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Review

Heavy Metals, Halogenated Hydrocarbons, Phthalates, Glyphosate, Cordycepin, Alcohol, Drugs, and Herbs, Assessed for Liver Injury and Mechanistic Steps

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Abstract

Aluminum, arsenic, cadmium, chromium, cobalt, copper, iron, lead, mercury, nickel, thallium, titanium, zinc, carbon tetrachloride, phthalates, glyphosate, alcohol, drugs, and herbs are under discussion having the potential to injure the human liver, but allocation of the injury to the hepatotoxicant as exact cause is difficult for physicians and requires basic clinical knowledge of toxicology details. Liver injury occurs at a variable extent depending on the dose, mostly reproducible in animal models that allow studies on molecular steps leading to the hepatocellular injury. These exogenous hepatotoxins may cause an overproduction of reactive oxidative species (ROS), which are generated during microsomal or mitochondrial oxidative stress from incomplete oxygen split and trigger the injury if protective antioxidant capacities are reduced. Primary subcelluar target organelles involved are liver mitochondria through lipid peroxidation of membrane structures and the action of free radicals such as singlet radical $^1O_2$, superoxide radical $HO•_2$, hydrogen peroxide H_2O_2, hydroxyl radical HO•, alkoxyl radical RO•, and peroxyl radical ROO•. They attempt covalent binding to macromolecular structural proteins. As opposed to inorganic chemicals, liver injury due to chemicals with an organic structure proceedes via the hepatic microsomal cytochrome P450 with its different isoforms. In sum, many exogenous chemicals may have the potential of liver injury triggerd by overproduced ROS leading primarily to impairment of mitochondial functions in the course of structural mitochondial membrane dearrangement. As clinical data were often incomplete, future clinical prototols should focus on meeting liver injury criteria, exclusion of alternative causes, a robust causality evaluation management, and obtaining liver histology if clinically indicated and of benefit for the patient.

Keywords: heavy metals; carbon tetrachloride; phthalates; glyphosate; cordycepin; alcohol; drugs; herbs; RUCAM

1. Introduction

Severe liver injury may be caused be a variety of chemicals of either exogenous or even endogenous origin, which are commonly the result of hereditary metabolic abnormalities leading to accumulation of toxic chemicals. As an example, toxic endogenous chemicals such as iron are found in severe liver diseases like primary hemochromatosis due to genetic overload of iron [1,2], Wilson disease caused by copper overload [3,4], metabolic diseases associated with disturbances of the porphyrin metabolism [5], or the broad spectrum of pediatric liver diseases based on genetic background [6]. As opposed, particular challenges in experimental studies and clinical practice are liver injuries caused by exogenous chemicals such as alcohol [7,8], occupational and household organic chemicals [9], pollutants and contaminants [10–12], nature based products like mushrooms [13], conventional drugs [8,14,15] and finally herbs including herbal medicines [8,15,16].

This review attempts to provide an overview on the abundant chemicals implicated in the development of liver injury and discusses mechanistic steps of the liver injury, which humans may suffer from under short-term conditions or during most of their life span, whereby the focus is on hepatic toxicity rather than on tumorigenicity. The selection of compounds analyzed was based on potential clinical priority.

2. Literature Search and Source Clinical

The PubMed database and Google Scholar was searched for articles by using the following key terms: Liver injury AND heavy metals, AND carbon tetrachloride, AND phthalates, AND glyphosate, AND alcohol, AND drugs, AND herbs. These terms were used alone or in combination. Limited to the English language with a few exemptions, publications from each search terms were analyzed for suitability of this review article. Publications were complemented from the large private archive of the authors. The final compilation consisted of original papers, consensus reports, and review articles with the most relevant publications included in the reference list of this review.

Table 1. Thresholds of ALT and ALP and typical features in patients with liver adaptation and liver injury.

Description	Thresholds of liver tests	Criteria and characteristic features
Liver adaptation	ALT $\leq$5 times of ULN and/or ALP $\leq$2 times of ULN	• Develops at low doses of a chemical • Presumably the majority of chemicals have the potency of causing rare but clinically not apparent liver adaptation • No signs of liver injury in histology • Normalization or stabilization of liver tests is commonly observed whether the chemical is stopped or continued
Idiosyncratic liver injury	ALT $\geq$5 times of ULN and/or ALP $\geq$2 times of ULN	• Develops at low doses of a chemical • Signs of liver injury found in histology • Cessation of use is mandatory and immediate • Worsening if chemical is continued • Most drugs cause idiosyncratic DILI, herbs rarely cause idiosyncratic HILI • Risk of acute liver failure • Develops at high dose of a chemical • Signs of liver injury found in histology • Cessation of use is mandatory and immediate
Intrinsic liver injury	ALT $\geq$5 times of ULN and/or ALP $\geq$2 times of ULN	• Caused by most chemicals but rarely by drugs or herbs • Risk of acute liver failure

Abbreviations: ALT, Alanine aminotransferase; ALP, Alkaline phosphatase; DILI, Drug induced liver Injury; HILI, Herb induced liver injury; ULN, Upper limit of the normal range.

3. Case Definitions

3.1 Hepatotoxicants and Hepatotoxins

In this article the focus is on exogenous toxins affecting the liver of humans and animals. The terms of hepatotoxicant and hepatotoxin may include compounds poisonous to the liver despite differences of their origin. More specifically, a toxicant is any toxic substance, man-made or naturally occurring, found as contaminant in the air, soil, water, or food [17]. As opposed, a toxin in a narrower sense is a poison produced naturally by an organism such as animals, plants, fungi, algae, or insects [18]. In a broader sense, both poisonous chemical are often termed uniformly as toxins without further specification. Exogenous chemicals are substances of any nature that are unrelated to the organism basal metabolism, yet they can interfere with metabolic processes causing harm [19].

3.2 Liver Adaptation or Tolerance versus Liver Injury

Interactions between chemicals and the liver may lead to liver adaptation, also known as tolerance, or to liver injury. However, the variability of chemical structures, mechanistic steps leading to modification of liver functions, and various target cells with different organelles makes it difficult to provide for all chemicals uniformity of liver tests (LTs) thresholds, liver histology findings, and clinical features, let alone the range of amounts taken up, the individual power of toxicity inherited in each chemical, and the specific susceptibility of the exposed individuals with their genetic polymorphisms. Best studied among all chemicals are drugs and herbs [20–22], allowing for general description of diagnostic criteria and clinical features.

Liver adaptation or tolerance are conditions whereby the liver takes care metabolically of small amounts without showing overt injurious effects known in some patients under treatment with drugs or herbal medicine [20,21]. Patients with liver adaptation commonly have alanine aminotransferase (ALT) values $<$5 times of the upper limit of the normal range (ULN) or alkaline phosphatase (ALP) $<$2 times of the ULN, whereas thresholds of real liver injury are above these values [21]. Of note, serum ALT and ALP activity increases are not necessarily liver specific as they are occasionally due to injury of other organs, facts to be considered for case evaluations. Thresholds and characteristic features of liver adaptation and injury caused primarily by drugs and herbs may be considered as framework of various chemicals allowing for refinement and are summarized (Table 1).

What really characterizes idiosyncratic liver toxicity (Table 1) is that it does not follow a clear dose-response and may appear at doses, which otherwise are well tolerated by the vast majority of individuals [20–22]. Intrinsic hepatotoxins act on any individual in a clear dose-response relationship.

3.3 Liver Injury Pattern

In analogy to drugs and herbs, which cause a hepatocellular, cholestatic, or mixed injury [22], other toxins exert these types of liver injury pattern, called also phenotypes. Criteria of liver injury pattern are primarily not necessarily based on liver histology obtained through invasive liver biopsy but rather than on analysis of serum LTs, using the

R (ratio), obtained by using multiples of ULN of ALT and ALP to be divided as ALT:ALP [22]. The liver injury is hepatocellular if R $\geq$5, the liver injury is cholestatic if R $\leq$2, and the liver injury is mixed if 2 $<$ R $<$ 5. However, if the liver injury pattern needs ascertainment or discrimination from other, treatable liver diseases by means of a liver histology, a liver biopsy may be considered if the patient will likely benefit from new data.

3.4 Dose Dependent Intrinsic DILI versus Dose Independent Idiosyncratic Liver Injury

Exogenous chemicals entering the liver cells in small amounts are usually well handled by the liver and metabolically degraded to harmless end products. However, larger amounts of potentially toxic chemicals can lead to severe liver injury, requiring differentiation between the dose dependent and dose independent liver injury, shown, for instance, for drug induced liver injury (DILI) [20,21] and herb induced liver injury (HILI) [16,21,22].

The dose dependent and thereby intrinsic liver injury is predictable, observed in animal models and humans, whereby mechanistic steps are commonly well known as based on results derived from animal studies easily transferrable to human conditions [20,21]. As opposed, the dose independent and thereby idiosyncratic liver injury is confined to susceptible humans, which is variable from one individual to the other due to their genetics [21]. This type of injury in not predictable and not reproducible in animals lacking human genetic characteristics.

3.5 Exclusion of Alternative Causes

In the clinic setting and office of practitioners, a variable number of patients show increased LTs of initially unknown cause to be further evaluated by the physicians. Whenever in humans a liver disease by toxins is suspected, other differential diagnoses have to be ruled out [22]. In the initial diagnostic stage, a careful past medical and exposure history including occupation background is mandatory and may help establish an initial diagnosis, followed by sophisticated specific diagnostic approaches.

3.6 Diagnostic Challenges

A number of exogenous chemicals like ethanol, heavy metals, and 1,2-unsaturated PAs cause histological signs of liver injury associated with normal or only marginal increases of LTs, presenting a particular challenge in clinical practice as valid routine laboratory tests are rarely available. For liver injury by these chemicals, diagnosis can be established by clinical features or liver histology, while specific diagnostic biomarkers are rarely available, except for intrinsic liver injury caused by paracetamol or pyrrolizidine alkaloids. In other words, normal or marginally increased LTs do not exclude liver injury by these chemicals.

4. General Mechanistic Considerations

4.1 Inorganic and Organic Chemicals

Potential hepatotoxic compounds present either with an inorganic or an organic chemical structure. Under ideal conditions, both types of chemicals, if taken up by humans, should leave the body immediately to prevent or at least reduce harmful accumulation. However, inorganic chemicals such as most heavy metals are problematic because they are not biodegradable and tend to hepatic bioaccumulation associated with the risk of injury, effects best attributed to the lack of mechanisms facilitating excretion or metabolic degradation [23]. Exogenous products with an organic chemical structure such as most conventional drugs may undergo metabolic modification through hepatic microsomal cytochrome P450 (CYP) via its isoforms [24–28] as phase I reaction (oxidation) [24]. They are only partly excreted via the urine or bile, either unchanged or following conjugation as phase II reaction [24,29], to make them water soluble [29], whereas conjugated hydrophilic metabolites can also leave the hepatocytes through excretion via the plasma membrane in a phase III reaction [24].

4.2 Reactive Oxygen Species as a Significant Intermediate Product

Both, inorganic and organic chemicals, share in the context of cellular oxidative stress the feature to generate ROS (reactive oxygen species), representing a bundle of important intermediate products responsible for mechanistic steps leading to liver injury [23,24,30,31]. Depending on the causing chemical, ROS includes a variety of toxic radicals such as singlet radical $^1O_2$, superoxide radical HO•$_2$, hydrogen peroxide H_2O_2, hydroxyl radical HO•, alkoxyl radical RO•, and peroxyl radical ROO• [32,33]. Some of these radicals may covalently bind to macromolecules and structural proteins or phospholipids of liver cell biomembranes such as microsomes [30,32,33] obtained from the endoplasmic reticulum by ultracentrifugation of liver homogenates, mitochondria, and plasma membranes, whereby lipid peroxides formed from unsaturated fatty acids initiate liver injury [33]. This occurs through damage of the membrane integrity leading to functional impairment. ROS is generated by parenchymal and non-parenchymal cells of the liver and at various subcellular sites within the hepatocyte [30,31,34]. If injurious ROS is generated by microsomes, it is microsomal oxidative stress, and if mitochondria are involved, mitochondrial oxidative stress is the commonly used term [32].

4.3 Hepatic Immune System, Bile Salt Export Inhibition, and Gut Microbiome

These topics are of specific relevance for DILI and are discussed below under 10. Synthetic drugs.

5. Heavy Metals

Heavy metals are commonly defined as elements, which are characterized by an atomic number of >20 and an atomic density >5 g cm^{-3}. Meeting these criteria, 84 heavy metals are presently known, but only part of these are relevant to human health with respect to potential risk of liver injury, worth to be discussed in this review article. Selected metals were considered in this report, virtually all were heavy metals with the exception of aluminum, which shows clinical and pathogenetic features similar to heavy metals but does not meet their classic criteria.

Patients experiencing intoxications by heavy metals often suffer from injuries of a variety of organs [23,35–38] including rare carcinogenicity [23,35,36], not discussed in the frame of the current article that focuses on the liver injured by heavy metals.

5.1 Aluminum

Aluminum (Al) is occasionally also termed as aluminium and can enter preferentially as aluminum phosphide the human body via inhalation of aerosols or particles, skin contact, vaccination, drinking water, ingestion of food or drugs like antacids, products containing aluminum like dialysis fluids or infusions, or occupational exposure to aluminum [37,38]. Aluminum is mostly excreted through the urine with low amounts leaving the human body via the bile [39].

Data on increases of serum ALT activities in patients with aluminum intoxications are limited or not reported [37,38,40]. In a single publication of 104 patients with aluminum poisoning, around 48% had increased serum ALT activities versus around 36% showed increased AST values but liver injury causality for aluminum was not verified using the Roussel Uclaf Causality Assessment Method (RUCAM) [40]. In more detail but without providing ULNs, means $\pm$ SD were provided for ALT (23.19 $\pm$ 26.78 U/L) and for AST (22.51 $\pm$ 15.98 U/L). As a consequence of this study, only part of the patients with aluminum phosphide intoxication experienced increased LTs [40], whereas in other studies on acute intoxications higher LT values were reported [41,42].

Histopathological changes of the liver in patients with aluminum phosphide poisoning include central venous congestion, degeneration, hemorrhage, sinusoidal dilation, bile stasis, centrilobular necrosis, Kupffer cell hyperplasia, infiltration by mononuclear cells and fatty change [43] shown as liver steatosis [37].

Mechanistic steps leading to experimental or clinical liver injury due to high amounts of aluminum phosphide, classified as prooxidant [37], are the consequence of biological oxidation processes causing destructive changes in the hepatocytes obviously triggered by the early generation of lipid peroxidation via reactive oxygen species (ROS) [37,38]. Their overproduction reduces hepatic glutathione content as well as the activities of glutathione S-transferase, and catalase, with the consequence that the liver is not any more sufficiently protected against toxic ROS produced via microsomal and mitochondrial oxidative stress [37,38]. As a result, aluminum produces ROS dependent oxidative injury to proteins and DNA in the hepatocyte. If the focus on liver injury is more on the rough endoplasmic reticulum, a reduction of protein synthesis is to be expected, whereas an injurious focus on the smooth endoplasmic reticulum will impair microsomal drug metabolizing enzymes and reduce the content of CYP isoforms [37]. Targeting liver mitochondria via mitochondrial stress will cause anaerobic respiration and impair both, the tricarboxylic acid cycle function and the electron transport chain reaction, leading to a reduction in ATP production through oxidative phosphorylation; changes are also described for iron depletion in the mitochondria and impaired homeostasis of calcium [37].

5.2 Arsenic

Arsenic is frequently found in the environment [36,44,45] including the atmosphere originating from natural sources such as volcanic eruptions and industrial processes [44]. This heavy metal is also a contaminant of soils [45], surface water [45] and ground water used as drinking water [44,46,47], and food [44,46]. In addition, arsenic was used in chemical warfare agents [44,45] and is a known component of some conventional or traditional medicines [36,44,45,48–51].

Prolonged and acute ingestion of arsenic leads to toxicity of many organs including the liver [44–47], associated with clinical features of arsenic poisoning [44–46,49,51]. Increased LTs such as ALT, AST or ALP have variably been published [49,50,52–54] with significantly higher values in patients with high arsenic exposure [53]. They were best documented, for instance, in two patients under a herbal, arsenic containing therapy with arsenical medication [54] with LT values meeting criteria of both, liver injury required for HILI (Table 1) and RUCAM based causality gradings of at least probable [22]. Evaluating the cases of these two patients in more detail, the first patient (68 years old, male) was on the arsenic containing remedy for 32 days and showed increased LT values of ALT 473 U/L, AST 498 U/L, and ALP 188 U/L, whereas the second patient (48 years old, female) was on the arsenic medication for 10 days and showed LT increases of ALT of 1122 U/L, AST 898 U/L, and ALP 364 U/L [54].

Light microscopical liver histology due to prolonged arsenic uptake by patients is characterized by liver fibrosis leading to clinical non-cirrhotic portal hypertension [55–58], whereby studies on the wedged hepatic vein pressure indicated that the obstruction to portal flow resided in the portal tracts [56]. Histology features of the liver include portal tract fibrosis [56–58] and an increase in the number of portal veins with thickening and hypertrophic walls in some reports [56,58]. A few inflammatory cells were described and mild fatty change in the surrounding hepatocytes [58],

a finding subsequently confirmed by electron microscopy [51].

Under experimental conditions using electron microscopy, at 24 hr. after acute arsenic exposure as well as following chronic arsenic use in rats resulted in hepatic necrosis and apoptosis [59,60]. In mice, prolonged application of arsenic resulted in an increased number of autophagosomes shown by transmission electron microcopy [60] and after a 7 day application hepatic mitochondrial damage was observed including organelle swelling, expansion of the matrix space, and fragmented or disorganized cristae [61].

Arsenic exists as metalloid (As^0) [36] and is found in the environment mainly as the inorganic As^{3+}, the arsenite [36,53], which is more toxic than As^{5+}, the arsenate [53]. Other forms include organic arsenic and arsine (AsH_3) [36]. The liver is the primary target organ not only for toxicity but also for the metabolism of arsenicals with its major metabolic pathway via methylation [36,53], which leads to the methylated intermediate of monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) [53]. Liver injury of arsenic is thought to result from its binding with sulfhydryl groups of enzymes and membrane proteins, conditions that lead to cross linkage [36,53,62,63]. Contributing factors of arsenic liver .injury are oxidative DNA, acquired tolerance to apoptosis, enhanced cell proliferation, altered DNA methylation, and genomic instability [53,64]. Most of these alterations were confirmed in an experimental model using zebrafish (*Danio rerio*) intoxicated with arsenic and evaluated for metabolomic changes [65]. In addition, ROS was implicated in the liver injury [36,65].

Arsenic speciation was analyzed in the liver of a patient with fatal arsenic intoxication [66]. Among the tested organs, the liver showed the highest arsenic concentration of total arsenic. As^{3+} was the predominant form, with a higher concentration of MMA compared with DMA. Several enzymes localized in the cytosol of the hepatocytes are involved in the metabolism of arsenicals: MMA is synthesized through the arsenite methyltransferase, whereas DMA is synthesized via the DMA methyltransferase [66,67].

5.3 Cadmium

Cadmium is found in the environment [23,35,68,69], municipal solid waste landfills [68,69], electronic waste [70], soils and plant parts such as leaves, stems, and roots [68] and is commonly used in the industry [23,35] as alloys, pigments, stabilizers, and batteries [35]. Human exposure may occur via inhalation including cigarette smoking as well as by food [35] such as contaminated plants and vegetables [68] like potatoes, grains, seed, or mushrooms, in addition to crustaceans, mollusks, shellfish, mussels, cocoa powder, and dried seaweed [35].

LTs such as ALT, AST, and ALP in humans following acute or prolonged exposure are reported with variable and mostly low values, often interpreted by the authors as signs of liver injury or liver disease [71–76]. However, published LT values commonly did not meet diagnostic criteria and thresholds of real liver injury as required (Table 1), and occasionally normal ranges of the listed parameters were not provided. Low LT values in these patients are either due to low cadmium exposure or may reflect liver diseases that have nothing to do with arsenic, as causality assessment using RUCAM was not performed. Based on association studies analyzing serum cadmium levels, there is also the proposal that cadmium may cause hepatic steatosis and fibrosis [76], classified as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) [71], now known as metabolic associated fatty liver disease (MAFLD) and metabolic associated steatohepatitis [77,78].

Light microscopy of the liver specimens obtained from patients with chronic cadmium exposure showed significantly more fibrosis and hemosiderosis as compared with nonexposed controls [79]. Fibrosis was periportal, portal, perisinusoidal, or bridging. Additional features included inflammation, hepatitis, and macrovesicular or microvesicular steatosis. These light microscopy changes in humans were grossly confirmed in corresponding animal models [80,81]. In other experimental microscopy studies diffuse hepatocellular degeneration and necrosis was observed, described also as hemorrhagic lesions and coagulative necrosis, associated with high plasma ALT activities and high cadmium concentration in the liver [82]. The reported vacuolar degeneration was not further discussed but may represent vacuoles corresponding to empty fat cells following staining with hematoxylin and eosin. Histopathological observations confirmed extensive parenchymal degeneration [83].

Transmission electron microscopy showed the presence of autophagosomes and dilated endoplasmic reticulum, which represents the microsomal fraction of the biochemists, and translucent vesicles and structural changes of the hepatic comprized the sinusoids and were replaced by large gaps [84]. Additional ultrastructural studies showed proliferation of the endoplasmic reticulum and lysosomes [83] and confirmed the presence of autophagosomes [82, 85].

The mechanism of liver injury caused by cadmium follows several steps: (1) the ionic form of Cd^{2+} binds to the protein metallothionenin (MT) to form the Cd metallothionenin (Cd-MT) complex, which allows for cadmium deposition in the liver and downgrades glutathione production; (2) this is associated with an increased oxidative stress and mitochondrial injury, leading to MAFLD due to lipid deposition in the liver; (3) finally, MASH may develop following autophagy modulation, a process that facilitates removal of damaged liver cell organelles [86].

5.4 Chromium

Chromium is a known pollutant with substantial accumulation, for instance, in municipal solid waste landfills

contaminating surface water, groundwater, soil, and plants in their vicinity [68]. In this context, the vegetable *Ipomoea aquatica*, commonly consumed by nearby villagers, was heavily contaminated by chromium, as its accumulation was elevated to much greater levels compared with WHO standards. In the grass *Pennisetum purpureum* with its deeper growth of the rhizome, even higher amounts in all plant parts were found than in *Ipomoea aquatica*. Consequently, there is a high risk that vegetables contaminated with chromium or contaminated grass via cattle may enter the food chain [68]. Pollution in the environment with various forms of chromium can be traced back to uses in the chemical industry, production of dyes, wood preservation, leather tanning, chrome plating, and alloys [87]. In addition, stainless steel contains up to 20% chromium, and occupational exposure to chromium among workers of the chromium industry may be high, whereas inhalation due to ambient pollution with small chromium containing particles due to emissions from cars may cause additional risks [87]. As medicine, chromium picolinate is used in some countries [88,89].

Little information exists on LT elevations in patients following exposure to chromium. However, in one patient with over-the-counter (OCT) use of chromium picolinate ranging from 1.200 to 2.400 mg daily for 4–5 months, substantial increases of LTs such as ALT and AST 15–20 times the ULN were recorded [89], meeting clearly the liver injury criteria (Table 1). Following cessation of the medication use, LTs returned to normal values documented before therapy initiation. In experimental studies using a rat model, potassium chromium application resulted in significant increases of serum ALT and AST activities compared with non-treated animals [90].

Liver histology of patients under chromium therapy is not available [89], as is of electroplating workers exposed to hexavalent chromium [90]. Light microscopy in the liver of rats exposed to potassium chromium showed hepatocytes with degenerated vacuolated cytoplasm, dilated congested portal veins, bile duct proliferation, and dilated sinusoids [91]. There are no data of electron microscopy of the liver in patients under chromium therapy [89] nor in workers exposed to chromium [90]. However, electron microscopical studies in experimental animal showed injurious changes of mitochondrial membranes with swelling, and cristae fracture [92,93] as well as dilatation of the rough endoplasmic reticulum in the hepatocytes, associated with injury of endothelial cells [92].

In line with mechanistic steps leading to hepatotoxicity by other heavy metals, the liver injury caused by chromium is primarily due to ROS generated from oxidative stress, which initiates apoptosis of liver cells [36,52,87,90,93]. There is now sufficient experimental evidence that chromium triggers apoptosis and promotes inflammation by inhibiting the deacetylation of SIRT1 [93], which stands for sirtuin as a member of a protein family involved in signaling metabolic regulation.

5.5 Cobalt

Cobalt is found with two valances, $Co^{2+}$and Co^{3+}, whereby Co^{2+} is the most commonly valance used in the chemical industry and the valance Co^{3+} is widely found in the nature as arsenides, oxides, or sulfides [94]. There are no cobalt data on municipal solid waste landfills [68], confirmed more recently [69]. For the general population, food is the primary exposure to cobalt [94–96], rarely also if used as cobalt containing medicine [97]. Patients with a metal-on-metal hip implant are at high risk of cobalt exposure [96,98]. Occupational exposure occurs via inhalation of dust [95] in workers of cobalt manufacturing industry producing cobalt superalloys [94,99] and among plate painters exposed to cobalt blue dyes [100].

Information on serum ALT activities in individuals exposed to cobalt is scattered, occasionally mild to moderate liver enzyme elevation were published, but, in general, lacking robust evidence of liver injury by cobalt led to the conclusion that hepatotoxicity in humans is an uncommon feature of cobalt toxicity due to a high toxicity threshold based on current literature [101]. In addition, ALT values were not provided even in a large Belgian epidemiological study of workers exposed to cobalt oxides, cobalt salts, and cobalt metal [102], confirmed by a similar study of Italy [103]. As opposed, serum ALT and AST activities were significantly increased following cobalt application at high doses [104–106].

Pathological light microscopy results of the liver in individuals exposed to cobalt are not available [95,96,98–103], missing even in a patient, who presented high blood cobalt levels, detectable metallic cobalt in hepatic macrophages, normal LTs, and no liver fibrosis or signs of liver injury [107]. In animals treated with cobalt, however, liver histology showed an infiltration of mononuclear cells and vascular congestion [105] as well as central vein displacement and injury of hepatocytes [104]. Data on transmission electron microscopy of the liver of cobalt exposed humans are not available, but in animals, apoptotic hepatocytes, nuclear malformation, and destroyed mitochondrial cristae of the hepatocytes prevailed [104].

Mechanistic steps leading to experimental liver injury by cobalt exposure are well studied in animals [105,106,108]. Cobalt initiates ROS generation via oxidative stress in isolated liver mitochondria, leading to permeability transition and apoptosis in hepatocyte cultures [108]. Other studies focused on increased lipid peroxidation and alteration of the antioxidant system with a decline of superoxide dismutase and glutathione peroxidase activities or reduced glutathione content in the liver as contributing factors of the liver injury [105].

5.6 Copper

Copper (Cu, from the Latin: cuprum) is commonly found in the environment [68,109–112] such as in solid waste fills [68], soil [68,112,113], drinking water [114], and the atmosphere [113] and is used in the agriculture [111,115] with preference in viticulture to protect grapes from downy mildew [115] and in industry like copper smelters, iron and steel production, and municipal incinerators [113]. Of note, overall environmental copper pollution is well documented using the attic dust approach, which provides some kind of an archive of historical air contamination by copper of the urban environment [109].

Serum activity of ALT was 51 U/L and of AST 190 U/L in a patient with prolonged exogenous copper exposure for 10 years, who used up to 8 mg copper daily dose for treating copper deficiency (human Swayback disease), whereby this iatrogenic copper overload led to liver transplantation due to compensated cirrhosis [116]. No LT values have been reported in patients with acute renal failure following copper sulphate intoxication [117]. Similarly, no ALT or AST values are available in copper workers. Wilson disease is a genetic disorder of the liver leading to hepatic copper accumulation [3,4], and the earlier termed Indian childhood diseases were in fact cases of Wilson disease rather than caused by exposure to exogenous copper in drinking water or milk as previously assumed by error [114]. Experimental studies in animals showed mostly unchanged or in rare cases slightly increased serum ALT and AST activities after application of high copper amounts [118–120].

In the patient with overdosed copper exposure, transjugular liver biopsy demonstrated upon light microscopy ongoing portal and segmental inflammation with ballooning degeneration of the hepatocytes as well as diffuse hepatocellular copper accumulation on rhodamine stain following prolonged copper overdose [116]. The diagnosis of cirrhosis was first established at the occasion of a laparotomy for umbilical hernia repair as evidenced by macroscopical morphology and later confirmed at the time of liver transplantation, showing both micro and macro nodules of the liver surface. Analysis of hepatic content of copper provided very high values, suggesting copper as the causative agent in this patient. In animals, however, overdosed copper administration caused no liver injury as assessed by light microscopy [119–121] or only minimal, partially dose dependent changes such as small vacuoles of hepatocytes, hepatocyte swelling, inflammatory cells, or sinusoidal congestion [118]. Electron microscopy data on the liver of the patient exposed to high amounts of copper were not available [116,117] but have been reported in animal studies as irregularly shaped nuclei, abundant mitochondria, and displayed cristae, and hepatocytes with inclusion of secondary lysosomes [120].

A broad range of proposals how copper causes mechanistically liver injury have been published, based on human or animal data [113,116]. However, clear pathogenetic concepts on copper hepatotoxicity from excess consumption are not well characterized [122]. Despite some uncertainties, copper injury is assumed as the result of the interaction between the reduced form of copper and oxygen, leading to ROS comprising superoxide anion, hydrogen peroxide, and hydroxyl radicals, all generated through oxidative stress and seemingly capable to trigger at least partially the injury [116]. However, copper hepatotoxicity is not just oxidative stress as zinc may be a major cofactor in various cellular processes of this copper liver injury [122]. There is also the note that free, unbound copper is the most toxic form of copper [122]. It remains to be establish whether animal models [123,124] including the goldfish model [121] or the zebrafish model [125] can contribute to close the existing mechanistic gaps of excess copper liver injury.

5.7 Iron

Iron (Fe from Latin: ferrum) is a pollutant found as the most abundant metal in the atmosphere [126,127] including as aeolian dust derived from iron containing Sahara sand considered as the primary iron source to the open ocean, with potential benefit by removing the major greenhouse gas CO_2 from the atmosphere [128]. There is also the notion on iron found in historical attic dust [109], the vicinity of steel fabrics [129,130], in surface or ground water [131], soil [132], and together with microplastics [133]. Fe data on municipal solid waste landfills were not available [68,134] including e-waste [70], and iron seemingly is not considered as pollution waste risk [69].

Increased serum ALT activities $>$40 U/L were reported in 10.8% among 7031 steelworkers in China as compared with AST in 2.8%, findings likely attributed to exogenous iron exposure [135]. This condition has to be differentiated from hereditary hemochromatosis, a human disease that is based on a genetic abnormality causing increased iron levels preferentially in the liver [1,2,136]. Increased serum ALT activities have variably been reported in different animal models following iron administration [137–144], occasionally causing activities of ALT $>$600 U/L associated with similar values of AST [137].

Liver histology data of steelworkers exposed to iron were not available [135], because there is no indication in the clinical context to perform a liver biopsy, an invasive diagnostic procedure with some health risks. However, in the liver of animals exposed to high iron amounts, several histology features have been presented upon light microcopy [137,139]. Iron excess leads to ballooning injury of hepatocytes, MASH, iron deposits in the reticuloendothelial system (RES) [137] rather than hepatocytes with only slight iron staining consistent with cytosolic ferritin [137,139]. Electron microscopy liver results of steelworkers have not been published, in line with expectations [135]. However, such ultrastructure data are available from animal models, providing variable results [137]. Key findings include an

increase in size and numbers of organelles such as mitochondria found in the hepatocytes, a borderline MAFLD, but no overt mitochondrial injury, hepatocyte necrosis or apoptosis was described in this particular animal model [137].

Experimental studies of animal models on mechanistic steps leading to liver injury by iron excess suggest a key role of hepatocellular oxidative stress generating ROS responsible for apoptosis, lipid peroxidation, and reduced superoxide dismutase activity [137,141], but studies on iron metabolism using the zebrafish model failed to contribute to new aspects on the mechanistic steps [125]. In addition, proteomic analysis in mice with hepatic iron overload suggests dysregulation of urea cycle, impairment of fatty acid oxidation, changes in the methylation cycle [139], and immune cell activation [137]. Free iron is extremely toxic to cells, but several protective mechanisms exist in the cells aiming to bind the iron, including the transferrin in the blood [145]. In more detail, the toxicity of iron in animal studies is related to the ability of ferrous iron to interact with H_2O_2 to generate the highly reactive hydroxyl radical, called the Fenton reaction [146]. However, whether the Fenton reaction is operative humans remains to be established.

5.8 Lead

Lead (Pb for Latin plumbum) is a common pollutant of the environment [69,147,148], caused, for instance, through the widespread use of leaded gasoline in developing countries [23,147] and lead harbored in municipal solid waste mills [69]. However, environmental lead pollution is not a modern issue but has a long historical background, as evidenced by approaches that quantified lead in the attic dust [109]. Occupational lead exposure is found in battery workers [149] and lead mine workers [150]. Lead is also found in paints, Indian beer, contaminated drinking water and food prepared in lead containing cooking pots [23]. It is a constituent of some herbal medicine as assessed in a systematic review article [151], confirmed more recently in herbal medicines especially of Ayurveda origin [152]. When ingested, the liver is the primary organ of lead accumulation [23].

Increased LTs of serum ALT and AST of variable extent have been described in humans following lead exposure [149,150,153–156]. In a patient under a therapy with herbs containing lead, serum activities of ALT were 230 U/L and AST 200 U/L [153], clearly meeting the criteria of liver injury (Table 1). Serum lead levels were increased in line with the suspected diagnosis of liver injury caused by lead, although HILI as alternative diagnosis was not definitively ruled because no causality assessment using RUCAM was performed for the used herbs. However, the patient experienced abdominal pains commonly found in patients with lead intoxication. Much higher LT values were found in another patients who by ingested by medication error 12 g of lead powder, which resulted in serum ALT activities of 4707 U/L, equivalent to 118 times the ULN considering the reported 40 U/L as the ULN, and AST of 5363 U/L, corresponding to 134 times the ULN based on the 40 U/L reported for the ULN [154], results in line with an established liver injury (Table 1). As opposed, occupational or population studies on prolong lead exposure led to marginally or even no increased ALT or AST values [149,150,154–156]. In animals, lead exposure caused mostly little, if any, change of serum ALT or AST activities [157–160].

Liver light microscopy data of humans exposed to lead were not available [149,150,153–156]. In an animal model of lead inhalation, the liver histology showed meganuclei and an increase in the inflammatory infiltrates [159]. More severe liver injury was observed in other animals following ingestion of lead acetate together with their diet, degeneration and necroses of hepatocytes prevailed with inflammatory cell infiltration, the central vein was dilated and congested along with nearby hemorrhage, hepatocytes appeared irregularly arranged, the liver architecture was disorganized, and the hepatocytes were large with foamy cytoplasm containing numerous vacuoles, nuclei were pycnotic [160]. Results on electron microscopy of the human liver exposed to lead have not been published [149,150,153–156], but ultrastructural data are available in animals exposed to lead [161,162]. Liver injury in rats treated with lead acetate prevailed in the centrilobular regions exposed to high levels of lead, reaching the liver via the gastrointestinal tract after oral intake [161]. Many particles were seen in the cytoplasm of the hepatocytes, likely the result of fragmented rough endoplasmic reticulum and indicating substantial cellular damage, in addition to endothel and Kupffer cells, which sequester electron-dense particles, possibly by endocytosis. In other studies, lead nitrate was mitogenic to hepatocytes, shown by a numerous binucleated hepatocytes [162]. The number of mitochondria was decreased in a dose dependent manner, necrotic changes such as cytoplasmic vacuoles displaced the mitochondria, which showed signs of degeneration.

Mechanistic steps involved in lead liver injury are challenging as is the risk evaluation. The liver is the main target organ in capturing lead, based on animal studies using electron scanning microscopy, coupled with a detection system of x-ray microanalysis involving energy dispersive spectrometry [163]. For risk estimation, quantitative lead analyses can also be done using various methods, shown in the liver [161], blood [148–150,155,156,161,164], soil [68,69], and water contaminated with lead, for which rapid detection of toxic metals in non-crushed oyster shells by portable x-ray fluorescence spectroscopy is feasible [165]. Regarding molecular issues and understanding of lead effects on the liver, consensus exist that ROS as product of oxidative stress plays a major role at initiation of the injury process [23,147,148,150,151,157,166,167]. Lead binds to sulfhydryl group of structural proteins any cy-

tosolic proteins such as glutathione, thereby reducing the antioxidant defense property and enhancing the lead toxicity [75,148,150,157,158,166] through lipid peroxidation of cell membranes such as of mitochondria or endoplasmic reticulum [23,75,148,150,151,164]. The high affinity of lead to protein sulfhydryl groups results in activity of a number of enzyme like catalase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, and superoxide dismutase [150,164], although some contrary results of increased enzyme activities have been reported but the reason of this discrepancy remained unexplained [152,158]. Studies on lead liver injury using the zebrafish model have not yet been done [125].

5.9 Mercury

Mercury, or Hg for formerly named hydrargyrum from the Greek words hydor, water, and argyros, silver [23] has been commonly used or is still rarely used in the industry as mercury barometers and thermometers, as electrodes for electrolysis, and in electronic switches [168]. In addition, with methylmercury and ethylmercury as organic mercury forms, it is or was widely used in agriculture for antifungal purposes in seed grains [168]. As a result, mercury can be detected in the atmosphere [169], solid municipal mills [69], historical attic dust [109], soil [69,170], and water [168]. Due to its worldwide use it became a global pollutant [171,172] with health hazards, shown also at the occasion of the 1956 Minamata tragedy in Japan caused by methylmercury [172,173]. Generated as byproduct during chemical acetaldehyde production in a fertilizer company [172], methylmercury was released for a long time via the Agana River [168] to the nearby shore of Minamata Bay and reaching finally the East China Sea [168,172]. This incident polluted as a classic case of environmental mercury poisoning the marine ecosystem, affecting also fishes such as shellfishes, and humans [172,173]. About 5000 residents who ate seafood from the area died or experienced health problems [172]. As a traditional medicine, mercury was used to treat various diseases or ailments [168], however, this approach is now under discussion regarding high risks and lacking efficacy. As thimerosal, mercury is still in use for the production of vaccines, but its use as amalgam in dentistry is now less common [168,169]. There are different ways of mercury entering the human body, via inhalation [168,169], skin [168,174], or ingestion [168,172,173].

Reported serum LT values due to mercury exposure continue to be inconsistent [175], also when correlating them with blood mercury levels, findings that require critical analyses [176,177]. In fact, respective data heavily depend on variables such as gender, and age, and alcohol use. As an example, there was no relationship between high blood mercury levels and LTs among females, while among males, a significant correlation existed between high blood mercury levels and increased serum activities of both, ALT and AST [175]. In another report mentioning in its title that mercury exposure is associated with a decrease of LTs, but this is obviously only one side of the coin, because it was detailed in the abstract and text that in woman, who drank alcohol more the 2 or 3 times per week, serum activities of ALT increased by 7.7% and of AST by 10.6%, while in men, ALT and AST activities showed little change as blood mercury levels increased [176]. The title of another publication implicates that blood mercury concentrations are associated with a decline in LTs in an elderly population, while in the abstract the conclusion is reached that mercury levels are associated with elevated LTs [177]. At first glance, more promising regarding issues of LTs and mercury exposure seemed the title of a case report, claiming acute liver failure as being induced by daily ingestion of a ceremonial Joss paper from Vietnam, which is typically painted with heavy metals including mercury [178]. On admission, her serum activities were for ALT 4891 U/L and AST 7565 U/L. As confounding variables, the patient had also consumed 1.3 g of acetaminophen and 800 mg of ibuprofen prior to presentation [178], but causality for these drugs and mercury was not verified by the updated RUCAM [22]. Clinical course was lethal, liver sections from autopsy showed steatosis, inflammation, and necrosis. Post-mortem examination concluded that the liver failure was due to a combination of acetaminophen use with concomitant mercury ingestion [178]. In animals exposed to mercury, serum activities of ALT were significantly increased by 56.0% and of AST by 14.3% [179], while in other studies ALT activity was only slightly enhanced [180].

Liver histology data of humans exposed to mercury and included in epidemiology studies were, as expected, not available [175–177]. In the patient with suspected mercury intoxication in connection with the consumption of the ceremonial Joss paper, liver histology was obtained during the clinical course through ultrasound guided liver biopsy, which revealed macrovesicular steatosis [178]. In animals, which had been exposed to mercury, liver histology showed deformed, degenerated, and necrotic hepatocytes [179]. Electron microscopy data of the liver are only available from animals exposed to mercury, showing extensive proliferation of the smooth endoplasmic reticulum and dilatation of the rough endoplasmic reticulum, while hepatic mitochondria lost their normal metrical density, cristae, and inner limiting membrane [181].

Mechanistic events leading to liver injury by mercury start with the generation of ROS, causing disruptions in the antioxidant system of defense and lipid peroxidation [23, 180]. Additional pathogenetic aspects are provided from results obtained from *Bryconamzonicus*, a freshwater fish [23] or Zebrafish gills after exposure to mercury chloride [182].

5.10 Nickel

Nickel is known for its environmental and human health toxicology [183,184]. Its extensive distribution in

the environment originates from natural sources and industrial production [184]. Nickel is found in municipal solid waste landfills [68], soil, water and atmosphere [68,184,185], herbal medicine [151], and as historical attic dust [109]. It is a preferred metal in the industry for products such as batteries, electroplating, alloy production, and as catalyst [183,184]. Nickel may enter the human body through inhalation [184] or the food chain via ingestion of plants, which easily take up this metal from contaminated soil [185]. In autopsies of humans not exposed occupationally to nickel, high amounts of this metal was found in the liver, more so in males as compared with females [186].

Increased LT values have been published in nickel-plating workers if compared with non-exposed workers [184]. Expressed as means $\pm$ SD, in exposed workers serum ALT activity was significantly higher when compared with non-exposed controls: 101.1 $\pm$ 6.5 U/L vs 30.1 $\pm$ 0.6 U/L, associated with similar increases of AST: 84.4 $\pm$ 5.7 U/L vs 21.4 $\pm$ 0.3 U/L [184]. Similar enhanced activities of ALT and AST were found in animal studies [187,188].

Liver histology of humans exposed to nickel is not available [184] but was described in animals as necrotic changes of hepatocytes and inflammatory cell infiltration [189]. In a patient with prior acute poisoning by carbonyl nickel, hepatic ultrastructural data have been published but results were confounded by postnecrotic cirrhosis [190]. More robust data of electron microscopy of the liver obtained from animals exposed to nickel showed additional details [191–193], focusing especially on diffuse dilatation of rough endoplasmic reticulum as the most prominent and consistent ultrastructural abnormality, whereas mitochondria remained unaffected [193].

The pathogenetic concept of nickel liver injury goes primarily back to ROS generated from oxidative stress leading to lipid peroxidation [23,194,195]. This is associated with a depletion of the hepatic glutathione levels [23] and nickel accumulation in the liver [196]. Finally, liver injury by nickel nanoparticles can also be triggered by nitrative stress, causing apoptosis and inflammation [197]. Evidence for the nitrative stress was provided by increased specific stress markers like iNOS (inducible nitric oxide synthase) or NO (nitric oxide) [197]. Studies with the zebrafish model failed to provide additional conceptual views on initiation or perpetuation the nickel injury [198].

5.11 Thallium

Thallium with preferential application in the industry [199,200] exerts a high degree of toxicity in humans [201–203] and animals [204]. This why thallium use in households has been forbidden in the US in 1965 and banned commercially in 1975 [199], a few countries subsequently also restricted its use [200]. Known as a historical environmental pollutant, thallium is found in the attic dust [109], while not analyzed in municipal solid waste landfills [68], but currently also detected in electronic waste [205], soil, water, and atmosphere [206], and a known contaminant in herbal medicine products [151] and edible plants [206]. In industry, thallium is used in the semiconductor branch, in optical lenses, and ^{201}Thallium chloride as one of its isotopes for cardiovascular scintigraphy to detect stenosing vascular heart disease [200]. Now forbidden in Western countries due to human poisoning risks, thallium sulfate was previously applied in the household as rodenticide and insecticide [199,200]. Thallium compounds are commonly odorless, colorless, and tasteless, and thus have been used in the past as poisons in suicides and in murders for criminal purposes [200–203], but also accidental poisonings have been reported in the literature [202].

Elevated serum activities of ALT in a range from 55.3 U/L to 117.8 U/L have been reported in patients accidentally intoxicated with thallium [202]. In another cohort of 34 thallium intoxicated patients with retrospective collection of incomplete laboratory data at time of admission, liver injury was described for half of the patients but actual LT values were not provided [203]. In thallium exposed animals, serum ALT activities a means $\pm$ SEM significantly rose to 84.4 $\pm$ 3.3 U/L compared with 29.3 $\pm$ 3.3 U/L in nontreated controls, associated with similar increased AST activities: 58.9 $\pm$ 2.0 U/L vs 23.8 $\pm$ 1.3 U/L [204].

Liver histology results of humans exposed to thallium are not available [201–203], also not mentioned in a thorough review article on thallium toxicity in humans [207] except in an autopsy study showing liver cell necrosis [208]. In exposed animals, main histology features include frequent hepatocyte necrosis and vacuolation [209]. At autopsy, liver ultrastructural results following thallium poisoning showed substantial injuries of the mitochondria and endoplasmic reticulum of several organs including the liver [208].

Mechanistically, experimental studies suggest that the liver is the storage site of thallium and hepatic mitochondria as one of the most important targets of the thallium liver injury [210]. Thallium exposure to animals caused impaired mitochondrial fatty acid metabolism [211]. Isolated mitochondria treated with thallium *in vitro* exhibited marked elevation in oxidative stress parameters, accompanied by increased mitochondrial ROS generation, adenosine triphosphate (ATP) depletion, impaired oxidation of reduced glutathione (GSH), and mitochondrial membrane potential (MMP) collapse [210]. Together with mitochondrial outer membrane rupture and swelling, disruption of the mitochondrial respiratory chain, these events trigger hepatocellular death signaling via opening of mitochondrial permeability transition pore [210].

5.12 Titanium

Titanium is in common use worldwide [212] and a known historical pollutant found in attic dust [109] but data on its possible contamination are not available in solid

waste landfills [68] or electronic waste [205]. At autopsy, titanium is found in some human organs including the liver [213] but there is overall lack of evidence that presence of titanium exerts major toxicity reaching the level of clinical relevance [212], except perhaps in patients with titanium implants and local complications [214]. Indeed, little information exists on clinical relevant toxicity of the liver due to titanium in humans [212–214], whereas, to overall surprise, abundant reports on experimental titanium liver injury still do overflood the scientific community, some of them are mentioned here as examples [215–219]. To complete the overview, titanium is found as titanium dioxide (TiO_2) in various foods, toothpaste, titanium implants, and pharmaceutical products [212] but is not listed as problematic impurity of herbal medicines [151]. Titanium uptake in humans occurs mostly by ingestion and rarely by inhalation or dermal route [212,213].

Increased LT values are not available in titanium exposed humans but have been reported in exposed animals [218,219]. For instance, if calculated as means ± SEM, serum ALT activity was significantly higher with 22.8 ± 0.8 U/L vs 14.5 ± 0.3 U/L in nonexposed animals as controls, results associated with higher serum activities of AST 45.8 ± 0.2 U/L vs 32.2 ± 0.2 U/L [218]. Nevertheless, these LT values are in line with low graded liver injury only and not suitable to be used as animal model of experimental titanium liver injury.

Liver histology of humans exposed to titanium is not available [212–214]. In experimental animals intoxicated with titanium, light microscopy revealed variable results including fatty degeneration of hepatocytes [215], hepatocyte apoptosis [217], massive focal degeneration of the hepatocytes associated with some cellular infiltration [218], or hepatocellular hydropic degeneration with spotty necrosis [219]. Electron microscopy of the liver derived from animals exposed to titanium showed vacuolation of mitochondria around nuclei in the hepatocytes [215].

Molecular considerations include a reduction of antioxidative mechanisms related to superoxide dismutase, catalase, and glutathione [216] and a theoretical role of ROS associated with cellular oxidative stress and lipid peroxidation, which trigger experimental liver injury by titanium [215,216]. Additional liver metabolomics analyses showed that 29 metabolites and two metabolic pathways changed significantly [215]. Most importantly and shown for the first time for experimental liver injury caused by heavy metals, titanium changed the diversity of gut microbiota and modified their metabolic functions, leading to increased generation of lipopolysaccharides (LPS). As endotoxins, they may trigger and perpetuate the liver injury [215] in a similar way as shown as gut-liver axis for clinical alcoholic liver disease in humans [32–34,220,221].

5.13 Zinc

Zinc is toxic in humans [222–224] and animals [225, 226], causing in addition concern as environmental pollutant [69,227]. It can be detected in water, soil, and plants of municipal solid waste landfills [68], electronic waste [205], and with high amounts in historical attic dust [109]. During the galvanization process in the iron and steel industry, zinc is required for coating steel or cast iron pieces, allowing protection against corrosion [228]. Zinc is commonly found in daily life products like toothpaste, jewelry, musical instruments such as tubas, automobiles, batteries, and as medicine for local treatment of diaper rush. As a contaminant, zinc is found in herbal medicines [151]. Intoxication by zinc can occur via inhalation, oral, or dermal route [222,223]. Occupational disease by prolonged exposure to zinc is rare, commonly does not affect the liver, and is mostly confined to pulmonary complications including pulmonary fibrosis or the acute respiratory distress syndrome (ARDS) [222]. Compared with other heavy metals, zinc is considered as relatively nontoxic to humans [223].

High LT values were reported in a patient who ingested 461 zinc containing coins: serum ALT activity was 341 U/L corresponding to 8.5 times of the ULN; serum AST activity was 1141 U/L equalizing to 28.5 times of the ULN [229]. These values of >5 times of the ULN verify that acute ingestion of high zinc amounts causes severe liver injury in humans (Table 1). In animals intoxicated with zinc, serum ALT, AST, and ALP activities were significantly higher as compared with untreated controls [230] and more specified for ALT and AST when expressed as means ± SD: ALT (158.0 ± 12.1 U/L vs 44.2 ± 3.9 U/L in untreated controls) and AST (240.1 ± 13.2 U/L vs 119.1 ± 12.5 U/L in controls) [231]. However, no changes of LTs were observed in another experimental study [232].

Liver histology obtained at autopsy of the patient intoxicated with zinc due to coin ingestion showed massive acute necrosis of hepatocytes associated with high zinc content in the liver [229]. In experimental studies, hepatocytes with darkly stained nuclei and vacuolated cytoplasm, proliferation of bile ducts, congested hepatic arteria with increasing thickness of its muscular layer, congested blood sinusoids, and mononuclear cellular infiltration prevailed [231]. Data of liver ultrastructure were not available in humans [229], but in animals intoxicated with zinc using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images, showing ZnO particles in the liver [230].

Pathogenetic details of experimental zinc liver injury focused on zinc oxide nanoparticles causing oxidative stress indicated by an increased lipid peroxidation, leading to DNA damage and apoptosis of hepatocytes [233]. Oxidative stress with augmented ROS generation is associated with reduced availability of protective antioxidant defense mechanisms [226]. Hepatic zinc toxicity is also characterized by alteration of mitochondrial metabolism and de-

creased ATP production in hepatocytes [234]. Interesting details of the zinc metabolism have been published using the zebrafish model [125].

6. Carbon Tetrachloride

Carbon tetrachloride is a powerful hepatotoxin in humans rather than a common pollutant [235–239] and was in use as effective solvent and cleaning agent in industrial manufactories, households, dry-cleaning textile laundries, fire extinguishers, precursor of refrigerants or rocket propellant [235], and surprisingly a human and animal medicine to treat hookworm disease [235,240]. Appreciated as experimental hepatotoxin, carbon tetrachloride was largely applied in animal studies [241–247] and found usable to establish a carbon tetrachloride animal model of toxic liver fibrosis [248].

Serum ALT and AST activities were commonly high in 12 patients with carbon tetrachloride intoxication, reaching partially values of 10,000 U/L or above [237], confirmed considering additional patients and publications [235]. Increased LT values were also published in carbon tetrachloride exposed animals [249,250] with maximum means ± SEM of serum ALT activity of 2910 ± 1320 U/L vs 30 ± 3 U/L in untreated controls and AST of 5010 ± 2070 U/L vs 98 ± 20 U/L [250].

Light microscopy following carbon tetrachloride intoxication in humans in historical cases showed often confluent centrilobular and midzonal liver cell necrosis [135], with similar data of massive hepatocellular centrilobular necrosis and fatty infiltration in exposed animals [251]. Electron microscopy of a patient acutely intoxicated with carbon tetrachloride showed in the hepatocyte injured mitochondria associated with proliferation of dilated smooth endoplasmic reticulum (Fig. 1, Ref. [235]) and highly vacuolated hepatocytes with swollen mitochondria and dilated rough endoplasmic reticulum in exposed animals [252].

Mechanistic steps involved in liver injury caused by the organic chemical carbon tetrachloride have well been studied since many decades [241–246] and are discussed by quoting relevant reports in detail recently [235]. Various aspects relate not only to carbon tetrachloride but are important for understanding of liver injury by many other organic chemicals. Briefly, carbon tetrachloride is basically an inert chemical [235,236,241–246]. Notably, total carbon tetrachloride excretion in breath after 1 h is as much as 33% of the dose taken up [235], and this is why for intoxicated patients forced ventilation is recommended to accelerate carbon tetrachloride removal via the lungs [235,236,238]. Agreement exists that only around 1% of the incorporated carbon tetrachloride is responsible for liver injury while 99% thereof will leave the body unchanged via the lungs (Fig. 2, Ref. [235]).

There is also consensus that the hepatic microsomal CYP2E1 is the preferred isoenzyme of CYP responsible for the conversion of carbon tetrachloride to toxic intermedi-

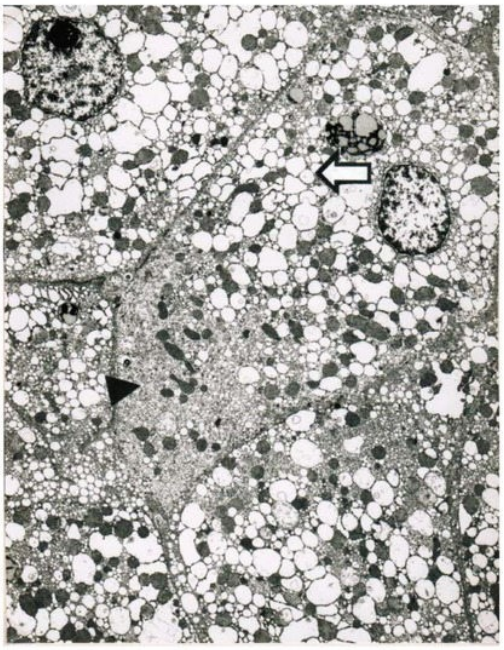

Fig. 1. Patient with poisoning by carbon tetrachloride ingestion (50 mL) and liver tissue specimen obtained on day 14 after intoxication for electron microscopy (5200-fold magnification). Key features include a striking proliferation of the smooth endoplasmic reticulum of the hepatocyte (►) with close by injured mitochondria, and in addition to a pronounced dilatation of the smooth endoplasmic reticulum presenting as dilated cisterns (⇦). Data are from a previous open access report [235], which provides additional pictures of liver electronic microscopy from other patients.

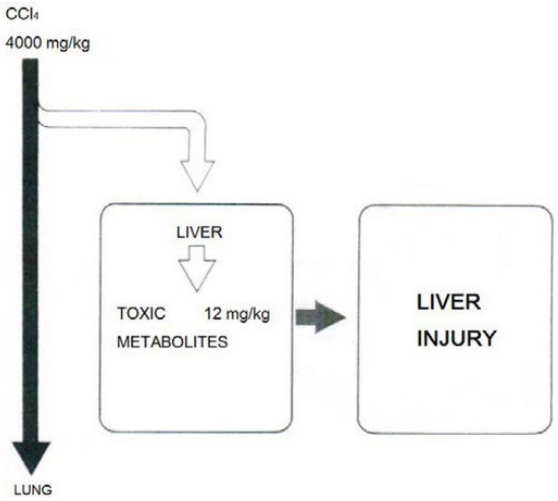

Fig. 2. Most of the ingested CCl_4 (carbon tetrachloride) will leave the human body via the lungs unchanged, little enters the liver to be metabolized to toxic intermediates causing the liver injury. This figure is derived from an open access publication [235].

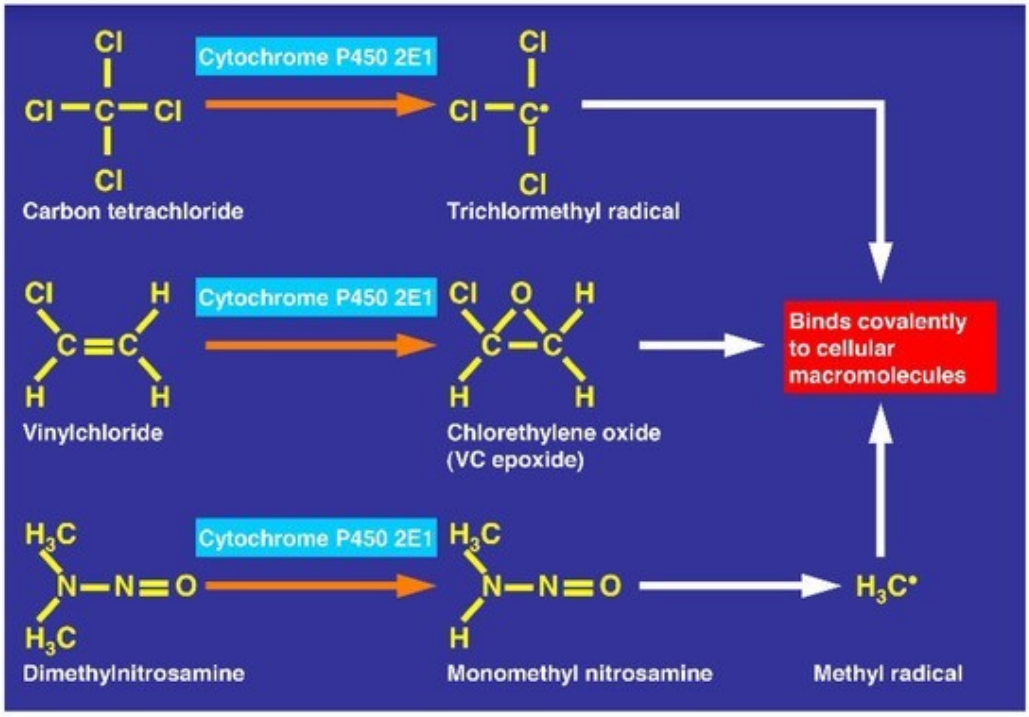

Fig. 3. Carbon tetrachloride is metabolized by cytochrome P450 2E1, now termed CYP2E1, similarly to other toxins such as vinylchloride and dimethylnitrosamine. This figure is derived from an earlier open access report [235].

ates that bind covalently to cellular macromolecules such as membrane proteins [235,244,245], in analogy to other toxins like vinyl chloride and dimethylnitrosamine (Fig. 3, Ref. [235]).

Carbon tetrachloride and ethanol share with CYP2E1 a common metabolic pathway that is induced by chronic alcohol consumption [235]. This induction is viewed as a risk factor for liver injury by acute carbon tetrachloride intoxication in animals, associated with increased covalent binding of $^{14}CCl_4$ metabolites to microsomal protein *in vitro* and an increased metabolism of $^{14}CCl_4$ to $^{14}CO_2$ [246]. These changes likely occur also in those patients with a history of chronic alcohol consumption prior to the acute carbon tetrachloride intoxication [235].

7. Phthalates

Phthalates represent esters of phthalic acid and are mainly used as plasticizers, commonly added to polymers such as plastics to make the material softer and more flexible, to increase its plasticity, to decrease its viscosity, or to minimize risk of friction during its handling in manufacture [253–255]. Lower molecular weight (LMW) phthalates are derived from C3-C6 alcohols, now being gradually replaced by high molecular weight (HMW) phthalates with more than C6 alcohols [255]. Whereas LMW phthalates are used in personal care products and cosmetics as fragrance carriers and solvent, HMW phthalates are preferentially added to polyvinyl chloride (PVC) plastic in food processing, packing, building materials, and medical devices. Of major concern, phthalates are environmental pollutants and represent a health risk to humans [253–255]. They are primarily exposed to HMW phthalates via ingestion of food contaminated by package and through inhalation in buildings containing PVC flooring. As primarily endocrine disruptors, phthalates cause most disturbingly substantial infertility in males and may contribute in upcoming decades to dramatic lower birth rates especially in countries with heavily exposed populations [256]. It seems, therefore, that in the future issues of global health, population growth stability, and economic burden is more negatively governed by exposure to phthalates rather than to heavy metals.

Increased serum activities of ALT in individuals exposed to phthalates were vaguely published [257–261] and not based on established criteria of liver injury (Table 1). In addition, ALT activities were mostly related to urine phthalate metabolites with positive or negative correlations. Thus, valid data of ALT were currently not available supporting phthalates being hepatotoxic in exposed humans, as opposed to animals treated with di(2-ethylhexyl)phthalate (DEHP) [262]: serum ALT activity 50.50 $\pm$ 1.71 U/L vs 25.00 $\pm$ 2.39 U/L in untreated controls, with all data given as means $\pm$ SEM; AST 191.83 $\pm$ 9.24 U/L vs 123.00 $\pm$ 7.38 U/L.

Liver histology was not available in humans exposed to phthalates [257–261]. In animals intoxicated with the phthalate metabolite DEHP, light microscopy examination of the liver showed an augmented eosinophilia in the cytoplasm of the hepatocytes [262]. Electron microscopy data

of hepatocytes obtained via liver puncture in dialysis patients exposed to DEHP revealed that peroxisomes outnumbered mitochondria [258]. In exposed animals, dilatation and destruction of the endoplasmic reticulum, loss of Golgi apparatus arrangement, glycogen depletion, size enlargement in mitochondria and fragmentation of their cristae, and cytoplasm filled with damaged mitochondria prevailed, associated with an increased number of peroxisomes [262]. These data were partially confirmed in another study showing a dramatic increased number of peroxisomes concomitantly with a minor increase of mitochondria [258], thereby classifying DEHP as a peroxisome proliferating chemical [258,262].

Considerations on mechanistic steps are not warranted, as little evidence exists that phthalates or their esters may harm the liver [257–261]. Unquestionably and since metabolites of phthalates are detected in the human urine [260], phthalates and their esters enter the human body and systematically reach any organ including the liver without injuring it.

8. Glyphosate and Cordycepin

Glyphosate, chemically *N*-phosphomethyl(glycine), is a known herbicide with potential environmental and human health risk [263,264]. Together with its primary metabolite aminomethylphosphonic acid, glyphosate can be detected in soils, water, plants, animals, food, and blood and urine of exposed humans. In 2017, the International Agency for Research on Cancer (IARC) classified glyphosate as "probably carcinogenic" in humans. However, other national agencies did not tighten their glyphosate restrictions and even prolonged authorizations of its use [263]. Whether glyphosate can cause non-Hodgkin lymphoma (NHL) is still a matter of debate [263–266], but authors, who had served as scientific board members of the US EPA FIFRA scientific advisory panel meeting that evaluated glyphosate, may have a conflict of interest when attempting to equalize epidemiological and experimental association with a causal association [265]. Instead, a causal link between glyphosate exposure and NHL may exists, but has not been rigorously studied in human populations, a balanced view highly appreciated [264]. Essentially, new NHL cases spontaneously develop in around 20 individuals per 100,000 persons in the USA with decreasing tendency within the last years [267], making indeed a causal attribution to glyphosate in NHL patients a difficult approach as claimed [265]. A little note in between and certainly outside of evidence based medicine, Hollywood star Jane Fonda just made public being treated for NHL, and it is unlikely that she was confronted with glyphosate in her garden or elsewhere, similar to many other NHL patients without any risky contact. Aside from the NHL issue but due to its systemic detection in the human body, glyphosate may theoretically enter various organs including the liver. In consideration of these aspects, a variety of reports focused on a possible hepatotoxic potential of glyphosate in both, humans [268] and animals [269,270].

High LTs have been observed in a farmer from India with acute liver failure following glyphosate poisoning, who acutely ingested 25 mL glyphosate and had a history of chronic low dosed exposure to glyphosate and alcohol use: serum activities of ALT were 1311 U/L and AST 1021 U/L [271]. This thereby meets basic requirements of a severe liver injury (Table 1). However, the diagnosis of glyphosate poisoning with acute fulminant hepatic failure (FHF), syn. acute liver failure (ALF), requires further analyses, let alone the fact that in up to 50% of worldwide published ALF cases, the cause remained unexplained [272]. For the reported case, it was vaguely suspected that the patient may have had underlying NAFLD, now termed MAFLD, and acute decompensation of liver function with fatal outcome following accidental ingestion of glyphosate [271], but data as published do not allow for MAFLD existence. In addition, the known high risk of HEV for both, the farmers [273,274] and the population in India [273,275], classified thereby the Indian farmer as a high risk patient of HEV infection, but this issue was largely circumvent in the published case report [271]. Gold standard for detection of ongoing acute or chronic HEV infection is testing for HEV-RNA by polymerase chain reaction (PCR) [276]. This important parameter was obviously not considered in the Indian farmer patient; instead, not further specified serological markers of infective hepatitis were globally reported as negative, while attempts to serological exclusion of HEV like by HEV-IgM and HEV-IgG were not explicitly mentioned [271]. In a study from India on hepatitis infections, it was noted that most of them were due to HEV, and ultrasound data suggested frequent abnormalities of the gallbladder as well as hepatosplenomegaly [275], both found in the patient under consideration in support of a possible HEV infection because toxic liver injury commonly does not cause splenomegaly. High serum activities of ALT and AST, as shown for the patient [271], are also supporting HEV as a diagnosis because they are characteristic features of HEV infections [277]. A review of original 131 patients intoxicated with glyphosate 138 $\pm$ 12 mL (means $\pm$ SEM) noted that among 119 evaluable cases only 33.6% of the patients had serum AST activities $>$40 U/L, indicating that glyphosate intoxication is commonly associated with a low grade of liver injury [278]. This is opposed to the extremely high LT values observed in the Indian farmer patient [271]. Thus, overall evidence suggest that this patient suffered from not recognized severe HEV infection, which could have well be treated with antiviral drugs, while the primarily assumed ALF due to glyphosate intoxication is highly questionable.

There is also uncertainty on a causal association in humans with MASH between detectable glyphosate or its primary metabolite aminomethylphosphonic acid (AMPA) in the urine and serum ALT activities [268]. Indeed, it seems

Fig. 4. ***Cordyceps militaris*** **(A) and its fruiting bodies (B).**

that in the control group, despite detectable glyphosate and its residual metabolite AMPA in the urine, MASH was excluded, in line with the view that a causal relationship between glyphosate use and MASH does not exist.

In studies with animals exposed to glyphosate, serum ALT activities rose significantly when compared with non-treated controls, whereby the increase was higher in animals treated with 25 mg/kg glyphosate compared with 50 mg/kg [279]. Serum AST activities remained virtually unchanged at the same dosage, but interestingly, with 100 mg/kg glyphosate, female rats showed higher ALT and AST values as compared with male rats.

Liver histology was not commonly available in humans intoxicated with glyphosate [271,278], except in a patient intoxicated with 500 mL glyphosate showing microvesicular steatosis in the hepatocytes preferentially in the centrilobular areas of the liver [279]. Published data in rats showed vacuolated cytoplasm, degeneration of some hepatocytes, and congestion of blood vessels including the central vein [280]. Not unexpected, ultrastructure data of the human liver following glyphosate intoxication were not published [271,278,279], but in animals profound changes such as fragmentation of cell organelles forming dense clusters including fragments of the rough endoplasmic reticulum were found [280].

Mechanistic views of steps leading to liver injury by glyphosate are primarily based on animal studies [263,269,280]. Consensus exists that glyphosate produces ROS in the hepatocytes, which in turn cause injurious changes of cell protein and structural membrane proteins via unsaturated lipid peroxidation under regulation of antioxidants, GSH, and catalase [269,280]. Glyphosate is a competitive inhibitor of the shikimate pathway that allows aromatic amino acid biosynthesis in plants, features explaining the herbicide property of glyphosate [263]. The shikimate pathway is absent in mammals including human, which may help explain the relative low systemic toxicity in rats and rabbits [278].

For reasons of completeness, with cordycepin, chemically a nucleoside analog 3'-deoxyadenosine, recently isolated from *Cordyceps militaris*, another herbicide has been described, which may become an alternative to glyphosate [281]. *Cordyceps militaris* is both, an edible fungus and a traditional Chinese medicine (TCM) with highly appreciated use in many Asian countries [282]. Efficacy as medicinal product was assumed but rarely proven using randomized controlled trials (RCTs). Toxicities such as liver injury in humans are not known, arguments that facilitate its broad use as herbicide [281]. Cordycepin was evaluated in animal studies for *in vivo* subacute toxicity and the Ames test. Following uptake, cordycepin is rapidly decomposed in humans through adenosine deaminase, as evidenced by a short half-life of about 1 min. This likely reduces the possible risk of toxicity and any tumor initiation [281]. By the way, *Cordyceps militaris* is an eye catcher (Fig. 4).

9. Alcohol

Alcohol is chemically ethyl alcohol C_2H_5OH, or in condensed form described as ethanol, whereby in the clinical context these terms often are used interchangeably [220]. It is a nature-based product from fermentation of glucose contained, for instance, in grapes. When consumed as alcoholic beverage in moderate amounts, it is commonly well tolerated [283]. Its misuse causes alcoholism as major disease [283,284], associated with various disorders including liver injury like alcoholic fatty liver disease (AFLD) [220], alcoholic steatohepatitis (ASH) or acute alcoholic hepatitis (AH) [283], alcoholic cirrhosis (AC) eventually associated with alcoholic hepatocellular carcinoma (AHCC) [33,34,220,285].

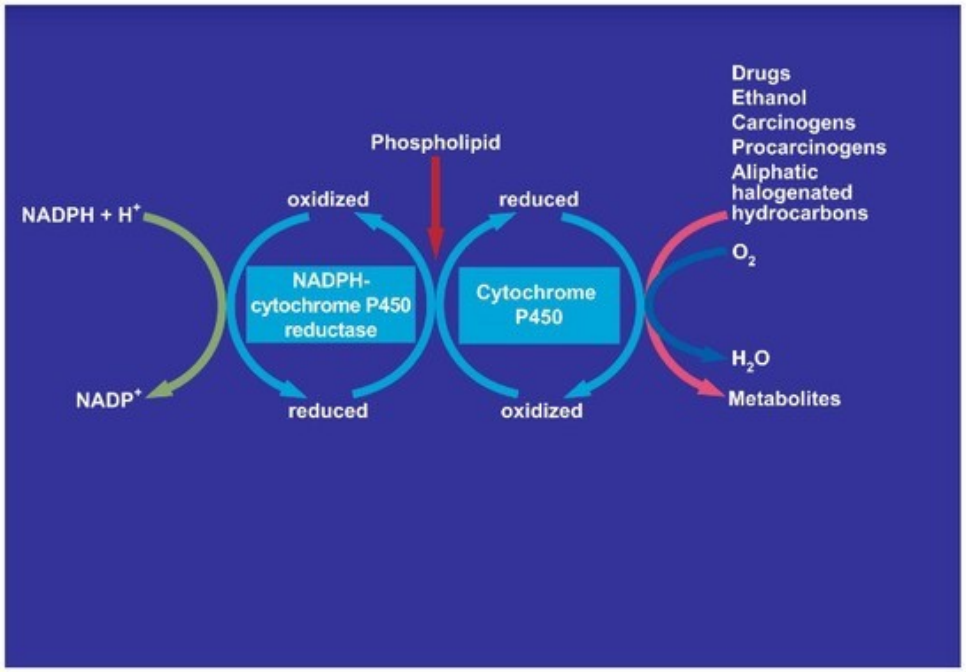

Fig. 5. Involvement of cytochrome P450 in the microsomal metabolism of various substrates including aliphatic halogenated hydrocarbons with carbon tetrachloride as example. The NADPH-cytochrome P450 reductase uses NADPH + H^+ and will itself be reduced, allowing the cytochrome P450 to be transferred from the oxidized state to the reduced state. The overall reaction needs also molecular oxygen and phospholipids. Figure is taken from an open access article [220].

Serum activities of ALT and AST are either within the normal range or only slightly increased in early stages of human alcoholic liver disease (ALD), but commonly with higher AST values as compared with ALT, considered an important diagnostic sign for suspected alcohol use whenever the cause of a liver disease remained clinically unclear [220,286]. Moderately higher serum activities were found for ALT and AST in animals exposed chronically to alcohol [286].

Light microscopy in human ALD is variable depending on the stage of the disease [220,283]. For instance, in AFLD macrovesicular steatosis of hepatocytes prevails [220]. For ASH, steatosis together with liver cell necrosis are characteristic features [220,283], with more frequent necrosis and inflammatory cell infiltration in acute AH [283]. AC presents with thick collagen strands around the central vein and coursing through the hepatic lobules, also involving the perisinusoidal and pericellular areas [220]. If associated with AHCC, typical tumor cells are found [285]. In animals, fatty liver is the most common stage of liver injury by prolonged alcohol administration, presenting fat within the liver cells [220]. Electron microscopy of human AFLD show injurious changes of liver mitochondria as well as a striking proliferation of the smooth endoplasmic reticulum within most hepatocytes [287]. Similar changes were observed in animal alcoholic fatty liver [288].

The pathogenesis of ALD includes the gut microbiome providing endotoxins [218,220], the low hepatic content of antioxidants [220], toxic effects of acetaldehyde [220], and the participation of the hepatic microsomal ethanol oxidizing system (MEOS), which metabolizes ethanol to acetaldehyde independently from alcohol dehydrogenase and catalase [7,33,220,289–291]. MEOS activity requires NADPH + H+ and molecular oxygen, generating ROS as byproduct. In addition to the NADPH dependent CYP reductase, CYP 2E1 is the major constituent of MEOS, which metabolizes not only ethanol but also other potentially hepatotoxic chemicals (Fig. 5, Ref. [220]).

10. Synthetic Drugs

Chemical drugs, or drugs in short as opposed to herbal drugs, are in worldwide use to treat hundreds of diseases, but they may lead to toxicity of several organs including the liver [15,22]. Until mid 2020, 81,856 case of DILI caused by various drugs were published with diagnosis assessed for causality applying the RUCAM [15], which represents a worldwide used diagnostic quantitative algorithm established on principles of artificial intelligence (AI) [292]. This kind of case analysis provides a good overview on current DILI [15], as opposed to many DILI databases, including specifically the US LiverTox, which contains cases that have not been qualified as real DILI due to missing alternative causes and circumventing RUCAM use [293]. Amoxicillin-clavunalate is among the top drugs causing DILI, considering only cases assessed for causality using RUCAM [294].

Serum ALT and AST activities with values >5 times of the ULN are commonly found in RUCAM based DILI cases [15,20], qualifying them principally for the RUCAM subscale of the hepatocellular injury [22,295]. More specifically, in a recent excellent cohort study from India on 1288 patients with RUCAM based idiosyncratic DILI, serum activities were determined as means: ALT 241 U/L (range 110–519) and AST 220 U/L (range 119–438) [296]. This study used a prospective and thereby proactive design that facilitated completeness of case data and thereby high RUCAM based causality gradings, an approach strongly recommended for using RUCAM [14,22,295]. Overall, LTs of ALT, AST, and ALP categorize DILI cases into hepatocellular injury or cholestatic/mixed injury that assign to one or the other RUCAM subscale [22]. As expected, data of serum ALT and AST activities are missing in animals as idiosyncratic DILI is not reproducible in animal models due to lack of genetics comparable to humans.

Liver histology data based on light microscopy are available and present a bundle of different findings but data were not obtained from DILI cases assessed by RUCAM and are thereby open for discussion [297]. Overall, liver histology features are unspecific and rarely of diagnostic value [295,298], this is why liver histology to be obtained by invasive liver puncture is not part of the diagnostic RUCAM algorithm [22]. In some reports, liver histology results were presented derived from animals, but such models are inappropriate as they do not reflect idiosyncratic DILI but mostly intrinsic DILI due to overdosed drugs. Ultrastructural hepatic changes of the liver obtained from patients have obviously not been published, and data of animals are missing, as expected.

Mechanistic challenges at the molecular level prevail when evaluating cases of idiosyncratic DILI as evidenced by differences of theories [14,28,299–303], and little in known with certainty [299] although many papers have been published on DILI since the nineties [302]. The general problem of presenting a uniform molecular basis for this form of DILI can be traced back to the unavailability of appropriate animal models that would correctly mimic the human disease with its genetic risk factors [28]. Consequently, mechanistic studies are confined to humans with RUCAM based idiosyncratic DILI, considering preferentially results of blood tests and liver histology [28,299].

Agreement exists that idiosyncratic DILI by several drugs is connected with specific HLA (human leucocyte antigen) genotypes and mediated by CD8 T cells of the adaptive immune system, a proposal supported by autoimmune parameters in the blood of patients, clinical features, and histology with monocytic infiltration [299]. The adaptive immune system requires prior activation of the innate immune system. Early steps in this process likely involves activation of antigen presenting cells by molecules such as danger associated molecular pattern molecules (DAMPs) [28,299]. Pathogenetic steps leading to the cholestatic DILI were rarely studied and focused on the impairment of the hepatocellular bile salt export pumps (BSEP) or functional modification of other bile acid transporters reported mostly from *in vitro* studies. In addition, the gut microbiome and the gut-liver axis may be involved in the occurrence of DILI [28]. Under discussion is a disturbed diversity of gut bacteria with increased production of endotoxins syn lipopolysaccharides (LPS) that may enter the portal circulation through a leaky gut, and if not cleared by the liver, reach the systemic circulation and the liver where they may trigger the injury.

Some few drugs exert their toxicity without metabolism involving CYP isoforms as opposed to more than half of the drugs, which are metabolized by various CYP isoforms causing idiosyncratic DILI shown in cases assessed for causality using RUCAM (Table 2, Ref. [302]) [28].

It is generally assumed that DILI is partially the result of ROS generated via microsomal or mitochondrial oxidative stress during exposure of a drug to the hepatocytes [8,299–301,303]. Other mechanistic theories focus on bile salt export inhibition [299] and gut microbiome [299,303], whereas the role of lipophilicity and drug doses is still controversially debated [303]. Except perhaps for serum glutamate dehydrogenase as a marker of liver mitochondrial injury, other biomarkers strongly recommended in the past years have meanwhile been retracted by EMA (European Medicine Agency) and the US FDA (Food and Drug Administration) due to falsified external study results [8].

11. Herbs

Herbs are found all around the world provided environmental conditions allow for appropriate plant growing. They are used, for instance, as medicinal herbal product such as regulatory approved herbal drug or traditional herbal medicine, or as ingredients of so called herbal dietary supplements although they do not supplement any balanced diet but help at least manufacturers [304]. As a reminder, HILI is often disqualified as DILI especially in China when herbs have been consumed and caused the liver injury, so a clear differentiation between HILI from DILI is needed and mixing cohorts of DILI with those of HILI is not acceptable. Medicinal herbal products are economically of importance for national treasuries and appreciated by consumers to be used for minor disorders or some kind of prophylaxis [305]. A single herb contains a bundle of phytochemicals, their number increases if herbs are consumed as mixtures [151,306,307]. Problematic are plants containing 1,2-unsaturated pyrrolizidine alkaloids (PAs) if they are used as traditional medicines [306]. Recognized as important issue, efficacy of medicinal herbal products is often poorly approved using RCTs [308], but efforts of continued improvements are recognizable as update of the International Society of Pharmacovigilance China Chapter [309] and quality improvement of RCT evaluations although many rele-

Table 2. Involvement of CYP isoforms in idiosyncratic DILI by various drugs as assessed in RUCAM based cases.

Drug	RUCAM based DILI cases (n)	Metabolized by CYP isoform
1. Amoxicillin-clavulanate	333	-
2. Flucloxacilllin	130	CYP3A4
3. Atorvastatin	50	CYP3A4/5
4. Disulfiram	48	CYP 2E1
5. Diclofenac	46	CYP2C8
6. Simvastatin	41	CYP3A4/5
7. Carbamazepine	38	CYP3A4/5
8. Ibuprofen	37	CYP 2C8/9
9. Erythromycin	27	CYP 3A4
10. Anabolic steroids	26	CYP2C19
11. Phenytoin	22	CYP 2C9
12. Sulfamethoxazole/Trimethoprim	21	CYP 2C9
13. Isoniazid	19	CYP 2E1
14. Ticlopidine	19	CYP 2C19
15. Azathioprine/6-Mercaptopurine	17	-
16. Contraceptives	17	CYP3A4
17. Flutamide	17	CYP1A2
18. Halothane	15	CYP2E1
19. Nimesulide	13	CYP 2C9
20. Valproate	13	CYP 2C9
21. Chlorpromazine	11	CYP 2D6
22. Nitrofurantoin	11	-
23. Methotrexate	8	-
24. Rifampicin	7	-
25. Sulfazalazine	7	-
26. Pyrazinamide	6	-
27. Natriumaurothiolate	5	-
28. Sulindac	5	CYP 1A2
29. Amiodarone	4	CYP 3A4
30. Interferon beta	3	-
31. Propylthiouracil	2	CYP/NA
32. Allopurinol	1	-
33. Hydralazine	1	-
34. Infliximab	1	-
35. Interferon alpha/ Peginterferon	1	-
36. Ketaconazole	1	-
37. Busulfan	0	-
38. Dantrolene	0	-
39. Didanosine	0	-
40. Efavirenz	0	CYP 2B6
41. Floxuridine	0	-
42. Methyldopa	0	CYP/NA
43. Minocycline	0	-
44. Telithromycin	0	CYP 3A4
45. Nevirapine	0	CYP 3A4
46. Quinidine	0	CYP 3A4
47. Sulfonamides	0	CYP/NA
48. Thioguanine	0	-

Listed are the top ranking 48 drugs implicated in causing 3312 idiosyncratic DILI cases with and without verified causality using RUCAM. The predominant CYP isoform involved in drug metabolism is listed. Abbreviations: CYP, Cytochrome P450; DILI, Drug induced liver injury. Table is taken from an earlier open access report [302].

Table 3. Selected suspected toxic compounds as suggested causes of liver injury due to use of herbal TCM.

Chinese name	Scientific name	Tentative hepatotoxic components
Ai Ye	*Artemisia argyi*	Volatile oil
Bi Ma Zi	*Rhicinus communis*	Ricin, toxic proteins
Cang Shan	*Xanthium*	Glycosides (kaurene), diterpenoids
Chang Shan	*Dichor febrifuga* Lour	Alkaloids (dichroine)
He Huan Pi	*Albizia julibrissin*	Glycosides (saponine)
He Shou Wu	*Polygonum multiflorum*	Anthraquinones
Huang Yao Zi	*Discorea bulbifera* L	Glycosides (steroids, diosgenin), diterpenoids-lactones
Ku Lian Zi	*Melia azedarach*	Glycosides (tetranortriterpenoids)
Lei Gong Teng	*Tripterygium wilfordii* hook F	Glycosides (tripterygium), diterpenoid-lactones
Qian Li Guang	*Senecio scandens*	Pyrrolizidine alkaloids
Shan Lu	*Phytolacca acinosa* Roxb.	Alkaloids (phytolaccine)
Xiang Si Zi	*Abrus precatorius*	Abrin

Data are derived from a published report in an open access journal [306].

vant publications are still only in Chinese language available [310]. While efficacy of herbal medicinal products remains an actual and largely intransparent clinical issue, adverse reactions including liver injury classified mostly as idiosyncratic and rarely as intrinsic HILI are well documented in worldwide published 12,068 RUCAM based cases [16], in addition to 11,160 RUCAM based HILI cases from the Asian region [311], for which RUCAM was used for causality assessment either in its now preferred updated version of 2016 [22] or as original of 1993 [312].

LTs are commonly increased in HILI, as evidenced by various published RUCAM based HILI cases [313–315]. As an example, in a large cohort of patients with RUCAM based HILI caused by different herbal TCMs, serum activities of ALT were with 935 ± 672 U/L (means ± SD) [313], meeting thereby classic criteria requirements of HILI (Table 1). In another large RUCAM based HILI cohort, the herbal TCM *Polygonum multiflorum* caused serum ALT activities up to 1947 U/L, a virtually perfect study as RUCAM was used and all HILI cases had a probable or even highly probable causality grading, all almost perfect except that the liver injury was not termed HILI but erroneously DILI commonly caused by synthetic drugs [314]. In animals exposed to *Polygonum multiflorum*, serum activities rose from 39.24 ± 7.58 U/L to 57.23 ± 48.70 U/L [316].

Liver histology in a patient with liver injury caused by *Polygonum multiflorum* consumption showed hepatocellular necrosis, lobular portal inflammation as well as enlargement and congestion in the sinusoid in the RUCAM based HILI case erroneously described as DILI rather than HILI [315], with similar results in another study [314]. In exposed animals, liver cell shrinking with disordered cord arrangement and karyopyknosis prevailed [316]. Ultrastructural changes are not available from the liver of patients injured by herbal TCMs including *Polygonum multiflorum*, the most commonly herbal medicine in China and other Asian countries [113–315], and were not reported in animal studies [316].

Discussions on molecular aspects of HILI is hampered by the fact that herbal products used as medicine may be unintentionally contaminated with heavy metals, aflatoxins, ochratoxin A, fumorisins, zearalenone, deoxynivalenol, pesticides, and herbicides, or deliberately adulterated with chemical drugs aiming to improve efficacy of the medicinal product, although the role of these impurities triggering HILI is uncertain [151]. This was discussed but not verified in cases of RUCAM based HILI caused by *Polygonum multiflorum* [315], Indian Ayurvedic medicines [317], or herbal TCMs in a prospective hospital study with a low HILI case frequency [318]. For reasons of transparency, a large list of phytochemicals as potential hepatotoxins contained in medicinal herbs was provided based on current expert views [151]. For herbal TCM, a list of tentative hepatotoxins is provided (Table 3, Ref. [306]).

A major challenge is related to the hundreds of herbs as potential culprits of HILI as no uniform pathogenetic concept for all herbs can be provided, but a number of excellent publications [319–325] has well covered mechanistic topics. This allows now to focus the discussion of mechanistic steps on HILI by *Polygonum multiflorum* [326–328] and herbs containing PAs [329–345].

Phytochemicals of *Polygonum multiflorum* such as emodin, quercetin, apigenin, resveratrol, gallic acid, kaempferol and luteolin have been identified as major potential culprits of the liver injury, using a computational systems toxicology approach [326]. Pathway enrichment analysis suggested that multiple interactions between hepatic apoptosis and metabolism might underlie HILI by *Polygonum multiflorum*. Various pathways have been identified in specific compounds, like glutathione metabolism, CYP metabolism, and the p53 pathways [326]. Other studies confirmed the hepatotoxic potential of anthraquinone derivatives such as the emodin-8-O-glucoside, which causes elevated ROS generation and a decrease of hepatic reduced glutathione content and mitochondrial membrane potential, indicating that its liver injury is linked

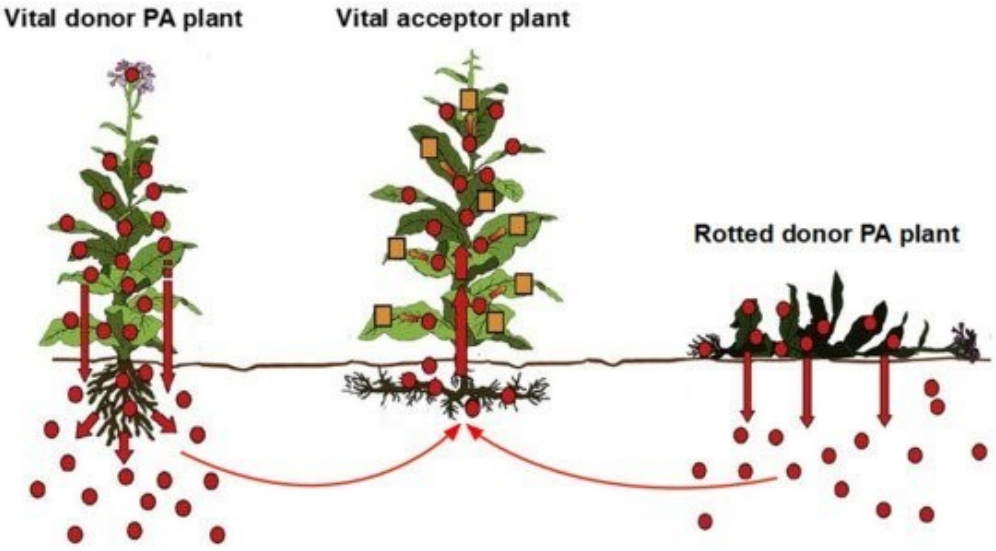

Fig. 6. System of horizontal natural product transfer of pyrrolizidine alkaloids (PAs). Vital donor PA plants biosynthesize, in their leaves, PAs, which may reach the soil through various mechanisms. Including, preferentially, the rhizome and roots extruding from PAs. In addition, PAs, located in leaves of rotted or rotting donor PA plants, enter the soil. Assisted by surface water, PAs contaminating the soil arrive at the rhizome of roots of a vital acceptor plant, and, following their uptake, reach aerial parts of the plants, shown as small red dots, whereas some PAs may undergo metabolic modification, shown as small yellow squares. Abbreviation: PA, Pyrrolizidine alkaloid. This figure was modified from earlier illustrations and suggestions of the group of Selmar [335,336] and is derived from a previous open access article [329].

to impaired mitochondrial unction [327]. Under more speculative consideration are also immune aspects of the liver injury caused by *Polygonum multiflorum* as well as genetic polymorphism including deficiency of enzymes involved in the metabolism of the phytochemicals because some patients have a history of familial adverse reactions to hepatic [328]. The enzyme deficiency likely facilitates accumulation of toxic intermediates during biotransformation, and herbal constituents and metabolites could function as haptens. They can covalently bind to macromolecular proteins and function as antigen, ready to initiate an immunological process of liver injury [328].

Molecular principles of PA liver injury, which is clinically termed as hepatic sinusoidal obstruction syndrome (HSOS) with 28 RUCAM based cases published in the past [329–331], are well described [329], because this dose dependent, intrinsic liver injury is not confined to humans but can perfectly be reproduced in animal models [329–334]. PAs are found in more than 6000 plant species growing in countries worldwide and are preferentially synthesized by the plants [329]. In analogy to the uptake of heavy metals from soil by plants growing nearby contaminated municipal waste landfills [68], PAs may also be taken up from the soil contaminated with PAs from vital or rotted PA containing plants via horizontal natural product transfer (Fig. 6, Ref. [329,335,336]).

Plants contain both, saturated and unsaturated PAs [329]. They have a typical heterocyclic structure in common, but differ in their potential toxicity, depending on the presence or absence of a double bond between C1 and C2. Fortunately, most plants contain saturated PAs without this double bond and are therefore not toxic for consumption by humans or animals [329,333]. In a minority of plants, however, PAs with this double bond between C1 and C2 exhibit strong hepatotoxic potentials. If consumed in error and in large amounts, plants with 1,2-unsaturated PAs induce metabolic breaking-off of the double bonds of the unsaturated PAs, generating PA radicals that may trigger severe liver injury [329] through a process involving microsomal CYP, with preference of its isoforms CYP 2A6, CYP 3A4, and CYP 3A5 [334]. This toxifying CYP dependent conversion occurs primarily in the endoplasmic reticulum of the hepatocytes, equivalent to the microsomal fraction. Toxified PAs injure the protein membranes of hepatocytes, and after passing their plasma membranes, more so the liver sinusoidal endothelial cells (LSECs), leading to life-threatening HSOS [329]. The detection of blood pyrrolizidine protein adducts provide strong evidence that in humans PA metabolites are formed as radicals covalently bound to proteins, shown in patients with established HSOS [329,331] assessed for causality using the updated RUCAM [22].

The structural diversity of PAs is overwhelming and impedes evaluating liver injury aspects to some extent [337–343]. PAs rarely occur in the free form of a pyrrolizidine base, but usually present as variable esters

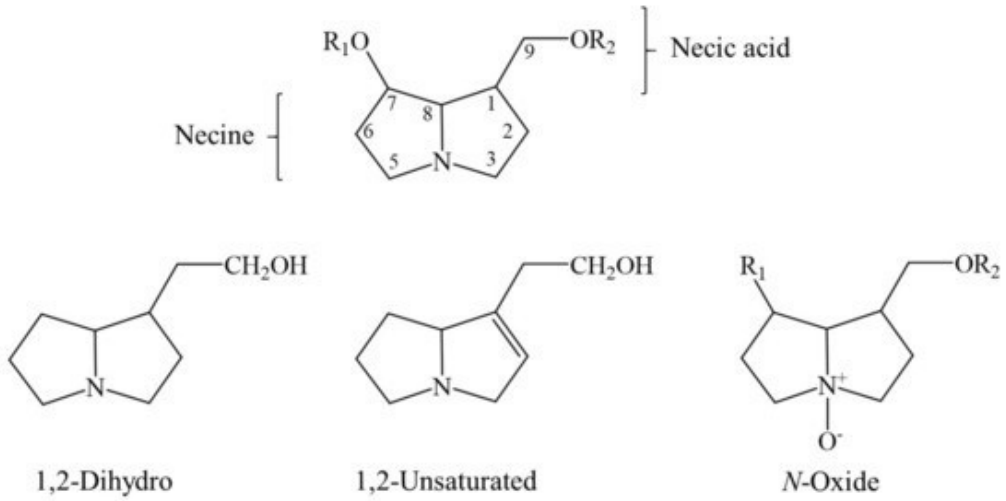

Fig. 7. Structure of a PA and its different forms. R_1 and R_2 correspond to different necic acids. Abbreviation: PA, Pyrrolizidine alkaloid. The figure was obtained from the open access report of Moreira *et al*. [337].

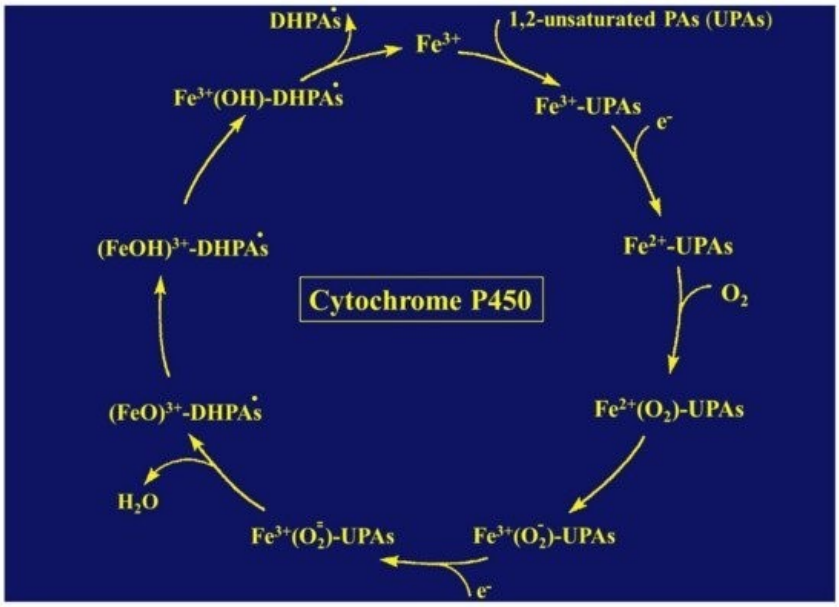

Fig. 8. Proposed catalytic cytochrome P450 cycle implicated in the toxification of 1,2-unsaturated PAs.

(mono-, di- or macrocyclic diesters), formed by a necine base, necine in short, and by one or several necic acids (mono- or dicarboxylic aliphatic acids), which finally determine the structural PA diversity [337]. PAs are found in the variable forms of tertiary bases of 1,2-dihydro and 1,2-unsaturated or *N*-oxides (Fig. 7, Ref. [337]) [329]).

Whenever exogenous compounds like 1,2-unsaturated PAs enter the catalytic CYP cycle to be oxidized, they first must bind to the Fe^{3+} of the oxidized CYP (Fig. 8) [28,329, 344,345].

In analogy to drugs, ethanol and other exogenous compounds, the 1,2-unsaturated PAs (UPAs) enter the catalytic cytochrome P450 cycle as substrate to be metabolized, shown on top of the cycle right side. After several steps, the metabolized UPA leaves the cycle as toxic radical DHPA•, whereby the 1,2-unsaturated PA loses its double bond and changes to pyrrole protein adducts. CYP stands for its various isoforms. As a reminder, regarding cytochrome P450, the term "P450" was used to describe a "pigment" with an absorption maximum at 450 nm within the ferrous-carbon monoxide complex of CYP in rat liver microsomes [345]. The figure was modified [344] and retrieved from an open access report [329].

More specifically, the first electron is provided to CYP by NADPH + H^+ via the NADPH CYP reductase and the reduced form of CYP with Fe^{2+} is generated, which finally becomes oxidized again after splitting off the oxidized substrate. CYP is then again free for the next substrate to be ox-

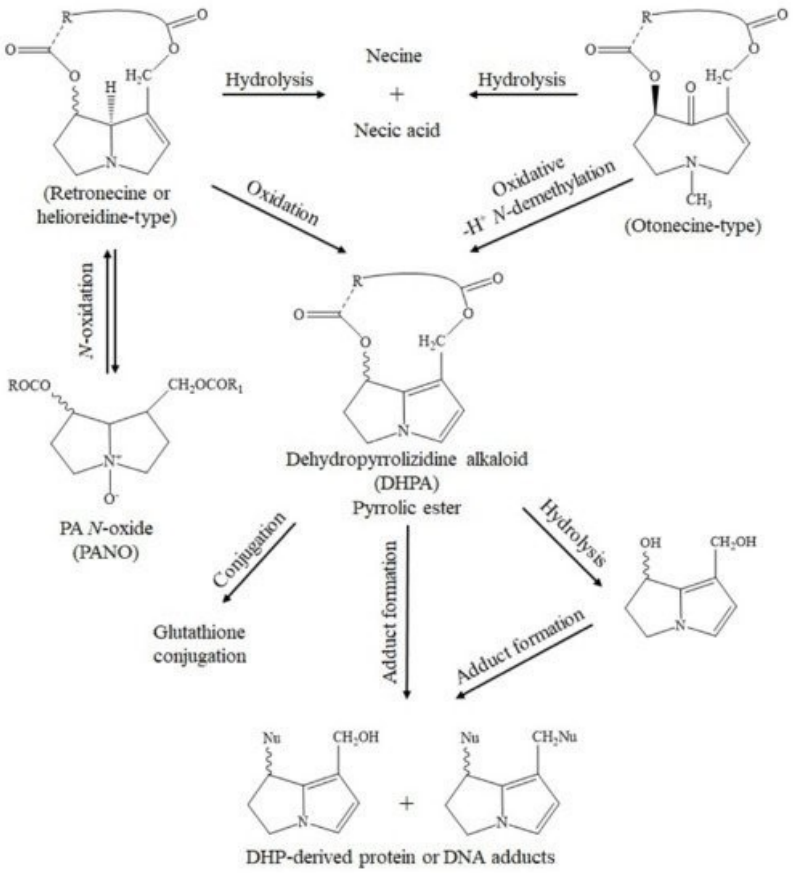

Fig. 9. Fate of 1,2-unsaturated PAs to adduct formation. Different steps are delineated leading from 1,2-unsaturated PAs to their adduct formation with proteins and DNA. DHPA is partially detoxified through conjugation with glutathione. Abbreviations: DHP, dehydronecine pyrrolizidine; DHPA, dehydropyrrolizidine alkaloid; PA, pyrrolizidine alkaloid. The figure was retrieved from the open access report of Schramm *et al*. [338].

Fig. 10. Photographies of selected PA containing plants. The pictures are derived from an earlier open access report [329].

idized (Fig. 7) [329,344]. Through introduction of molecular oxygen, a multi-compound reactive complex emerges, facilitated by inclusion of another electron that, commonly, is provided through the NADPH CYP reductase or a similar but NADPH-independent reductase [329].

Under normal conditions, such as drug metabolism leading to drug oxidation, this CYP-dependent enzymatic process proceeds smoothly, especially in drug metabolism, but occasionally, and most likely, in connection with the metabolism of 1,2-unsaturated PAs, much ROS is gener-

ated from incomplete split of oxygen (Fig. 7), leading to liver injury, but CYP concomitantly modifies the chemical structure of 1,2-unsaturated PA types [329], as shown for some examples (Fig. 9, Ref. [338]).

The retronecine-, heliotridine-, and otonecine-types of PAs actually lose their double bond between C1 and C2 and receive other conformational modifications, while the resulting dehydropyrrolizidine alkaloid (DHPA) or dehydronecinepyrrolizidine (DHP) are parts of the adduct formation with proteins and DNA (Fig. 8). It is conceivable that parts of the DHPA and DHP in the liver remain free of any adduct attached, leave the liver cell, are taken up by neighboring LSECs, or enter the systemic circulation before they dock with other cellular constituents, including DNA at organs outside of the liver [329]. These mechanistic steps close up the metabolic fate of injurious 1,2-unsaturated PAs provided by plants as shown with a few examples (Fig. 10, Ref. [329]).

12. Conclusions

Humans are exposed to a variety of chemicals with the potential of liver injury if incorporated and reaching the liver, which normally modifies the chemical structure of selected compounds to make them ready for elimination via urine or bile. Nonmodified compounds remaining in the liver cause on overproduction of ROS through microsomal or mitochondrial oxidative stress, which in turn triggers via lipid peroxidation the liver injury if antioxidant systems like reduced glutathione, catalase, or superoxide dismutase are not available in sufficient amounts. Human liver injury is commonly dependent on the dose of the toxin except for drugs and herbal medicines that may cause dose independent liver injury. Diagnosis of human liver injury by exogenous chemicals like heavy metals or phthalates used in form of esters as plasticizers remains challenging. In future clinical protocols, liver tests analyzed in the serum should follow required minimum threshold criteria, confounding alternative causes must sufficiently be excluded by careful clinical approaches including causality assessment using the updated RUCAM, and obtaining liver histology may be helpful possibly obtainable from patients provided a clinical indication is given.

Author Contributions

RT wrote the draft; TDX provided the figures and edited the first draft by adding new aspects. Both authors agreed to the final version to be submitted.

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Conflict of Interest

The authors declare no conflict of interest. RT is serving as one of the Guest editors of this journal. We declare that RT had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to VJ.

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