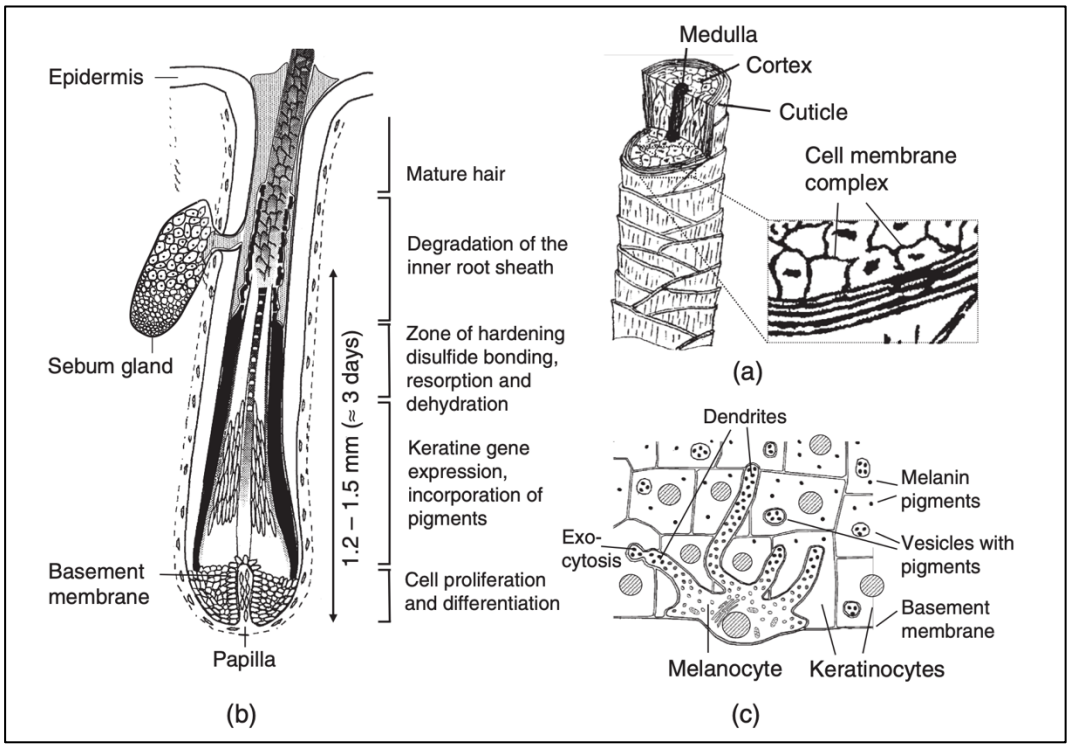


Figure 8. *Structure of hair shaft (a), pilosebaceous complex structure (b), and formation of pigment by melanocytes (c)* (101)

Human hair consists of approximately 65-95% protein, 15-35% water, and 1-9% lipids; pigmentation of the hair shaft represents 0.1-5% of the total mass of the hair (100). The differences in hair color are due to the variation in the type and quantity of melanin. Follicular melanogenesis (also called pigment formation) occurs in organelles named melanosomes, which are present within specialized cells, the melanocytes (102). This process occurs exclusively within the hair follicle and is regulated by enzymes, receptors, and proteins during the anagen phase of active hair growth (103). Four types of melanin determine hair color: eumelanin, pheomelanin, and their oxidation products, oxymelanin and oxypheomelanin.

Generally, darker hair colors (black and brown) contain predominantly eumelanin, while lighter shafts are associated with more oxymelanin. Pheomelanin makes hair red (104).

Hair growth is influenced by several factors, such as age, sex, pregnancy, metabolic and genetic disorders, nutrition, and seasonal changes (105). Hair follicles are organs unique, as they continuously follow phases of activity and degeneration. All the hair follicles are formed during the prenatal period, in fact, after birth, no more production of hair follicles occurs.

The three phases of the hair follicle life cycle are recognized as anagen phase (hair production), catagen phase (involution/degeneration of the follicle lower), and the telogen phase ("resting" phase) (106).

The anagen or growth phase lasts 2 to 4 years in men and 5 to 6 in women. In healthy hair, approximately 85-90% of the hair is in this stage.

The catagen or transitional phase is the stage of progressive suspension of the vital functions of the hair, lasting about 3 weeks, the time needed for the bulb to detach to the collar and lose the internal epithelial sheath. The growth of the hair during the catagen phase is much slower.

Telogen or resting phase lasts 2/4 months, when the hair is still into the follicle, but vital activities have entirely ceased. Nourishment and oxygenation have ended, and the hair can be easily removed by modest traction because of the weak fixation on the follicle. In healthy people, approximately 9-14% of the hair is in the telogen phase (107).

The growth rate varies among the different body hair such as head, pubic, axillary, chest, arm, leg, beard (Table 4).

Hair Type	***Mean/Range (cm/month)***
Head	0.60 – 3.36
Pubic	0.60 – 0.90
Axillary	0.87 – 1.00
Beard	0.75 – 1.20
Body	0.30
Chest	0.66 – 0.96
Arm Hair	1.05

Leg Hair	0.39 – 1.05

Table 4. *Published Growth Rates for Different Human Hair Types (99).*

Most head hair growth rates reported in the literature vary from 0.6 to 1.5 cm per month, resulting in an average growth rate of 1 cm/month, acknowledged by the Society of Hair Testing (SoHT) (108).

On the other hand, the average growth rate of pubic is reported to range from 0.6 to 0.9 cm/month.

4.2.2. Mechanism of drug incorporation and drug stability

Hair testing has gained increasing attention over the years, especially for the retrospective investigation of chronic drug abuse, due to its unique ability to serve as a long-term storage site of foreign substances with a detection window of up to months and years. However, the actual mechanisms of drug or analyte incorporation and the factors affecting their stability in the hair are not yet completely understood (101).

Three mechanisms of incorporation of drugs or substances into the hair are currently recognized: passive diffusion from blood capillaries, deposition by diffusion from sebum and sweat, and external contamination [107], as shown in Figure 9.

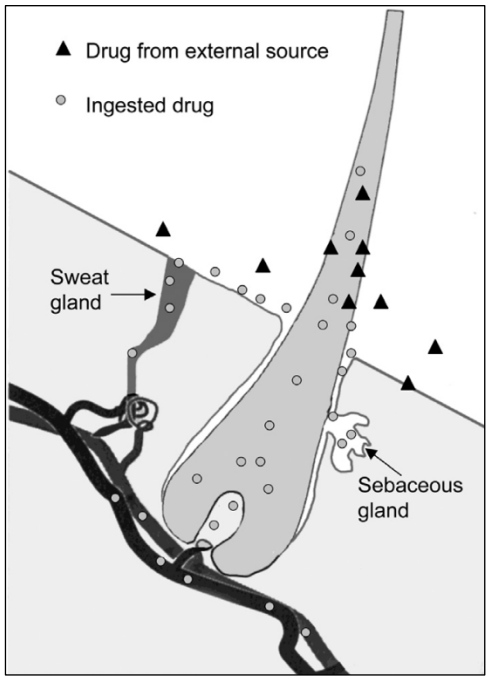


Figure 9. *Incorporation of drugs by different mechanisms.* (99)

From the structural point of view, many factors may influence the drug and analyte incorporation: pKa, structure, size, lipophilicity, protein binding capacity of the compounds, as well as the melanin content of hair (109,110).

For example, basic drugs, such as amphetamines and cocaine, are incorporated into hair to a greater extent than neutral or acidic drugs (benzodiazepines and cannabinoids) given the pH gradient from plasma, pH 7.3, and even more acidic conditions inside the hair cells (pH 3-6) which provides advantageous conditions for the incorporation of basic drugs (111).

In addition, natural hair pigmentation affects the incorporation of substances, in particular basic substances, such as cocaine, opiates, and amphetamines, which are incorporated more easily into darker hair (which contains more eumelanin, darker pigment) than lighter hair, which contain less eumelanin (112).

External contamination of the hair matrix following passive exposure to substances, in particular proximity to subjects who smoke (e.g., heroin, crack cocaine, cannabis) or manipulate drugs, should be taken into consideration, in particular, in children and infants who live in families where substance abuse is prevalent (113), as well as for the fetus if the mother continues to misuse during pregnancy (114).

Retention and stability of drugs in hair are considered good. Even if the distal cuticle in long hair becomes more susceptible to damage by mechanical stress and increasingly penetrable for drug elimination, studies focused on long hair showed the presence of drug in distal segments even years after ceased consumption (101).

However, strong cosmetic treatments like bleaching or permanent waving affect the hair structure, allowing for partial or complete loss of drug substances and risking false negative results (115).

4.2.3. Post-mortem toxicological analysis of hair

Sampling

Sampling and storing postmortem hair samples can be challenging, depending on the condition of the deceased's hair. Preferably, hair samples should be dry and collected identifying the proximal end, even if this is not possible in all cases.

The sample should be cut from the area at the back of the head, called vertex posterior, where the lowest portion of telogen hair and a relatively uniform rate of hair growth are acknowledged. The sample length and size needed varies among laboratories, depends on the analytical method, and reflects information according to the time course of drug use (116).

Bloody hairs are common in cases of road accidents or assaults, and even in the case of decomposition, heavy staining due to body fluids is regularly found. Bloody and soiled hair samples require careful decontamination procedures compared to standard washing protocols used on hair samples from living people (108).

Hair samples should be stored under dark and dry conditions at room temperature in a labeled paper envelope in order to avoid the potential extraction of lipophilic substances from the hair by plastic materials (101).

Decontamination

Decontamination is a crucial phase of hair analysis. Indeed, analytical noise/ background could produce false positive results due to hair care products, sweat, sebum, and other material residues (even biological) present on hair. In addition, washing removes external environmental contamination without compromising levels of drugs from the hair matrix (117).

However, there is no consensus regarding pre-analytical washing procedures.

Hair washing should remove external adulterants, without extracting drugs from the hair matrix. Solvents are commonly utilized, including dichloromethane, acetone, methanol, water, or sequential decontamination with different organic solvents (118). In any case, the wash procedure used can strongly affect the results of hair analysis (119).

Cutting and grinding

Before extraction, the hair sample must be pulverized or cut into small pieces. Cutting hair with scissors should obtain 1–3 mm fragments, but this procedure is time-consuming, particularly when a considerable number of samples should be analyzed. Automated grinding homogenizer and process have been developed and optimized to accelerate sample preparation, obtain more homogenous matrices (powdering), and reach a better extraction harvest. Moreover, several studies

demonstrated a significantly higher detectable concentration of a studied compound when analyzing pulverized rather than cut hair samples, leading also to lower background noise (120).

Extraction

A procedure compatible with almost all drug substances is methanol extraction (5–18 h) in an ultrasonic thermostatic bath, leading to swelling and drug liberation via diffusion due to the strong degradation of neutral and lipophilic compounds. The methanol extract can be directly injected, especially in the case of drugs at high concentrations, with the disadvantage of a high impurity level inside the system. Therefore, a secondary clean-up procedure, a liquid/liquid extraction (LLE), or solid-phase extraction (SPE) is generally recommended.

Basic drugs (cocaine and its metabolites, amphetamines, opiates, methadone) are well extracted by aqueous or phosphate buffer, whereas drugs that are stable under alkaline conditions (cannabinoids, antidepressants, neuroleptics) are extracted by aqueous NaOH (121,122).

Detection

Low drug concentration and small hair sample size can be problems for drug screening methods. Immunoassay (IA) kits may be available for a limited number of drugs (or metabolites) and be affected by a lack of sensitivity and specificity. Sensitivity should be adequate to ensure that no false negative results are reported, whereas positive results must be confirmed by chromatographic techniques.

The combination of liquid or gas chromatography coupled with mass spectrometry is currently used in hair analysis. The advantages of GC–MS include high resolution of the capillary and high specificity of electron impact ionization mass spectra, with sufficient accuracy at very low concentrations for volatile. However, samples require derivatization steps prior to GC–MS analysis. Alternatively, LC systems coupled with tandem mass spectrometry can be used, even as a valid alternative for screening methods (123).

Practical applications of postmortem hair sample analysis

Although hair samples are not a first-choice matrix in forensic postmortem toxicology, hair collection during autopsy has increased over the last few years. Even if the leading value of hair analysis in postmortem forensic toxicology is to support death diagnosis, excluding or proving chronic substance abuse, analysis of hair shaft finds many practical applications in practical postmortem forensic casework, such as giving information about pathological alterations of organs due to chronic abuse, providing evidence for tolerance in cases of opiates overdose, explaining withdrawal symptoms or contributing to the identification of an unknown corpse.

Lifestyle information regarding the deceased is not always available for the interpretation of pathological alterations observed during the autopsy and to classify the cause of death correctly. In these cases, drug findings in hair segments may be helpful, for example, regarding cases of severe cardiovascular alteration due to chronic amphetamine abuse or in cases of sudden unexplained death due to chronic use of steroids (124,125).

Regarding opioid death and interpretation of postmortem opioid concentrations, a factor of utmost importance is whether evidence could support chronic abuse or not. As a matter of fact, tolerance to opioids can increase after chronic abuse: opioid concentrations lethal for a naïve person could be harmless for long-term abusers. Hair testing, given the wide detection window, could accurately identify chronic or single opioid administration (116).

In fatal traffic or work-related accidents, hair analysis may provide essential information if the victim survived several days or if blood was not immediately collected. In case of a negative blood drug result, a positive hair result may be an indication of impairment due to withdrawal symptoms. Even negative results could be crucial in interpreting the manner and dynamics of death (116).

Hair analysis can also be used as a tool to identify sub-populations (e.g., psychiatric patients or drug abusers) prior to final identification by DNA (126).

Pharmaceutical drugs, drugs of abuse, and ethanol are often used to achieve personal criminal profit (i.e., criminal assault, child abuse, robbery) nowadays. In these cases, hair testing can identify incidents in which people try to govern other behavior illegally (127).

5. EXPERIMENTAL PART

5.1. INTRODUCTION AND AIM

Nowadays fentanyl and its derivatives, including both pharmaceutical and non-pharmaceutical fentanyls, represent the largest group among synthetic opioids (14). Fentanyls are highly lethal even at low doses, with nearly sudden deaths occurring due to decreased respiratory drive and cardiac arrest or, rarely, an anaphylactoid reaction. Fentanyls-related deaths have been reported in recreational use/abuse and therapeutic practice. Furthermore, fatalities from tampering with pharmaceutical products so that they can be smoked, snorted, injected, or taken orally have been reported (13).

In the United States, from 2013 to 2019, the age-adjusted rate of deaths involving synthetic opioids increased by 1,040% (128). Of them, about 50% was caused by fentanyl or derivatives alone or in association with other drugs (72). Moreover, in 2021, there were more than 80,000 opioid overdose deaths in US. Most of those deaths were mainly driven by synthetic opioids (primarily fentanyls) (24).

In addition, since the mid-2010s, an increasing number of unintentional overdose deaths involving opiates and/or fentanyl showed the presence as adulterant of xylazine, a veterinary drug able to worsen hypotension, central nervous system, and respiratory depression caused by opiates (129,130).

On the basis of above evidence, it looks necessary to monitor the diffusion of fentanyls and adulterants among the population to understand their role in the overdose-risk environment and possibly prevent fentanyls overdose fatalities.

In this frame, hair testing can provide essential information regarding previous intake/exposure to xenobiotics. The use of hair analysis has gained attention over the years, especially for the retrospective investigation of chronic drug abuse and the unique ability of this matrix to serve as a long-term storage site for xenobiotics (131).

The present study has been carried out in collaboration with the Division of Forensics (Dept of Pathology, University of Alabama at Birmingham (UAB), AL, U.S.) discusses the forensic toxicological value of the results of fentanyl, fentanyl analogs, and xylazine determination in hair from 250 post-mortem cases with

different causes and manners of deaths also to verify the usefulness of extensive hair analysis in forensic pathology and epidemiological field.

The analytical procedure of hair testing was developed and validated by Prof. Rossella Gottardo and Dr. Marco Ballotari of the laboratory of Forensic Toxicology of the Dept. of Diagnostics and Public Health of the University of Verona.

5.2. MATERIAL AND METHODS

5.2.1. Hair samples

Hair samples (n = 250) were obtained from all postmortem cases under the jurisdiction of the Jefferson County Coroner/Medical Examiner Office from January 2023 to March 2023.

Hair collection was performed by me during the research scholar period spent at UAB following the SoHT guidelines (108).

Briefly, hair from scalp (n=191) and, when they were not available, from pubis (n=59), were cut by scissors as close as possible to the skin. In case of head hair, the proximal end was flagged with a piece of aluminum foil. After the collection, the samples were put in paper envelopes and stored at room temperature in their original packing until the analysis, which was performed at laboratory of Forensic Toxicology of the Dept. of Diagnostics and Public Health of the University of Verona.

The post-mortem cases included drug overdose deaths, homicides, suicides, traffic accidents, and natural deaths. The biological samples (blood, urine, vitreous humor, as well as bile or tissues in selected cases) of these cases underwent toxicological analysis at the UAB laboratory for the most common drugs of abuse through immunoassay screening tests on urine or bile and GC-MS confirmation analysis on blood or tissues.

The cases were anonymized by using an alphanumerical code.

5.2.2. Standard solutions and reagents

Fentanyl, norfentanyl, ß-hydroxy fentanyl, acetyl fentanyl, acetyl norfentanyl, despropionyl para-fluorofentanyl, 4-aminophenyl-1-phenethylpiperidine (4-ANPP), carfentanil, norcarfentanil, ocfentanil, furanyl fentanyl, U-47700 were obtained from Comedical (Trento, Italy) as certified standard methanolic solutions

at concentrations between 0.02 an 0.05 mg/mL, while fentanyl-D5, used as internal standard (IS), was purchased from Cerilliant (Round Rock, TX, USA). Xylazine and xylazine-D6 were purchased as powder (10-100 mg) from Merck (Darmstadt, Germany), and a stock solution was obtained by diluting the powder in methanol to a final concentration of 1 mg/mL. LC–MS grade, methanol, acetonitrile, hydrochloric acid (HCl), formic acid were purchased from VWR International (Radnor, PA, USA).

5.2.3. Hair sample preparation and extraction procedure

Hair samples were washed twice with the non-ionic aqueous solution 3% Tween (*w/v*) and taken to dryness at 60°C. About 50 mg were then manually cut into small segments with scissors and spiked with 20 µL of a mixture of the two deuterated standards to a final concentration of 200 pg/mg for fentanyl-D5 and 5 ng/mg for xylazine-D6, added with 1 ml of 0.1M HCl, and maintained overnight at 45 ◦C.

The digested samples were then neutralized with 230 µL of 0.1M NaOH and centrifuged at 4000 g for 5 min. The supernatant was separated and eluted through a HyperSep Verify-CX solid phase extraction (SPE, 130 mg) cartridge (Restek, France, Glastron, Inc.). The cartridges were activated with 2 mL methanol and equilibrated with 2 mL phosphate buffer (pH 9) prior to the sample transfer, then sequentially washed with 2 mL bidistilled water, 3 mL 0.1M HCl, and 3.5 mL methanol. The analytes were then eluted from the column with 2 mL dichloromethane-isopropanol mixture (8:2 *v/v*) with 2% ammonia solution.

The eluate was dried under nitrogen stream at 60°C and reconstituted in 50 µL of solvent A, prior to the injection into the LC–MS/MS system.

5.2.4. Instrumentation and analytical conditions

Separations were performed by using a model I-Class ACQUITY UPLC system (Waters, Milford, MA, USA) provided with a Restek Force Biphenyl 2.1 × 50 mm, 1.8 µm and an UltraShield UHPLC PreColumn Filter 0.2um frit kept at 45°C. Mobile phase A was composed of water and formic acid 0.1%, while mobile phase B consisted of pure acetonitrile. The injected samples were eluted with a linear gradient from 5 to 70% of solvent B, lasting 5 min. The column was then washed with 90% of phase B and then the starting conditions were restored in 2 min and

kept for 3 min to allow system re-equilibration. The flow rate was set at 0.4 mL/min. An injection volume of 2 µL was used in all experiments.

The liquid chromatograph was coupled with an API 6500 QTrap mass spectrometer equipped with an IonDrive Turbo Spray V ion source (AB Sciex, Framingham, MA, USA). The instrument was operated in the positive-ion mode with the following optimized parameters: curtain gas (CUR, nitrogen): 30 L/h; IonSpray Voltage (IS): 5500 V; source temperature (TEM): 600°C; Ion Source Gas 1 and Gas 2 (GS1-GS2, air): 60 and 70 L/h, respectively.

The analyses were performed in multiple reaction monitoring (MRM) mode using optimized transitions and mass parameters reported in Table 5.

Analyte	Precursor ion (Da)	Daughter ion (Da)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention time (min)
Fentanyl	337.2	**188.1**	44	6	31	12	2.85
		105.0	50	6	46	8	
Norfentanyl	233.0	**84.1**	48	7	25	9	1.75
		151.1	30	7	16	9	
ß-hydroxy fentanyl	353.0	**335.1**	10	4	25	11	2.57
		186.1	10	4	36	17	
Acetyl fentanyl	323.0	**188.1**	16	4	31	11	2.54
		105.0	50	4	41	8	
Acetyl norfentanyl	219.0	**84.1**	41	8	24	9	1.38
		105.0	21	8	21	10	
Despropionyl para-fluorofentany	299.1	**188.1**	16	5	25	11	2.91
		105.0	10	5	40	14	
4-ANPP	280.9	**188.1**	19	6	25	19	2.84
		105.0	19	6	41	15	
Carfentanil	395.1	**335.1**	10	6	25	9	3.08

		363.4	27	6	19	9	
Norcarfentanil	290.9	**231.2**	26	4	18	16	1.92
		259.1	14	4	20	12	
Ocfentanil	371.2	**188.1**	20	5	31	13	2.53
		105.0	28	5	55	15	
Furanyl fentanyl	375.2	**188.3**	38	4	50	20	3.05
		105.0	33	4	20	9	
U-44700	330.2	**286.0**	46	5	24	24	2.72
		206.0	31	5	30	15	
Xylazine	221.0	**90.0**	33	10	30	13	2.06
		148.2	22	10	51	11	
Fentanyl-D5 (IS)	342.5	**188.1**	44	6	31	12	2.84
		105.0	50	6	46	8	
Xylazine-D6 (IS)	226.0	**90.0**	34	5	31	11	2.04
		153.2	56	5	42	10	

Table 5. *Multiple-reaction monitoring (MRMs) transitions, optimized mass parameters and retention times of the studied compounds and their internal standards using the ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) system (quantifier transitions are in bold)*

To assess the precursor ions and relative product ion transitions of the analytes, standard solutions of each compound at a concentration of 1 µg/mL were infused directly in the ionization source. The mass parameters were fine-tuned in order to achieve the maximum sensitivity by optimizing the declustering potential (DP), the entrance potential (EP), the collision energy (CE), and the cell exit potential (CXP). MRM mode was used for the quantitative determination of the target compounds. The optimized analytical conditions allowed for a good chromatographic separation of the analytes obtaining their elution in less than 5 minutes per run.

A total ion chromatogram for each analyte at a concentration of 1000 pg/mg was reported in Figure 10.

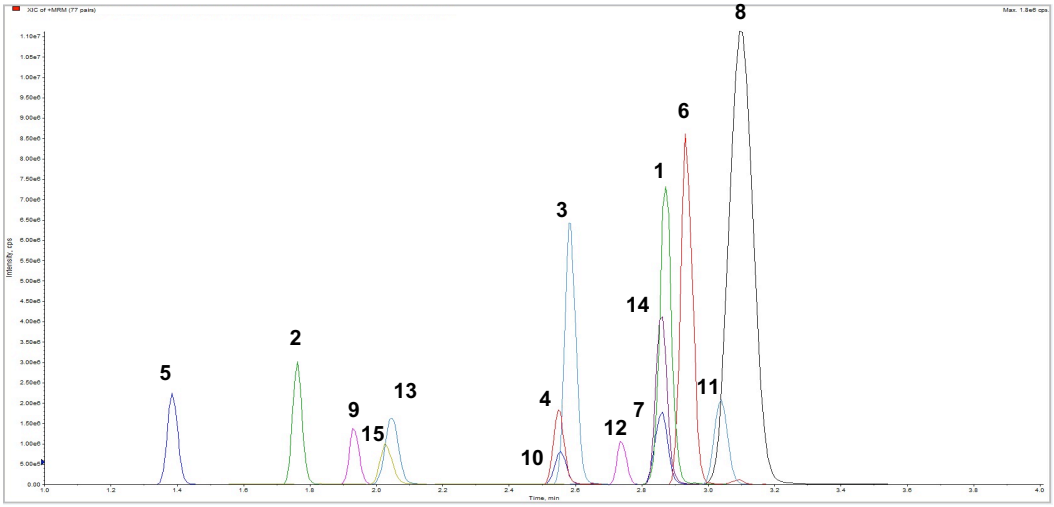


Figure 10. *Extract ion chromatograms of a mixture of the studied analytes at a concentration of 1000 pg/mg (1: fentanyl; 2: norfentanyl; 3: ß-hydroxy fentanyl; 4: acetyl fentanyl; 5: acetyl norfentanyl; 6: despropionyl para-fluorofentanyl; 7: 4-ANPP; 8: carfentanil; 9: norcarfentanil; 10: ocfentanil; 11: furanyl fentanyl; 12: U-44700; 13: xylazine) and relative ISs (14: fentanyl-D5; 15: xylazine-D6)*

5.2.5. Method validation

The analytical method was validated according to international guidelines for the quantitative analysis in forensic toxicology (132,133) in terms of interferences (selectivity), linearity, sensitivity (limit of detection [LOD] and limit of quantification [LOQ]), intra- and inter-day precision, accuracy (bias), matrix effect (ME) and extraction recovery (RE), carryover, and processed sample stability.

Selectivity was assessed by testing 20 different drug-free (negative) hair samples with and without the internal standards, to evaluate interferences from the matrix or from the internal standards at the retention times of any of the transitions selected for the analysis of the compounds of interest.

To assess the *linearity*, solutions of the analytes at different concentrations have been prepared and injected on five different days in the range of the expected concentrations. At least six different non-zero levels and five replicates per concentration in separate runs were tested. The calibration curves for the analytes

were created by weighted regression analysis of the normalized peak areas (analyte area/IS area).

The *limit of detection (LOD)* was assessed over multiple runs, and expressed as the analyte concentrations associated with chromatographic peaks with a S/N ≥ 3.

The *limit of quantification (LOQ)* for all the studied compounds was calculated as the lowest amount of analyte that can be quantitatively determined with an acceptable (< 20%) precision and accuracy (bias), corresponding to the lowest non-zero calibrators.

Intra- and interday precision were calculated in five different days using replicates of three chosen QC levels (low, medium, high) for each compound. The intraday assessments were expressed in terms of percent relative standard deviation (intra- or interday RSD%), and considered acceptable below 20%. The inaccuracy of the method was expressed as intra- and interday bias, or percentage deviation from the expected value, within ±15%.

The *enhancement or suppression of analyte ionization* was studied following the post-extraction addition approach (134). To assess the matrix effect (ME), different sets of samples were prepared, and the analyte peak areas of neat standards (set A) were compared to matrix samples fortified with neat standards after extraction (set B) or processing (set C). Matrix effect was analyzed in three replicates and evaluated at three concentration levels. In addition, the extraction recovery (RE) was also determined and calculated as following reported: ME (%) = (B/A) × 100; RE (%) = (C/B) × 100. For the evaluation of analyte carryover, different blank matrix samples (n=10) were analyzed immediately after a high-concentration sample.

The processed *sample stability* was evaluated by reinjecting every 2 h low and high QCs over an entire analysis day. The average responses at each time interval were compared to the time zero responses, and the samples were considered stable within ±25%.

No *interferences* from the matrix or from the internal standards at the retention times of any of the studied compounds were observed.

Linearity was assessed in the range of 3-10,000 pg/mg for fentanyl, norfentanyl, ß-hydroxy fentanyl, and between 9-10,000 pg/mg for 4-ANPP; in the range of 3-1,200 pg/mg for acetyl fentanyl, acetyl norfentanyl, carfentanil, norcarfentanil, ocfentanil,

furanyl fentanyl, and U-47700; between 18.8-1,200 pg/mg for despropionyl para-fluorofentanyl. Linearity for xylazine was evaluated in the range of 62.5-10,000 pg/mg. The calibration model was accomplished via a linear, non-forced, and 1/x2 model for all the analytes.

The *LOQs* for all the studied compounds were determined using to the lowest non-zero calibrators, while the LODs were calculated to be 0.9 pg/mg for fentanyl, norfentanyl, ß-hydroxy fentanyl, acetyl fentanyl, acetyl norfentanyl, carfentanil, norcarfentanil, ocfentanil, furanyl fentanyl, and U-47700; 2.7 pg/mg for 4-ANPP, and 5.6 pg/mg for despropionyl para-fluorofentanyl; 18.8 pg/mg for xylazine (Table 6).

Intra- and interday precision and bias for all the tested levels over the 5-day period were within the acceptable criteria of ±15%.

The mean *matrix effect* of the analytes tested at two different concentration levels in triplicates ranged within the acceptable range of 75-125%, and >50% for the extraction recoveries. Deuterated internal standards were employed to compensate for these evaluations.

No evidence of *carryover* was shown by injecting blank samples after high concentration levels, and good *sample stability* was determined for the low, and high QCs (within ±25% of target concentration).

Analyte	LOD (pg/mg)	LOQ (pg/mg)
Fentanyl	0.9	3
Norfentanyl	0.9	3
ß-hydroxy fentanyl	0.9	3
Acetyl fentanyl	0.9	3
Acetyl norfentanyl	0.9	3
Despropionyl para-fluorofentanyl	5.6	18.8
4-ANPP	2.7	9
Carfentanil	0.9	3
Norcarfentanil	0.9	3
Ocfentanil	0.9	3
Furanyl fentanyl	0.9	3

U-44700	0.9	3
Xylazine	18.8	62.5

Table 6. *LODs, and LOQs for each target analyte detected.*

5.2.6. Data analysis

The *geospatial distribution* of the population was studied by converting home addresses into latitude and longitude coordinates and entering data in QGIS software (Quantum Geographic Information System), creating heat maps of Jefferson County which represented the magnitude of events.

Fisher's exact test was used to evaluate any association between fentanyl hair-positivity and the means of death.

Multivariable logistic regression was applied to identify different risk factors for fentanyl hair positivity.

Nonparametric Kruskal-Wallis tests was used to evaluate the fentanyl blood concentrations at the time of death on the basis of the different hair fentanyl concentrations. For multiple comparisons, *Dunn test* was applied

All statistical analyses were performed using Stata software version 18.0, StataCorp, College Station, TX.

5.3. RESULTS AND DISCUSSION

5.3.1. Study population

From January to March 2023, 250 cases fell under the jurisdiction of the Jefferson County Coroner/Medical Examiner Office. After the Coroner's investigation, Medical Examiners performed 180 autopsies (72%) and 70 external examinations (28%).

The population included 177 males (70.8%) and 73 females (29.2%), with ages ranging from 3 weeks to 92 years old (mean = 48.85 yo; SD = 17.3 yo). The age distribution is shown in Figure 11.

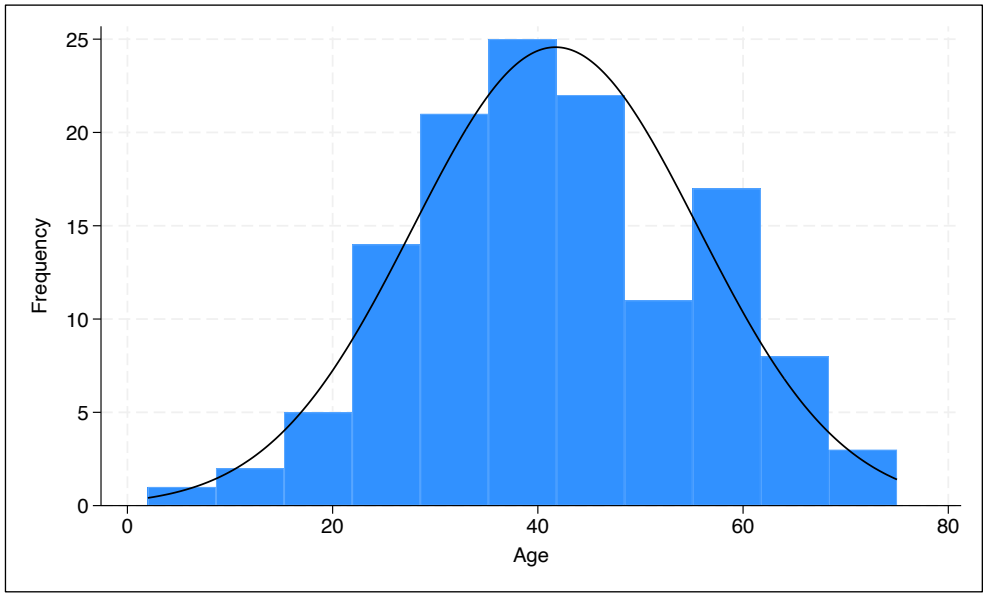


Figure 11. *Age distribution in the studied population.*

Geographical distribution of the cases on the basis of home address is shown in Figure 12.

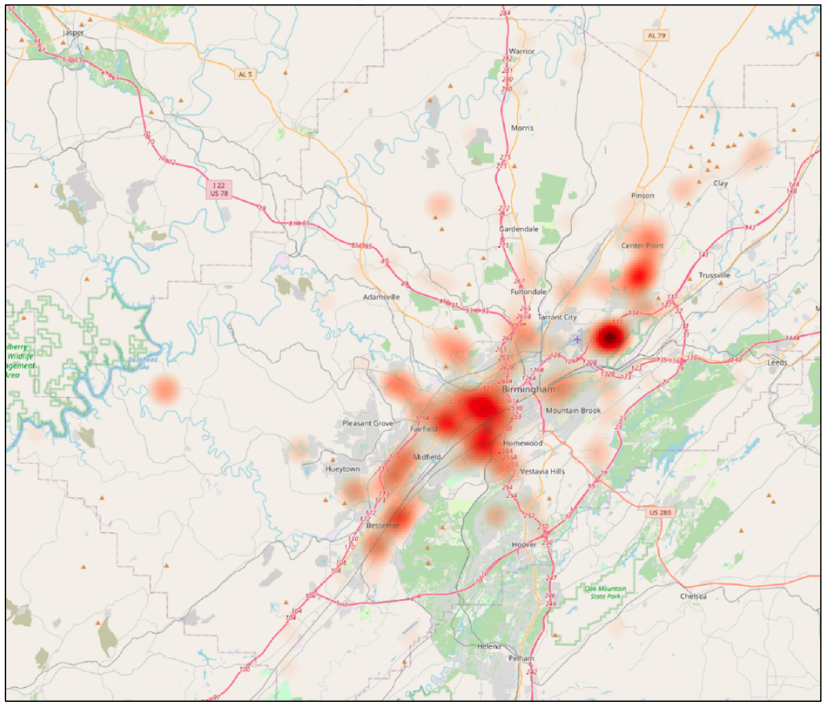
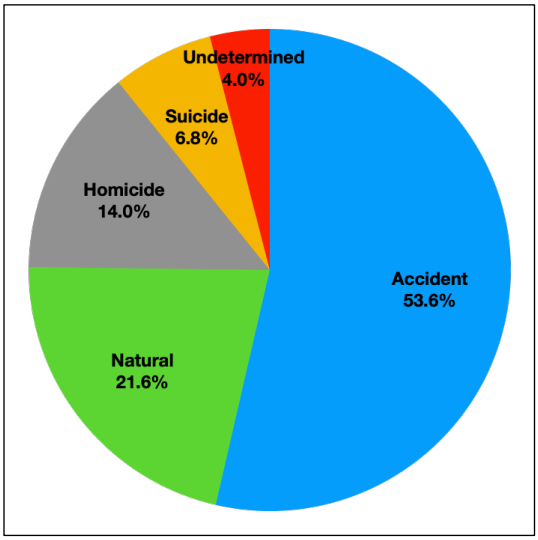


Figure 12. *Geospatial data of the studied population obtained using QGIS software.*

The manner of death was stated as accident n = 134, natural n = 54, homicide n = 35, suicide n = 17, and undetermined n = 10 (Figure 13 and Table 7).



Manner of Death	*n*
Accident	134
Natural	54
Homicide	53
Suicide	17
Undetermined	10

Figure 13 and Table 7. *Manner of death in the studied population.*

Regarding the means of death, the most represented were drug-opioid (n = 84), gun (n = 48), and drug (n = 17). Figure 14 shows all the means of death.

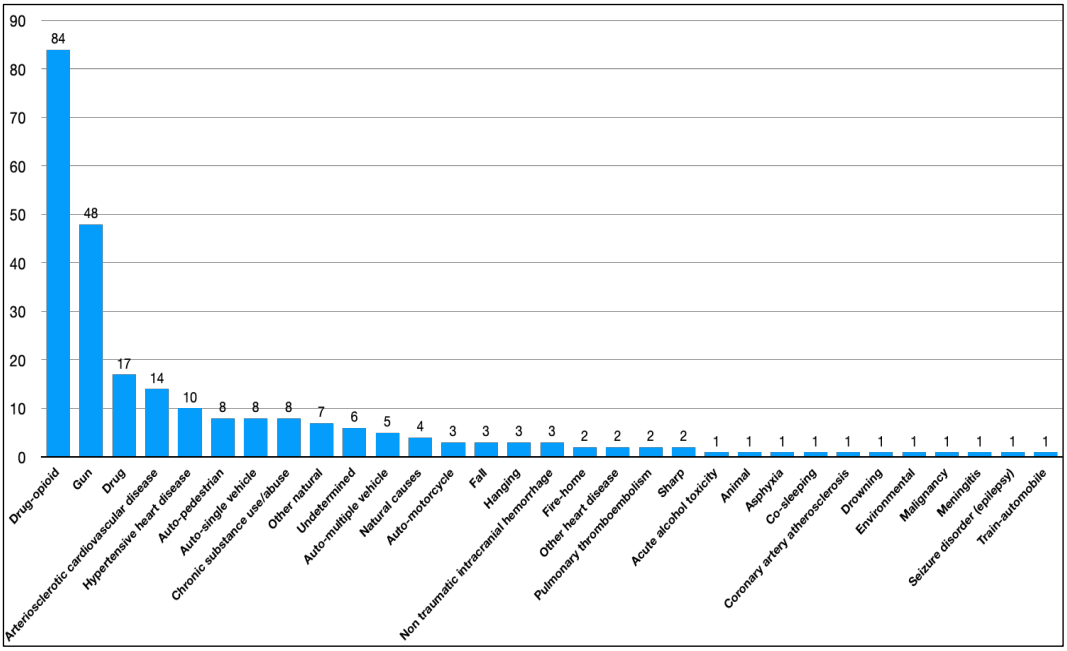


Figure 14*. Means of death of the studied population.*

5.3.2. Toxicological Analysis

Toxicological analyses were carried out in 237 cases out of 250. Of them, 87 cases tested negative through screening or confirmation analyses.

The remaining 150 cases tested positive for at least one xenobiotic. In 10 of these cases blood was not available because of the state of the corpse. Consequently toxicological analysis was performed on tissues or purge.

Table 8 shows the xenobiotics identified in blood, while Table 9 those identified in tissues or purge, being fentanyl the third most identified compound after nicotine and caffeine.

Analyte	*n*	*Mean (mg/L)*	*Min (mg/L)*	*Max (mg/L)*
Nicotine	105	Presence (not quantified)		
Caffeine	91	Presence (not quantified)		
Fentanyl	84	0.037	<LOQ	0.610
Cotinine	83	Presence (not quantified)		
Cocaine	57	0.117	<LOQ	1.9
Ecgonine methyl ester	45	Presence (not quantified)		
Methamphetamine	39	0.507	<LOQ	>1.0
Amphetamine	21	0.103	<LOQ	0.483
THC-COOH	21	0.09	0.009	0.416
Alprazolam	16	0.048	<LOQ	0.416
Cocaethylene	16	0.029	<LOQ	0.092
4-ANPP	15	Presence (not quantified)		
Diphenhydramine	13	0.24	<LOQ	1.81
Hydrocodone	10	0.03	<LOQ	0.054
Morphine	7	0.134	0.008	0.277
Xylazine	7	0.078	<LOQ	0.373
Dextromethorphan	6	0.23	<LOQ	0.238
Nortriptyline	6	0.176	<LOQ	0.392
Promethazine	6	0.14	<LOQ	0.615
Codeine	5	0.052	<LOQ	0.219
Fluoxetine	5	0.485	0.042	2.068
Bupropion	4	Presence (not quantified)		
Doxylamine	4	0.065	<LOQ	0.108
Lidocaine	4	Presence (not quantified)		
Nordiazepam	4	0.09	<LOQ	0.207
Oxycodone	4	0.107	<LOQ	0.317
Tramadol	4	0.224	<LOQ	0.396
Amitriptyline	3	0.172	<LOQ	0.258
Carbamazepine	3	3.35	2.7	4
Cyclobenzaprine	3	0.031	<LOQ	0.043
Diazepam	3	<LOQ	<LOQ	<LOQ
Fluorofentanyl	3	Presence (not quantified)		
Methadone	3	0.478	0.087	1.23
6-MAM	2	0.016	0.008	0.025

Acetaminophen	2	305	<LOQ	>600
Citalopram	2	0.051	<LOQ	0.077
Clonazepam	2	<LOQ	<LOQ	<LOQ
Dicyclomine	2	Presence (not quantified)		
Dihydrocodeine	2	Presence (not quantified)		
EDDP	2	Presence (not quantified)		
Ketamine	2	Presence (not quantified)		
Mirtazapine	2	0.054	0.048	0.06
Norketamine	2	Presence (not quantified)		
Norsertraline	2	Presence (not quantified)		
Quetiapine	2	1.764	0.248	3.28
Sertraline	2	0.064	0.028	0.101
Chlorpheniramine	1	Presence (not quantified)		
Chlorodiazepam	1	0.306	0.306	0.306
Gabapentin	1	6.5	6.5	6.5
Hydromorphone	1	0.006	0.006	0.006
Metoclopramide	1	Presence (not quantified)		
Metoprolol	1	0.661	0.661	0.661
Naloxone	1	Presence (not quantified)		
Norfentanyl	1	0.001	0.001	0.001
Phenobarbital	1	1.85	1.85	1.85

Table 8. *Compounds identified through toxicological analysis of blood.*
N: number of observations; Mean: mean concentration; Min: minimum concentration; Max: maximum concentration.

Matrix	***Compound***	***n***	***Concentration (mg/L)***
Brain	***Amphetamines***	3	Presence (not quantified)
	Cocaine	1	0.488
	Cocaethylene	1	0.125
	Diphenhydramine	1	<LOQ
	Fentanyl	2	Presence – 0.234
	Nicotine	1	Presence (not quantified)
Chest purge	***Amphetamines***	2	Presence (not quantified)
	Caffeine	1	Presence (not quantified)
	Cocaine	1	0.200
	Ecgonine methyl ester	1	Presence (not quantified)
	Fentanyl	2	Presence – >0.040
	Hydroxyzine	1	0.123
	Xylazine	1	13.660
Liver	***4-ANPP***	1	Presence (not quantified)
	Cocaine	2	<LOQ – 0.887
	Cocaethylene	2	<LOQ – 1.030
	Ecgonine methyl ester	1	Presence (not quantified)
	Fentanyl	1	>0.067
	Nicotine	2	Presence (not quantified)

Table 9. *Compounds identified through toxicological analysis of tissues or cadaveric fluids.*

Fentanyl toxicity was recognized as cause of death in 82 of the 250 cases. In 78 cases fentanyl was detected in blood and in the remaining 4 cases in tissues or fluids (2 brain, 1 purge, 1 liver).

It is worth mentioning that in these fentanyl-related deaths, other compounds were detected along with fentanyl as reported in Figure 15. Cocaine and methamphetamine were the illicit drugs more frequently co-detected.

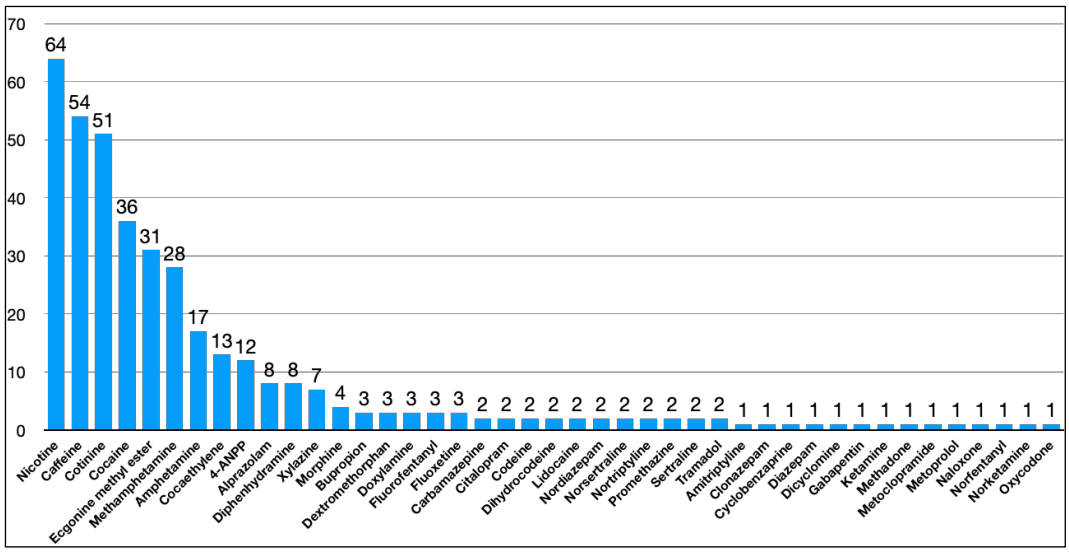


Figure 15. *Drugs detected in blood or tissues in cases of fentanyl-related death.*

In addition, in 6 cases fentanyl was detected in blood without contributing to the cause of death. In these cases, death was attributed to:

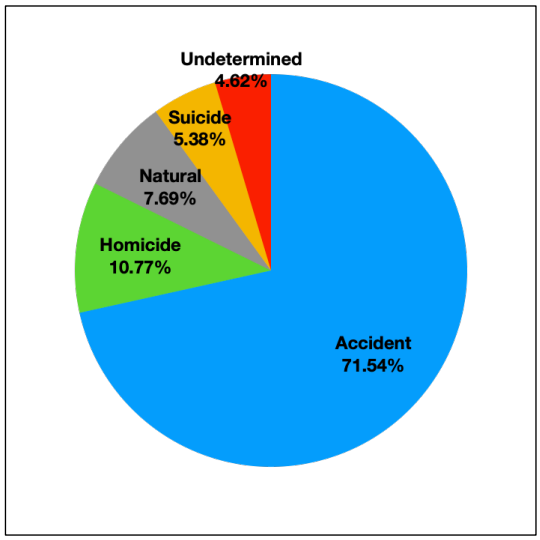
- Cocaine toxicity (blood fentanyl < LOQ);
- Heart rupture (blood fentanyl = 0.035 mg/L);
- Gunshot wound of head (blood fentanyl = 0.014 mg/L);
- Gunshot wound of head (blood fentanyl = 0.610 mg/L);
- Cocaine toxicity (blood fentanyl < LOQ);
- Blunt force injuries due to motor vehicle accident (blood fentanyl = 0.034 mg/L).

5.3.3. Hair testing

Hair testing was performed in all 250 cases. **In 129 out of 250 cases (51,6%), hair samples tested positive for fentanyl, fentanyl analogs, and adulterants**,

counting 92 males (71.31%) and 37 females (28.69%), with a mean age of 41.72 years old (SD = 13.90 yo).

The manner of death in the subpopulation with positive hair testing was stated as accident n = 92, homicide n = 14, natural n = 10, suicide n = 7, and undetermined n = 6 (Figure 16, Table 10).



Manner of Death	*n*
Accident	92
Homicide	14
Natural	10
Suicide	7
Undetermined	6

Figure 16 and Table 10. *Manner of death in the subpopulation with positive hair testing.*

Regarding the means of death of the subjects who tested positive on hair, the most represented were drug-opioid (n = 76), gun (n = 19), and drug (n = 13). Figure 17 shows all the means of death.

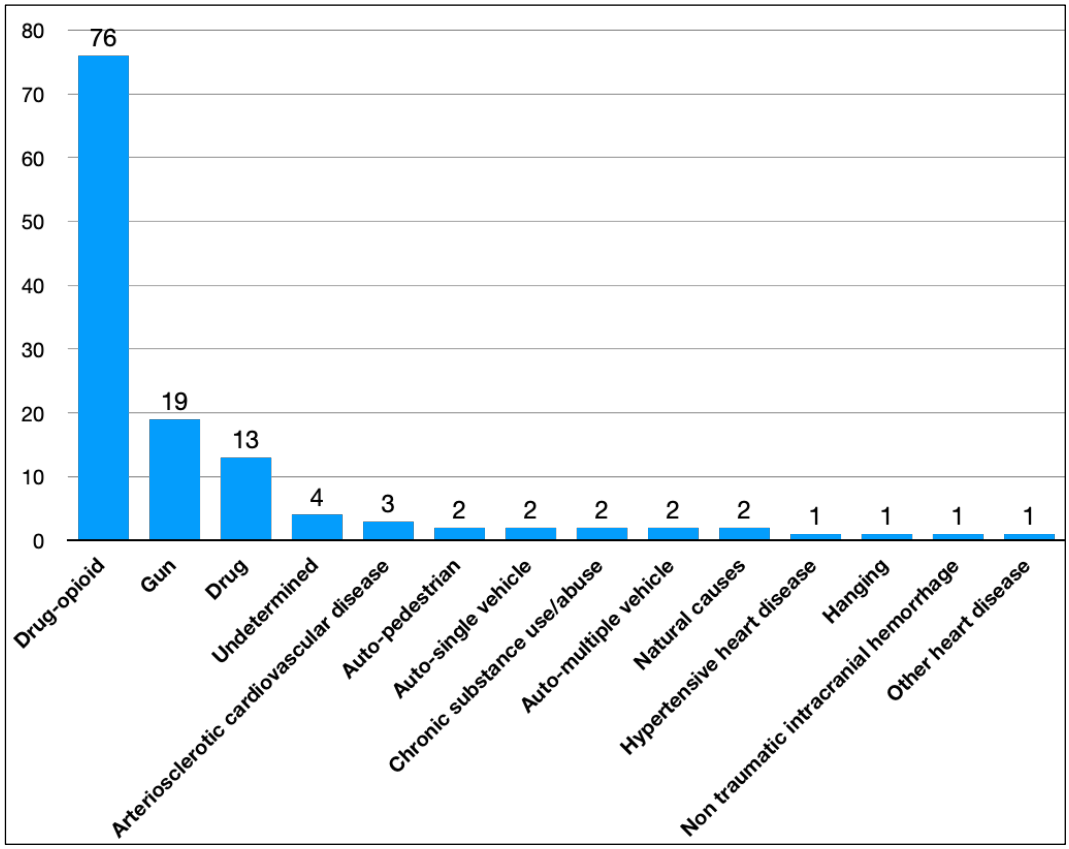


Figure 17*. Means of death of the hair-fentanyls-positive sub-population*

The hair concentrations of fentanyl, fentanyl analogs and xylazine are summarized in Table 11 and Figure 18.

Analyte	*n*	*Mean (pg/mg)*	*Min (pg/mg)*	*Max (pg/mg)*
Fentanyl	125	1,697.02	<LOQ	>10,000
Norfentanyl	15	482.29	<LOQ	>10,000
Acetyl fentanyl	16	104.15	<LOQ	431
ß-hydroxy fentanyl	42	380.28	<LOQ	2,350
Despropionyl para-fluorofentanyl	26	129.05	<LOQ	1,385
4-ANPP	83	516.18	<LOQ	8,401
Xylazine	52	1,357.58	<LOQ	>10,000

Table 11. *Compounds detected through toxicological analysis of hair.*
Mean: mean concentration; Min: minimum concentration; Max: maximum concentration

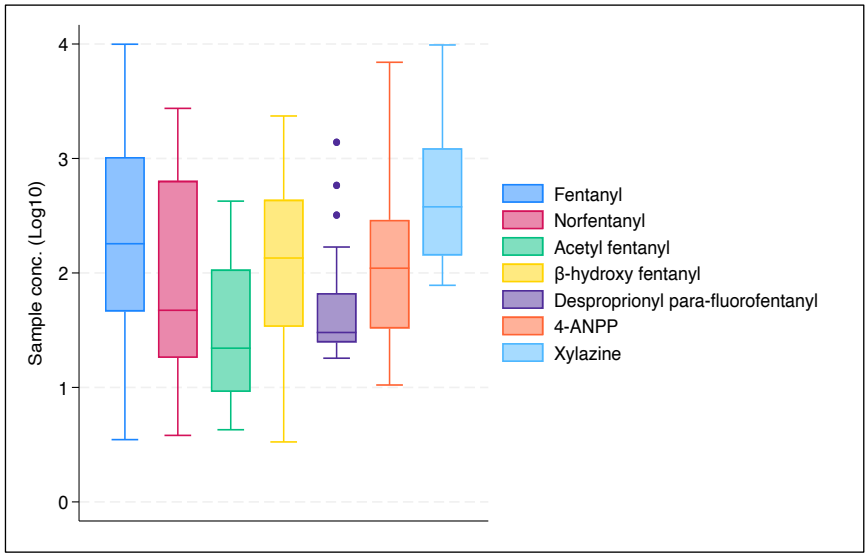


Figure 18. *Data distribution for the detected compounds in processed samples expressed as Log10 concentrations (performed by Stata software version 18.0, StataCorp, College Station, TX).*

Interestingly, fentanyl was detected in almost all cases (125/129 cases) usually with analogs (101/125 cases). The analogs included: norfentanyl (n = 83, 66.4 %), acetylfentanyl (n = 16, 12.8 %), ß-hydroxy fentanyl (n = 42, 33.6 %), despropionyl para-fluorofentanyl (n = 26, 20.8%), 4-ANPP (n = 83, 66.4 %).

In 51 out of 125 cases the adulterant xylazine was identified (n = 51, 40.8 %).

Four cases tested negative for fentanyl but tested positive for norfentanyl (n = 3), xylazine (n = 1).

The geographical distribution of all positive hair samples considering home addresses is shown in Figure 19, while figure 20 shows the geographical distribution of xylazine-positive hair samples.

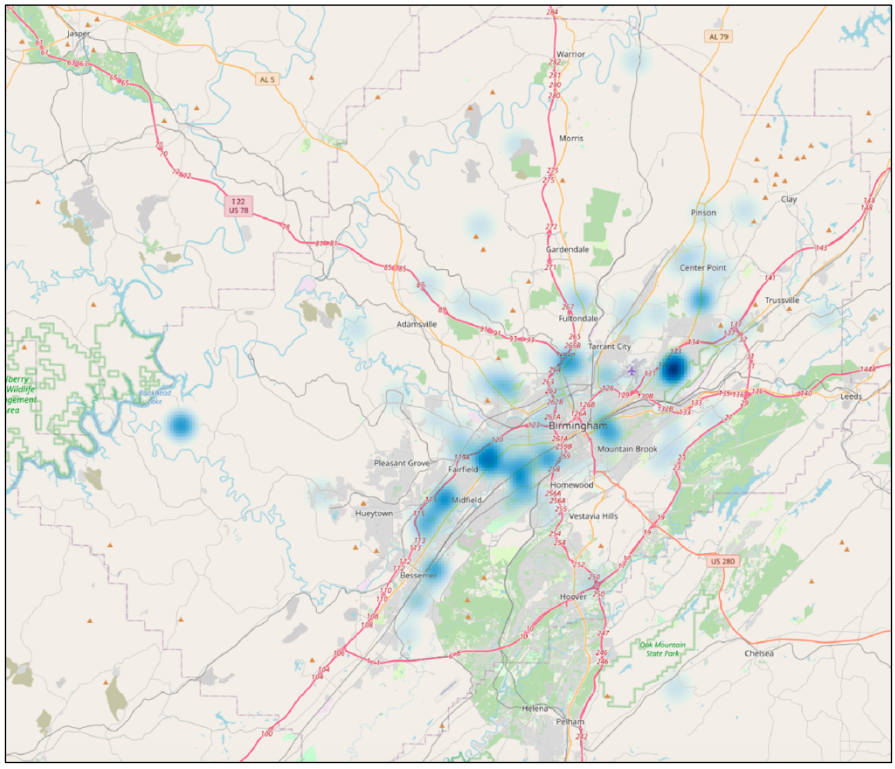


Figure 19. *Geospatial distribution of fentanyls and adulterant positive hair samples obtained using QGIS software.*

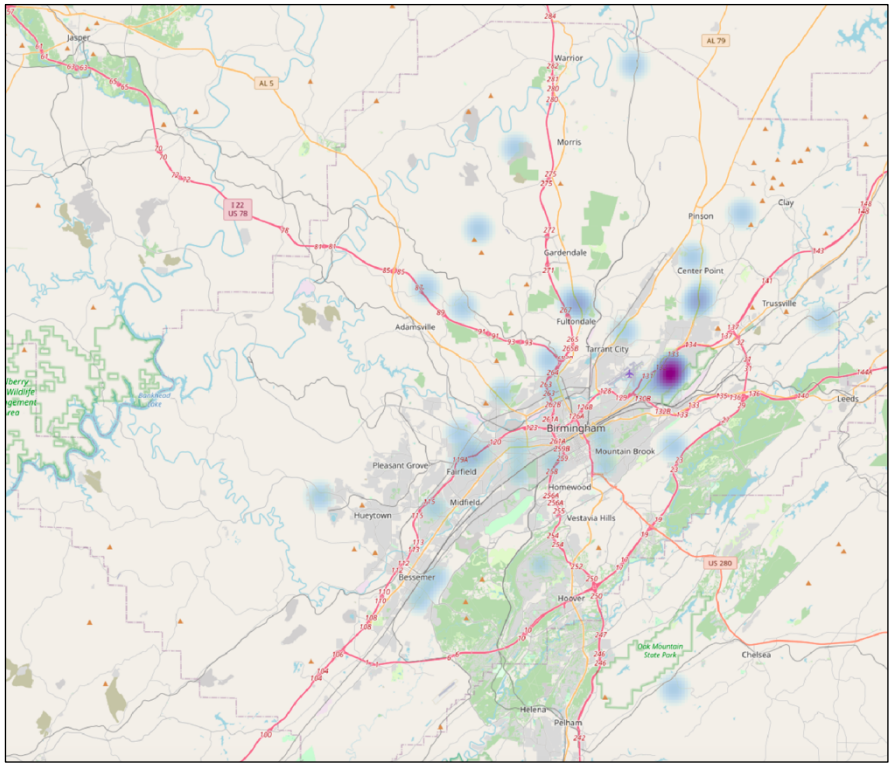


Figure 20. *Geospatial distribution of xylazine-positive hair samples obtained using QGIS software.*

The concentration ratio maps of Jefferson County shows several areas of elevated hair-positive subjects, where Lewisburg, Fairfield, Midfield, West End, Avondale, and East Lake were the most represented (Figure 19).
On the contrary, the maps showing the concentration ratio of xylazine-hair-positive subjects demonstrate a high incidence in the East Lake neighborhood (Figure 20). This finding supports the hypothesis that people coming from East Lake are more likely to access drugs adulterated with xylazine.

To the best of our knowledge, this is the first study carried out systematically on all the post-mortem cases under the jurisdiction of a coroner/medical examiner office in a certain time interval in a region with high spread of fentanyls and adulterants. Indeed, the studies in literature report only sporadic cases and, in any case, carried out in regions where the use of new synthetic opioids is not prevalent. The studies retrieved in literature are summarized in Table 12.
It is worth mention that the hair fentanyls concentrations reported in literature, ranging from tens to thousands picograms per milligram, are similar to those determined in our study.

Authors (year) ref.	***Country***	***Real cases tested***	***Hair positive cases***	***Analysis type***	***Fentanyls***	***Concentration (pg/mg)***
Moore et al. (2008) (135)	U.S.	9	4	Mass	Fentanyl	12 – 1930
Staeheli et al. (2016) (136)	Switzerland	1	1	Mass	Butyrfentanyl	11,000
Guerreri et al. (2017) (137)	Sweden	2	2	Segmental	Fentanyl	N.D.
Ramírez Fernández et al. (2019) (138)	Belgium	2	2	Segmental	Fentanyl Norfentanyl	1.4 – 88 3.1
Gulier et al. (2019) (139)	France	1	1	Segmental	Carfentanyl	54 – 166
Larabi et al. (2020) (140)	France	1	1	Segmental	Acetyl fentanyl Fentanyl Methoxyacetyl fentanyl 3-fluorofentanyl Carfentanyl Furanyl fentanyl	1 3 – 760 500 – 600 25 – 150 2.5 – 3 15 – 500
Freni et al. (2020) (141)	Italy	20	2	Segmental	Fentanyl Furanylfentanyl Acetyl fentanyl Methoxyacetyl fentanyl Methoxyacetyl norfentanyl	8.2 – 12.8 136.7 – 195.8 1.0 – 1.4 259.9 – 479.6 17.1 – 32.7

					Ocfentanil 4-ANPP	4.1 – 11.1 Positive
Allibe et al. (2021) (142)	France	1	1	Segmental	Ocfentanyl	37 – 150
Neukamm et al. (2023) (143)	Germany	1	1	Mass	Fentanyl Norfentanyl	770 11

Table 12. *Postmortem hair analysis reported in the literature.*

The group with positive hair (n = 129) was overlapping sex-wise with the total study population (male ~ 70%, female ~ 30 %).

In contrast, considering the age at the time of death, data showed a decrease in the average age from 48.8 to 41.7 years old, with a net reduction of 7.1 years compared with the general population included in the study. This finding is reflected in literature data, displaying that young age is a risk factor for drug exposure and death (144).

Considering the manner of death, nearly 70 % of positive-hair-subject deaths were certified as accidental deaths, where about 96% were unintentional (accident) drug poisoning deaths. These included cases where too much of a drug was taken accidentally and the wrong drug was taken in error. These data are consistent with the evidence that drug overdose is the leading cause of accidental death in the United States (145).

The prevalence of accidental death in the positive-hair population is clearly related to the fact that subjects exposed to xenobiotics, particularly opioids, have a higher risk of dying from a drug overdose (146,147).

The second most represented manner of death was homicide, accounting for ~ 11% of positive-hair cases. It is well known that drug exposure and addiction are closely related to involvement with crime (148).

As above reported, fentanyls detected included: fentanyl and its metabolites (norfentanyl, ß-hydroxy fentanyl, despropionyl fentanyl → 4ANPP), acetyl fentanyl, despropionyl para-fluorofentanyl. In many cases also xylazine was identified.

Acetyl fentanyl is an illicit synthetic opioid that is four to five times more potent than heroin (149). Despropionyl para-fluorofentanyl is a metabolite of fluoro isobutyl fentanyl, a precursor in the synthesis of para-fluorofentanyl and a potential impurity of para-fluorofentanyl preparations (150).

The fentanyls identified in the studied population overlap with those detected across the U.S. (151).

Xylazine is a nonopioid veterinary anesthetic and sedative that is increasingly detected in the illicit drug supply in the United States. This adulterant is reported to increase the risk of fatal intoxication by acting on a different receptor than opioids (α-2 adrenergic receptor) and making opioid antagonists completely ineffective in cases of overdose. For these reasons, xylazine spread represents a major public health concern (152).

To the best of our knowledge, this is the first time that xylazine has been detected in hair. Chronic consumption/exposure to this compound could be related to unintentional intake due to unknown adulteration of drug supply or to the intentional research of long-lasting effects of drug supply, as reported in the literature (153).

The identification of xylazine in 52/129 (40%) cases of hair-positive population, suggests a high adulteration of synthetic opioid supply in the area.

These data confirm **the usefulness of systematic hair testing in postmortem investigation**, providing helpful information with potential substantial repercussions on public health. The systematic search for synthetic opioids and adulterants can provide a **qualitative representation of drug spread in near real-time in a specific area** (in this case, the county). Indeed, toxicological analysis of traditional matrices at the time of death may not return some critical information. As matter of fact, xylazine was present in the hair of approximately 40% (n = 52) of the positive cases compared to only 4 deaths in which xylazine was detected in blood and therefore attributed to xylazine toxicity.

In addition, identifying substances in the hair absent in the blood at the time of death can provide **data consistent with chronic organs and tissue pathologic alterations** typical of those substances. For example, ulcers caused by the chronic use of xylazine could be objectively confirmed by hair positivity in cases in which xylazine was absent at the time of death.

In this respect, our record shows two subjects presenting multiple large ulcers (some with eschar formation) on the back, forearms, and legs. Toxicological analyses performed on hair tested positive for xylazine supporting the chronic exposure to

this compound as the cause of the skin lesions, despite the absence of xylazine in cadaveric blood.

Finally, the study of the **geographical distribution of positivity can identify the neighborhoods at the most significant risk of addiction** and, consequently, at the greatest risk of drug-related crime. Risky neighborhoods identified by post-mortem hair testing may be modified through social development policies such as targeted infrastructure improvements or other community development strategies, such as park-making or building renovation.

In order to examine in depth the possibilities offered by hair testing in post-mortem cases, subjects who tested positive for fentanyls in hair were grouped according to the means of death.

Drug- and drug-opioid-related deaths were taken into consideration in comparison with other means of death.

Seventy percent of subjects positive for fentanyl in hair died for drug overdose. The remaining 30% died for causes other than drug toxicity, as shown in Figure 21. It is patent that the most frequent events in this second group were gun violence-related deaths.

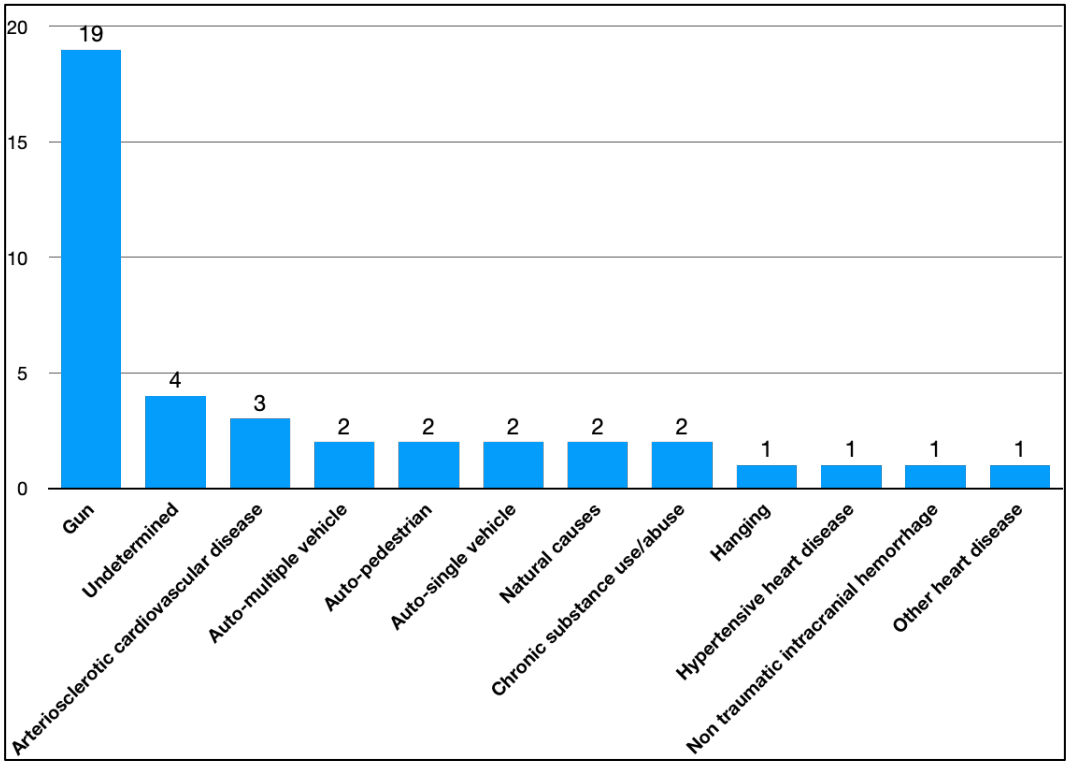


Figure 21. *Means of deaths other from drug-related of hair-fentanyls-positive subjects.*

In addition, the **univariable analysis** among hair testing and the different means of death demonstrate a significantly strong association between means of death (drug and drug-opioid toxicity) and fentanyls positive hair testing ($p < 0.001$) (Table 13). It means that people dead from overdose (all types of overdoses, opioid- and non-opioid-related) are more likely to test positive on hair fentanyls analysis. On the other hand, the association between people who died from overdoses due to drugs other than opioids and hair fentanyls positivity suggests a condition of polydrug abuse in the periods preceding death, which is an emerging public health issue (154). Overall, about one third of fentanyl positive cases in hair died for a cause different from drug intoxication. These results are not surprising given the wide spread of fentanyl and analogs in the studied geographic area.

Means of death	***Hair negative***	***Hair positive***	***Total***
Drug toxicity	4 *3.30 %*	13 *10.08 %*	17 *6.80 %*
Drug-opioid toxicity	8 *6.61 %*	76 *58.91 %*	84 *33.60 %*
Other deaths	109 *90.09 %*	40 *31.01 %*	149 *59.60 %*
Total	121 *100.00 %*	129 *100.00 %*	250 *100.00 %*

Table 13 *Association between means of death (drug and drug-opioid toxicity) and fentanyls positive hair testing ($p < 0.001$).*

A **logistic regression model** was used to estimate the risk related to fentanyls hair positivity by entering sex, age, and means of death, as shown in Table 14.

Variable	***Odds ratio***	***95% CI***	***p-value***
Age	0.97	0.95 – 0.99	**0.003**
Gender M	1.10	0.53 – 2.32	0.835
Drug	9.30	2.82 – 30.76	**< 0.001**
Drug-opioid	29.96	12.60 – 71.22	**< 0.001**

Table 14. *Logistic regression analysis of hair-positive risk.*

The multivariable logistic regression shows that older age is a protective factor for hair fentanyls positivity risk (Odds Ratio = 0.97).

Regarding the means of death, drug-related dead people have 10 times more probability of testing positive for hair analysis than non-drug-related deaths, and in the drug-opioid-related deaths group, this risk increases by 30 times.

Lastly, the population with fentanyl in blood, hair positivity, and death related to fentanyls was divided into three equal parts (tertiles) based on **hair fentanyl concentration** (Group 1: 3 – 64 pg/mg; Group 2: 65 – 472 pg/mg; Group 3: 473–10,000 pg/mg). The division of the population into tertiles based on hair fentanyl concentration stems from the lack in the literature of data reporting the post-mortem concentrations of novel synthetic opioids in large populations. As previously mentioned, studies on the topic are scarce, and the sample size is limited, often including only one case.
The distribution of fentanyl blood concentration among groups is reported in Table 15.

	Blood fentanyl concentration median [range] mg/L	***n***
Group 1 (3 – 64 pg/mg)	0.004 [0.002-0.039]	18
Group 2 (65 - 472 pg/mg)	0.016 [0.003-0.088]	31
Group 3 (473 - 10,000 pg/mg)	0.020 [0.002-0.224]	26

Table 15. *Distribution of blood fentanyl concentration between 3 groups of hair fentanyl concentration in cases where fentanyl was the cause of death.*

Data analysis demonstrates a statistically significant difference between group 1 and the other two groups. In particular, group 1 with lower hair fentanyl concentrations showed low blood fentanyl concentrations compared to both groups with higher hair fentanyl concentrations, which additionally presented high blood fentanyl concentrations ($p < 0.001$) (Figure 22).

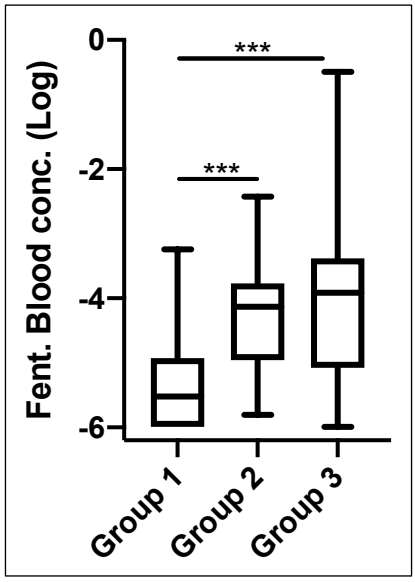


Figure 22. *Graphical representation of the uqnatitative correlation betwwem fentanyl blood concentrations and fentanyl hair concentrations. ***: $p < 0.001$.*

These data would suggest that recurrent consumption/exposure to fentanyl could lead to tolerance in subjects who would, therefore, requiring higher blood concentrations of fentanyl to cause death.

It could be true, even if it is necessary to take into account the specific features of post-mortem hair testing.

Firstly, although some studies have reported a correlation between blood and hair concentration for some xenobiotics, the scientific community agrees that there is only semi-quantitative correlation between the dose consumed and the concentration of xenobiotics in the hair. In fact, the hair incorporation of compounds is influenced by several factors such as melanin content, type of drug, cosmetic treatment, etc.

In addition, in the post-mortem period, biological fluids, such as blood, sweat, and putrefactive fluid, can soak the hair for an indefinite time, allowing compounds present in such fluids to be incorporated into the hair, distorting the result.

6. CONCLUSION

Hair analysis is rarely performed in the context of postmortem forensic pathological investigations because of reasons of cost, and, sometimes, due to the lack of specialized skills in in the forensic toxicological labs.

This study presents the results of hair testing carried out on 250 post-mortem cases from Jefferson County Coroner/Medical Examiner's Office, Birmingham, Al, USA. To the best of our knowledge, this is the first study conducted systematically on the population under the jurisdiction of the US coroner/medical examiner system in a specific time interval.

The opportunity to apply hair testing on a large non pre-selected population provided the possibility to verify its usefulness of this approach in post-mortem context.

Given the constantly increasing diffusion of new synthetic opioids, particularly in North America, and the related social cost of the emergency, the attention was focused on the search for fentanyls, the most represented class of synthetic opioids. Consistently with the epidemiological data, hair testing showed the consumption/exposure to these compounds in more than half of the cases, regardless of the cause and means of death.

In addition, the recent concern of the use of xylazine as drugs adulterant led us to develop a LC-MS/MS method suitable, in terms of both analytical sensitivity and specificity, to determine this substance in hair. The following application of the method to the real cases of the study allowed for demonstrating the wide spread of xylazine as adulterant. Indeed, xylazine was found in about 40% of fentanyl hair positive cases. Interestingly, the vast majority of the xylazine positive cases came from the same geographical area, i. e. East Lake, confirming the usefulness of hair testing also for epidemiological purposes.

7. REFERENCES

1. Duke J. Anesthesia secrets. Elsevier Health Sciences, 2011.
2. Fürst S, Hosztafi S. Pharmacology of poppy alkaloids. In: Bernàt J, editor. Poppy: The Genus Papaver. CRC Press, 1998;291–318.
3. Fürst S, Hosztafi S. The chemical and pharmacological importance of morphine analogues. Acta Physiol Hung 2008;95(1):3–44.
4. Kumar V. Handbook on Opium: History and Basis of Opioids in Therapeutics. Academic Press, 2022.
5. Loeser JD. Bonica's management of pain. Lippincott Williams & Wilkins Philadelphia, 2001.
6. Fox III CJ, Hawney HA, Kaye AD, Menon S, Kunnumpurath S. Opioids: Pharmacokinetics and pharmacodynamics. In: Vadivelu N, Urman RD, Hines RL, editors. Essentials of pain management. Springer, 2010;91–103.
7. Karch SB, Drummer O, Garavan F. Karch's pathology of drug abuse. 5th ed. CRC press, 2015.
8. Kasper D, Fauci A, Hauser S, Longo D, Jameson J, Loscalzo J. Harrison's principles of internal medicine. 19th ed. Mcgraw-hill New York, NY, USA:, 2015.
9. Christie MJ. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. Br J Pharmacol 2008;154(2):384–96.
10. Pergolizzi Jr J V, Raffa RB, Rosenblatt MH. Opioid withdrawal symptoms, a consequence of chronic opioid use and opioid use disorder: Current understanding and approaches to management. J Clin Pharm Ther 2020;45(5):892–903.
11. Tabarra I, Soares S, Rosado T, Gonçalves J, Luís Â, Malaca S, et al. Novel synthetic opioids–toxicological aspects and analysis. Forensic Sci Res 2019;4(2):111–40.
12. Stanley TH. The fentanyl story. J Pain 2014;15(12):1215–26.
13. Pichini S, Solimini R, Berretta P, Pacifici R, Busardò FP. Acute intoxications and fatalities from illicit fentanyl and analogues: an update. Ther Drug Monit 2018;40(1):38–51.
14. Suzuki J, El-Haddad S. A review: fentanyl and non-pharmaceutical fentanyls. Drug Alcohol Depend 2017;171:107–16.
15. Kuczyńska K, Grzonkowski P, Kacprzak Ł, Zawilska JB. Abuse of fentanyl: An emerging problem to face. Forensic Sci Int 2018;289:207–14.
16. Ciccarone D. Fentanyl in the US heroin supply: A rapidly changing risk environment. Int J Drug Policy 2017;46:107–11.
17. Watanabe S, Vikingsson S, Roman M, Green H, Kronstrand R, Wohlfarth A. In vitro and in vivo metabolite identification studies for the new synthetic opioids acetylfentanyl, acrylfentanyl, furanylfentanyl, and 4-fluoro-isobutyrylfentanyl. AAPS J 2017;19:1102–22.

18. Kritikos PG, Papadaki SP. The history of the poppy and of opium and their expansion in antiquity in the eastern Mediterranean area. 1967.
19. Kramer JC. Opium rampant: medical use, misuse and abuse in Britain and the West in the 17th and 18th centuries. British Journal of Addiction to Alcohol & Other Drugs 1979;74(4):377–89.
20. Masic I, Skrbo A, Naser N, Tandir S, Zunic L, Medjedovic S, et al. Contribution of Arabic medicine and pharmacy to the development of health care protection in Bosnia and Herzegovina-the First Part. Medical Archives 2017;71(5):364.
21. Althofer J, Musgrove B. "A ghost in daylight": drugs and the horror of modernity. Palgrave Commun 2018;4(1):1–11.
22. Kamienski L. Shooting up: a short history of drugs and war. Oxford University Press, 2016.
23. Levy N, Sturgess J, Mills P. "Pain as the fifth vital sign" and dependence on the "numerical pain scale" is being abandoned in the US: why? Br J Anaesth 2018;120(3):435–8.
24. UNODC. United Nations Office on Drugs and Crime. World Drug Report 2023. 2023. https://www.unodc.org/unodc/en/data-and-analysis/world-drug-report-2023.html.
25. Carod-Artal FJ. Psychoactive plants in ancient Greece. Neurosciences and History 2013;1(1):28–38.
26. Stebbins GL. Edgar Anderson 1897-1969. National Academy of Sciences, Washington. 1978.
27. Merlin MD. Archaeological evidence for the tradition of psychoactive plant use in the old world. Econ Bot 2003;57(3):295–323.
28. Micheli I. Cultural and Linguistic Transition explored. Proceedings of the ATrA closing workshop. Trieste, May 25-26, 2016. EUT-Edizioni Università di Trieste, 2017.
29. Guo L, Winzer T, Yang X, Li Y, Ning Z, He Z, et al. The opium poppy genome and morphinan production. Science (1979) 2018;362(6412):343–7.
30. Smith RK, Stacey RJ, Bergström E, Thomas-Oates J. Detection of opium alkaloids in a Cypriot base-ring juglet. Analyst 2018;143(21):5127–36.
31. Salavert A. Agricultural dispersals in mediterranean and temperate Europe. In: Shugart H, editor. Environmental sciences: Oxford research encyclopedia. Oxford University Press, 2017;1–43.
32. Krikorian AD. Were the opium poppy and opium known in the ancient Near East? J Hist Biol 1975;:95–114.
33. Rosso AM. Poppy and opium in ancient times: remedy or narcotic? Biomedicine International 2010;1(2):81–7.
34. Prioreschi P, Heaney RP, Brehm E. A quantitative assessment of ancient therapeutics: poppy and pain in the Hippocratic Corpus. Med Hypotheses 1998;51(4):325–31.
35. Cilliers L, Retief FP. Poisons, poisoning and the drug trade in ancient Rome. Akroterion 2000;45(1):88–100.

36. Julyan M, Dircksen M. The ancient drug opium. Akroterion 2011;56(1):75–90.
37. Spivack BS. AC Celsus: roman medicus. J Hist Med Allied Sci 1991;46(2):143–57.
38. Karaberopoulos D, Karamanou M, Androutsos G. The theriac in antiquity. The lancet 2012;379(9830):1942–3.
39. De Vos P. The “Prince of Medicine”: Yūḥannā ibn Māsawayh and the Foundations of the Western Pharmaceutical Tradition. Isis 2013;104(4):667–712.
40. Abboud T. Al Kindi: The Father of Arab Philosophy. The Rosen Publishing Group, Inc, 2006.
41. Batchelor DAF. Al-Biruni: Outstanding “modern” scientist of the Golden Age of Islamic Civilisation. In: Wain A, Kamali M, editors. The architects of Islamic civilisation. Pelanduk Publications, 2018.
42. Tashani O, Johnson M. Avicenna’s concept of pain. Libyan Journal of Medicine 2010;5(1):5253.
43. Pagel W. Paracelsus: An Introduction to Philosophical Medicine in the Era of the Renaissance. Karger Medical and Scientific Publishers, 1982.
44. Latham RG. The Works of Thomas Sydenham. Sydenham Society, 1848.
45. Maehle A-H. Opium: Explorations of an ambiguous drug. In: Maehle A, editor. Drugs on trial: Experimental pharmacology and therapeutic innovation in the eighteenth century. Rodopi, 1999;141–52.
46. Dikötter F, Laamann L, Xun Z. Narcotic culture. a social history of drug consumption in China. British Journal of Criminology 2002;42(2):317–36.
47. Zheng Y. The social life of opium in China, 1483–1999. Mod Asian Stud 2003;37(1):1–39.
48. Wright A. Not Just a" Place for the Smoking of Opium": The Indian opium den and imperial anxieties in the 1890s. J Colon Colon Hist 2017;18(2):13.
49. Stahnisch FW. François Magendie (1783–1855). J Neurol 2009;256(11):1950–2.
50. Kane HH. The Hypodermic Injection of Morphia: Its History, Advantages, and Dangers. Chas L Beringham & Co, 1880.
51. Aurin M. Chasing the Dragon: The Cultural Metamorphosis of Opium in the United States, 1825-1935. Med Anthropol Q 2000;14(3):414–41.
52. Terry CE, Pellens M. The Opium Problem, Bureau of Social Hygiene. Inc, New York 1928;:53–93.
53. Quinones MA. Drug abuse during the Civil War (1861–1865). International Journal of the Addictions 1975;10(6):1007–20.
54. Hubbard FH. The opium habit and alcoholism. AS Barnes & Company, 1881.
55. Fernandez H, Libby TA. Heroin: Its History, Pharmacology & Treatment. Hazelden, 2011.

56. Jones MR, Viswanath O, Peck J, Kaye AD, Gill JS, Simopoulos TT. A brief history of the opioid epidemic and strategies for pain medicine. Pain Ther 2018;7:13–21.
57. Larson MJ, Wooten NR, Adams RS, Merrick EL. Military combat deployments and substance use: Review and future directions. J Soc Work Pract Addict 2012;12(1):6–27.
58. Edmonds-Seal J, Prys-Roberts C. Pharmacology of drugs used en neuroleptanalgesia. Br J Anaesth 1970;42(3):207–16.
59. Lowenstein E, Hallowell P, Levine FH, Daggett WM, Austen WG, Laver MB. Cardiovascular response to large doses of intravenous morphine in man. New England Journal of Medicine 1969;281(25):1389–93.
60. Harbaugh RE, Coombs DW, Saunders RL, Gaylor M, Pageau M. Implanted continuous epidural morphine infusion system: Preliminary report. J Neurosurg 1982;56(6):803–6.
61. Porter J, Jick H. Addiction rare in patients treated with narcotics. N Engl J Med 1980;302(2):123.
62. Leung PTM, Macdonald EM, Stanbrook MB, Dhalla IA, Juurlink DN. A 1980 letter on the risk of opioid addiction. New England Journal of Medicine 2017;376(22):2194–5.
63. WHO. Cancer Pain Relief. 1986.
64. Portenoy RK, Foley KM. Chronic use of opioid analgesics in non-malignant pain: report of 38 cases. Pain 1986;25(2):171–86.
65. Van Zee A. The promotion and marketing of oxycontin: commercial triumph, public health tragedy. Am J Public Health 2009;99(2):221–7.
66. Zawilska JB. An expanding world of novel psychoactive substances: opioids. Front Psychiatry 2017;8:110.
67. CDC. Understanding the Opioid Overdose Epidemic. 2023.
68. CDC. Vital signs: overdoses of prescription opioid pain relievers - United States, 1999-2008. MMWR Morb Mortal Wkly Rep 2011;60(43):1487–92.
69. Rudd RA, Paulozzi LJ, Bauer MJ, Burleson RW, Carlson RE, Dao D, et al. Increases in heroin overdose deaths-28 states, 2010 to 2012. Morbidity and Mortality Weekly Report 2014;63(39):849.
70. Gladden RM, Martinez P, Seth P. Fentanyl law enforcement submissions and increases in synthetic opioid–involved overdose deaths - 27 states, 2013–2014. Morbidity and Mortality Weekly Report 2016;65(33):837–43.
71. O'Donnell JK, Gladden RM, Seth P. Trends in deaths involving heroin and synthetic opioids excluding methadone, and law enforcement drug product reports, by census region - United States, 2006–2015. Morbidity and Mortality Weekly Report 2017;66(34):897.
72. O'Donnell JK, Halpin J, Mattson CL, Goldberger BA, Gladden RM. Deaths involving fentanyl, fentanyl analogs, and U-47700 - 10 states, July–December 2016. Morbidity and Mortality Weekly Report 2017;66(43):1197.
73. Drug Enforcement Administration. National drug threat assessment. 2019.

74. CDC National Center for Health Statistics. Wide-ranging online data for epidemiologic research (WONDER). 2021. http://wonder.cdc.gov.
75. Peterson AB, Gladden RM, Delcher C, Spies E, Garcia-Williams A, Wang Y, et al. Increases in fentanyl-related overdose deaths—Florida and Ohio, 2013–2015. Morbidity and Mortality Weekly Report 2016;65(33):844–9.
76. O'Donnell J, Gladden RM, Mattson CL, Kariisa M. Notes from the field: overdose deaths with carfentanil and other fentanyl analogs detected—10 states, July 2016–June 2017. Morbidity and Mortality Weekly Report 2018;67(27):767.
77. Drummer OH, Gerostamoulos J. Postmortem drug analysis: analytical and toxicological aspects. Ther Drug Monit 2002;24(2):199–209.
78. Baselt RC. Disposition of Toxic Drugs and Chemicals in Man, 8th Edition. 2009.
79. Drummer OH, Odell MS. The forensic pharmacology of drugs of abuse. 1st ed. Hodder Education Publishers, 2001.
80. Moriya F, Hashimoto Y. Medicolegal implications of drugs and chemicals detected in intracranial hematomas. J Forensic Sci 1998;43(5):980–4.
81. Flanagan RJ, Connally G, Evans JM. Analytical toxicology: guidelines for sample collection postmortem. Toxicol Rev 2005;24:63–71.
82. Moffat AC, Osselton MD, Widdop B, Watts J. Clarke's analysis of drugs and poisons. Pharmaceutical press Chicago, 2011.
83. Karch SB. Postmortem toxicology of abused drugs. CRC Press, 2007.
84. Pigaiani N, Bertaso A, De Palo EF, Bortolotti F, Tagliaro F. Vitreous humor endogenous compounds analysis for post-mortem forensic investigation. Forensic Sci Int 2020;310:110235. https://doi.org/10.1016/j.forsciint.2020.110235.
85. Bévalot F, Cartiser N, Bottinelli C, Fanton L, Guitton J. Vitreous humor analysis for the detection of xenobiotics in forensic toxicology: a review. Forensic Toxicol 2016;34:12–40.
86. Bévalot F, Cartiser N, Bottinelli C, Guitton J, Fanton L. State of the art in bile analysis in forensic toxicology. Forensic Sci Int 2016;259:133–54.
87. Tassoni G, Cacaci C, Zampi M, Froldi R. Bile analysis in heroin overdose. J Forensic Sci 2007;52(6):1405–7.
88. Morley SR, Bolton J. Variation in postmortem liver sampling: implications for postmortem toxicology interpretation. J Clin Pathol 2012;65(12):1136–7.
89. Jenkins AJ. Drug testing in alternate biological specimens. Humana Press, 2008.
90. Dinis-Oliveira RJ, Carvalho F, Duarte JA, Remião F, Marques A, Santos A, et al. Collection of biological samples in forensic toxicology. Toxicol Mech Methods 2010;20(7):363–414. https://doi.org/10.3109/15376516.2010.497976.
91. Colucci AP, Aventaggiato L, Centrone M, Gagliano-Candela R. Validation of an extraction and gas chromatography-mass spectrometry quantification

method for cocaine, methadone, and morphine in postmortem adipose tissue. J Anal Toxicol 2010;34(6):342–6.
92. Williams KR, Pounder DJ. Site-to-site variability of drug concentrations in skeletal muscle. Am J Forensic Med Pathol 1997;18(3):246–50.
93. Hargrove VM, Molina DK. Morphine concentrations in skeletal muscle. Am J Forensic Med Pathol 2014;35(1):73–5.
94. Watterson J. Challenges in forensic toxicology of skeletonised human remains. Analyst 2006;131(9):961–5.
95. Cartiser N, Bévalot F, Fanton L, Gaillard Y, Guitton J. State-of-the-art of bone marrow analysis in forensic toxicology: a review. Int J Legal Med 2011;125:181–98.
96. Suzuki O, Hattori H, Asano M. Nails as useful materials for detection of methamphetamine or amphetamine abuse. Forensic Sci Int 1984;24(1):9–16.
97. Buffoli B, Rinaldi F, Labanca M, Sorbellini E, Trink A, Guanziroli E, et al. The human hair: from anatomy to physiology. Int J Dermatol 2014;53(3):331–41.
98. Paus R, Arck P, Tiede S. (Neuro-) endocrinology of epithelial hair follicle stem cells. Mol Cell Endocrinol 2008;288(1–2):38–51.
99. Kintz P, Salomone A, Vincenti M. Hair analysis in clinical and forensic toxicology. Academic Press, 2015.
100. Harkey MR. Anatomy and physiology of hair. Forensic Sci Int 1993;63(1–3):9–18.
101. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. Clinica chimica acta 2006;370(1–2):17–49.
102. Prota G. The role of peroxidase in melanogenesis revisited. Pigment Cell Res 1990;3:25–31.
103. Slominski A, Wortsman J, Plonka PM, Schallreuter KU, Paus R, Tobin DJ. Hair follicle pigmentation. Journal of Investigative Dermatology 2005;124(1):13–21.
104. Prota G. Melanins, melanogenesis and melanocytes: looking at their functional significance from the chemist's viewpoint. Pigment Cell Res 2000;13(4):283–93.
105. Randall VA, Ebling FJG. Seasonal changes in human hair growth. British Journal of Dermatology 1991;124(2):146–51.
106. Blume-Peytavi U, Whiting DA, Trüeb RM. Hair growth and disorders. Springer Science & Business Media, 2008.
107. Wosicka H, Cal K. Targeting to the hair follicles: current status and potential. J Dermatol Sci 2010;57(2):83–9.
108. Cooper GAA, Kronstrand R, Kintz P. Society of Hair Testing guidelines for drug testing in hair. Forensic Sci Int 2012;218(1–3):20–4.
109. Joseph Jr RE, Su T-P, Cone EJ. In vitro binding studies of drugs to hair: influence of melanin and lipids on cocaine binding to Caucasoid and Africoid hair. J Anal Toxicol 1996;20(6):338–44.

110. Knörle R, Schniz E, Feuerstein TJ. Drug accumulation in melanin: an affinity chromatographic study. J Chromatogr B Biomed Sci Appl 1998;714(2):171–9.
111. Stout PR, Ruth JA. Comparison of In Vivo and In VitroDeposition of Rhodamine and Fluorescein in Hair. Drug Metabolism and Disposition 1998;26(10):943–8.
112. Kronstrand R, Ahlner J, Dizdar N, Larson G. Quantitative analysis of desmethylselegiline, methamphetamine, and amphetamine in hair and plasma from Parkinson patients on long-term selegiline medication. J Anal Toxicol 2003;27(3):135–41.
113. Pichini S, García-Algar O, Alvarez A-T, Mercadal M, Mortali C, Gottardi M, et al. Pediatric exposure to drugs of abuse by hair testing: monitoring 15 years of evolution in Spain. Int J Environ Res Public Health 2014;11(8):8267–75.
114. Kintz P. Contribution of in utero drug exposure when interpreting hair results in young children. Forensic Sci Int 2015;249:314–7.
115. Skopp G, Pötsch L, Moeller MR. On cosmetically treated hair—aspects and pitfalls of interpretation. Forensic Sci Int 1997;84(1–3):43–52.
116. Kintz P. Value of hair analysis in postmortem toxicology. Forensic Sci Int 2004;142(2–3):127–34.
117. Romano G, Barbera N, Spadaro G, Valenti V. Determination of drugs of abuse in hair: evaluation of external heroin contamination and risk of false positives. Forensic Sci Int 2003;131(2–3):98–102.
118. Balıková MA, Habrdová V. Hair analysis for opiates: evaluation of washing and incubation procedures. Journal of Chromatography B 2003;789(1):93–100.
119. Paulsen RB, Wilkins DG, Slawson MH, Shaw K, Rollins DE. Effect of four laboratory decontamination procedures on the quantitative determination of cocaine and metabolites in hair by HPLC-MS. J Anal Toxicol 2001;25(7):490–6.
120. da Rosa Chagas AG, Spinelli E, Fiaux SB, da Silva Barreto A, Rodrigues SV. Particle-size distribution (PSD) of pulverized hair: a quantitative approach of milling efficiency and its correlation with drug extraction efficiency. Forensic Sci Int 2017;277:188–96.
121. Moeller MR, Fey P, Wennig R. Simultaneous determination of drugs of abuse (opiates, cocaine and amphetamine) in human hair by GCMS and its application to a methadone treatment program. Forensic Sci Int 1993;63(1–3):185–206.
122. Moore C, Guzaldo F, Donahue T. The determination of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in hair using negative ion gas chromatography-mass spectrometry and high-volume injection. J Anal Toxicol 2001;25(7):555–8.
123. Kintz P. Hair analysis in forensic toxicology: an updated review with a special focus on pitfalls. Curr Pharm Des 2017;23(36):5480–6.

124. Beránková K, Habrdová V, Balíková M, Strejc P. Methamphetamine in hair and interpretation of forensic findings in a fatal case. Forensic Sci Int 2005;153(1):93–7.
125. Thiblin I, Rajs J, Lindquist O. Cause and manner of death among users of anabolic androgenic steroids. J Forensic Sci 2000;45(1):16–23.
126. Gaillard Y, Pépin G. Screening and identification of drugs in human hair by high-performance liquid chromatography—photodiode-array UV detection and gas chromatography—mass spectrometry after solid-phase extraction a powerful tool in forensic medicine. J Chromatogr A 1997;762(1–2):251–67.
127. LeBeau MA, Mozayani A. Drug-facilitated sexual assault: a forensic handbook. Academic Press, 2001.
128. Ahmad FB, Rossen LM, Sutton P. Provisional drug overdose death counts. National center for health statistics 2021;12.
129. Johnson J, Pizzicato L, Johnson C, Viner K. Increasing presence of xylazine in heroin and/or fentanyl deaths, Philadelphia, Pennsylvania, 2010-2019. Injury Prevention 2021;27(4):395–8. https://doi.org/10.1136/injuryprev-2020-043968.
130. Friedman J, Montero F, Bourgois P, Wahbi R, Dye D, Goodman-Meza D, et al. Xylazine spreads across the US: A growing component of the increasingly synthetic and polysubstance overdose crisis. Drug Alcohol Depend 2022;233. https://doi.org/10.1016/j.drugalcdep.2022.109380.
131. Benhalima I, Castex E, Dumont G, Gish A, Hakim F, Allorge D, et al. Hair analysis interpretation in post-mortem situations: Key considerations and proposals to overcome main hurdles. Leg Med 2022;56:102032.
132. AAFS Standards Board 2019. Standard Practices for Method Validation in Forensic Toxicology. 2019.
133. LeBeau MA. ANSI/ASB standard 036 for method validation in forensic toxicology has replaced SWGTOX's version. J Anal Toxicol 2020;44(4):414.
134. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 2003;75(13):3019–30. https://doi.org/10.1021/ac020361s.
135. Moore C, Marinetti L, Coulter C, Crompton K. Analysis of pain management drugs, specifically fentanyl, in hair: Application to forensic specimens. Forensic Sci Int 2008;176(1):47–50. https://doi.org/10.1016/j.forsciint.2007.06.027.
136. Staeheli SN, Baumgartner MR, Gauthier S, Gascho D, Jarmer J, Kraemer T, et al. Time-dependent postmortem redistribution of butyrfentanyl and its metabolites in blood and alternative matrices in a case of butyrfentanyl intoxication. Forensic Sci Int 2016;266:170–7. https://doi.org/10.1016/j.forsciint.2016.05.034.

137. Guerrieri D, Rapp E, Roman M, Druid H, Kronstrand R. Postmortem and toxicological findings in a series of furanylfentanyl-related deaths. J Anal Toxicol 2017;41(3):242–9. https://doi.org/10.1093/jat/bkw129.
138. Ramírez Fernández M del M, Wille SMR, Jankowski D, Hill V, Samyn N. Development of an UPLC–MS/MS method for the analysis of 16 synthetic opioids in segmented hair, and evaluation of the polydrug history in fentanyl analogue users. Forensic Sci Int 2020;307. https://doi.org/10.1016/j.forsciint.2019.110137.
139. Gaulier JM, Richeval C, Phanithavong M, Brault S, Allorge D, Dumestre-Toulet V. A case report of carfentanil-related fatality in France. Toxicologie Analytique et Clinique 2019;31(4):323–31. https://doi.org/10.1016/j.toxac.2019.01.002.
140. Larabi IA, Martin M, Fabresse N, Etting I, Edel Y, Pfau G, et al. Hair testing for 3-fluorofentanyl, furanylfentanyl, methoxyacetylfentanyl, carfentanil, acetylfentanyl and fentanyl by LC–MS/MS after unintentional overdose. Forensic Toxicol 2020;38(1):277–86. https://doi.org/10.1007/s11419-019-00502-0.
141. Freni F, Moretti M, Radaelli D, Carelli C, Osculati AMM, Tronconi L, et al. Determination of fentanyl and 19 derivatives in hair: Application to an Italian population. J Pharm Biomed Anal 2020;189. https://doi.org/10.1016/j.jpba.2020.113476.
142. Allibe N, Paysant F, Willeman T, Stanke-Labesque F, Scolan V, Eysseric H. Ocfentanil testing in hair from a fatality case: Comparative analysis of a lock of hair versus a single hair fiber. Forensic Sci Int 2021;326. https://doi.org/10.1016/j.forsciint.2021.110937.
143. Neukamm MA, Halter S, Auwärter V, Schmitt G, Giorgetti A, Bartel M. Death after smoking of fentanyl, 5F-ADB, 5F-MDMB-P7AICA and other synthetic cannabinoids with a bucket bong. Forensic Toxicol 2023. https://doi.org/10.1007/s11419-023-00666-w.
144. Jalal H, Buchanich JM, Sinclair DR, Roberts MS, Burke DS. Age and generational patterns of overdose death risk from opioids and other drugs. Nat Med 2020;26(5):699–704. https://doi.org/10.1038/s41591-020-0855-y.
145. Schiller EY, Goyal A, Mechanic OJ. Opioid overdose. 2017.
146. Lyons RM, Yule AM, Schiff D, Bagley SM, Wilens TE. Risk Factors for Drug Overdose in Young People: A Systematic Review of the Literature. J Child Adolesc Psychopharmacol 2019;29(7):487–97. https://doi.org/10.1089/cap.2019.0013.
147. Siegler A, Tuazon E, Bradley O'Brien D, Paone D. Unintentional opioid overdose deaths in New York City, 2005–2010: A place-based approach to reduce risk. International Journal of Drug Policy 2014;25(3):569–74. https://doi.org/https://doi.org/10.1016/j.drugpo.2013.10.015.
148. Nurco DN. A Long-Term Program of Research on Drug Use and Crime. Subst Use Misuse 1998;33(9):1817–37. https://doi.org/10.3109/10826089809059323.

149. Lozier MJ, Boyd M, Stanley C, Ogilvie L, King E, Martin C, et al. Acetyl Fentanyl, a Novel Fentanyl Analog, Causes 14 Overdose Deaths in Rhode Island, March–May 2013. Journal of Medical Toxicology 2015;11(2):208–17. https://doi.org/10.1007/s13181-015-0477-9.
150. Watanabe S, Vikingsson S, Roman M, Green H, Kronstrand R, Wohlfarth A. In vitro and in vivo metabolite identification studies for the new synthetic opioids acetylfentanyl, acrylfentanyl, furanylfentanyl, and 4-fluoro-isobutyrylfentanyl. AAPS J 2017;19:1102–22.
151. Prekupec MP, Mansky PA, Baumann MH. Misuse of novel synthetic opioids: a deadly new trend. J Addict Med 2017;11(4):256.
152. Alexander RS, Canver BR, Sue KL, Morford KL. Xylazine and Overdoses: Trends, Concerns, and Recommendations. Am J Public Health 2022;112(8):1212–6. https://doi.org/10.2105/AJPH.2022.306881.
153. Friedman J, Montero F, Bourgois P, Wahbi R, Dye D, Goodman-Meza D, et al. Xylazine spreads across the US: A growing component of the increasingly synthetic and polysubstance overdose crisis. Drug Alcohol Depend 2022;233:109380. https://doi.org/https://doi.org/10.1016/j.drugalcdep.2022.109380.
154. Font-Mayolas S, Calvo F. Polydrug definition and assessment: the state of the art. Int J Environ Res Public Health 2022;19(20):13542.