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SCIENTIFIC REPORT

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Specific effects on liver relevant for performing a dietary cumulative risk assessment of pesticide residues

European Food Safety Authority (EFSA) | Anna F. Castoldi | Tamara Coja | Anna Lanzoni | Kyriaki Machera | Luc Mohimont | Monica Nepal | Camilla Recordati | Federica Crivellente

Correspondence: pesticides.mrl@efsa.europa.eu

The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

According to the 'EFSA-SANTE Action Plan on Cumulative Risk Assessment for pesticides residues', EFSA initiated a retrospective cumulative risk assessment (CRA) of the effects of pesticide residues on the liver. For this CRA, EFSA identified the following liver-specific effects in accordance with the International Harmonisation of Nomenclature and Diagnostic Criteria (INHAND): (1) hypertrophy due to enzymatic induction, liver; (2) fatty change and/or phospholipidosis, hepatocellular; (3) degeneration/cell death, hepatocellular; (4) porphyria, hepatocellular, biliary duct; (5) cholestasis, hepatocellular, biliary duct; (6) preneoplastic and neoplastic changes, hepatocellular; (7) neoplastic changes, biliary duct. In addition, as gallbladder is part of the extrahepatic biliary system and can be affected by hepatic toxicity, the following specific effects in the gallbladder were defined: (1) erosion/ulceration, gallbladder (2) calculi, gallbladder and (3) neoplastic changes, gallbladder. Histopathology was considered as the most appropriate source of evidence together with the increase in relative liver weight, and a list of indicators was defined and will be used to collect information on these specific effects as included in the assessment reports of the different active substances used as plant protection products. The criteria for inclusion of active substances/metabolites into cumulative assessment groups (CAGs) were also defined, together with the hazard characterisation methodology and the lines of evidence for assessing CAG-membership probabilities. While primary indicators define the specific effect, secondary indicators and other endpoints (named ancillary endpoints) are considered not sufficiently informative to indicate a specific effect but are rather contributing to the overall evidence; these will be collected only for a limited number of substances (i.e. risk drivers based on hazard and exposure considerations) for determining the likelihood of the substances truly belonging to the CAGs (CAG-membership probabilities). Considering that it is not considered appropriate to establish CAGs for acute liver effects, CRAs on the liver will be only focused on chronic exposure. The process of data extraction and actual establishment of the CAGs is beyond the scope of this report. This part of the CRA process was outsourced and will be the subject of a separate report.

KEYWORDS

cumulative assessment groups, cumulative risk assessment, gallbladder, liver, pesticide residues, specific effects

CONTENTS

SUMMARY

EFSA initiated a retrospective cumulative risk assessment (CRA) of the effects of pesticide residues on the liver in line with the EFSA-SANTE Action Plan on CRA for pesticides residues. The EFSA internal mandate was focused on the identification of the liver-specific effects of relevance for the CRA of pesticide residues and this activity was performed with the support of a Working Group (WG) of experts. EFSA identified the following liver-specific effects in accordance with the International Harmonisation of Nomenclature and Diagnostic Criteria (INHAND): (1) hypertrophy due to enzymatic induction, liver; (2) fatty change and/or phospholipidosis, hepatocellular; (3) degeneration/cell death, hepatocellular; (4) porphyria, hepatocellular, biliary duct; (5) cholestasis, hepatocellular, biliary duct; (6) preneoplastic and neoplastic changes, hepatocellular; (7) and neoplastic changes, biliary duct. The following specific effects were identified in the gallbladder: (1) erosion/ulceration, gallbladder (2) calculi, gallbladder and (3) neoplastic changes, gallbladder. Histopathology was considered as the most appropriate source of evidence together with the increase in relative liver weight, and a list of primary indicators (i.e. toxicological endpoints measurable in regulatory studies) describing the different specific effects was compiled together with their synonyms. In addition, a list of secondary indicators (e.g. information on the consequence or progression following the primary toxic insult) was provided. While primary indicators trigger the inclusion of a substance in the respective cumulative assessment group (CAG), the secondary indicators only consist of supportive evidence of the soundness of this inclusion. Other liver ancillary endpoints routinely collected in toxicological studies (gross pathology findings, clinical pathology parameters, microsomal liver enzymes) were not considered by the WG as indicators since not sufficiently informative to indicate a specific effect; however, some ancillary endpoints were collected for risk drivers in order to facilitate assessment of CAG-membership probability (i.e. the probability that an AS/metabolite is actually causing the respective specific effect).

The WG also defined the criteria for inclusion of the active substances (AS)/metabolites into CAGs with the main one being the finding of at least one statistically significant and/or biologically relevant change in a primary indicator of a specific effect in a regulatory toxicological study. Each substance was characterised for its potency by the assignment of an overall no observed adverse effect level (NOAEL) and an overall lowest observed adverse effect level (LOAEL) for the respective specific effect based on the totality of the available oral studies showing primary indicators of that specific effect.

Finally, for risk drivers the WG established lines of evidence (LoE) for the assessment of the probability that a substance included in a CAG is causing the respective specific effect, together with their strength (high, medium, low) to assess the contribution of each LoE to the certainty that a substance is causally linked to the effect under consideration (CAG-membership probabilities).

Considering that the design of the required toxicological studies investigating the effects on liver is not suitable to identify the possible effects occurring after acute exposure, the establishment of acute CAGs for liver effects was not considered appropriate; therefore following CRA will be focused on chronic effects only.

Based upon the output of the WG and the feedback received from a public consultation, the specific effects identified, their respective indicators and ancillary endpoints, the criteria for CAG establishment and hazard characterisation methodology, and lines of evidence for CAG-membership probabilities are finalised.

The subsequent step of this work, related to data extraction and establishment of the CAGs, has been outsourced to Austrian Agency for Health and Food Safety (AGES, Austria) under the Framework Partnership Agreement (FPA) GP/EFSA/PREV/2023/03. The data will be collected in a template developed by AGES. The outcome of this work and the composition of the CAGs (separate report) will be published in the EFSA website.

1 | INTRODUCTION

Cumulative risk assessment (CRA) has been defined as the analysis, characterisation and possible quantification of the combined risks to health or the environment from multiple agents or stressors (U.S. EPA, 2003). It differs from most assessments, which primarily consider the effects of one agent or stressor in isolation.

In the European Union, Regulation (EC) No 1107/2009 concerning the placing on the market of plant protection products (PPPs)[1] and Regulation (EC) No 396/2005 on maximum residue levels (MRLs) in or on food and feed of plant and animal origin require thorough consideration of cumulative and the potential synergistic effects of residues of PPPs.

To comply with these provisions, EFSA and the Panel on PPPs and their residues (PPR Panel) started in 2007 the development of the necessary methodologies to carry out CRA of pesticide residues. This methodological development included a tiered approach for the assessment of cumulative risks of pesticides residues (EFSA PPR Panel, 2008), a guidance on the use of probabilistic methodology for modelling dietary exposure to pesticide residues (EFSA PPR Panel, 2012) and a procedure to establish cumulative assessment groups (CAGs) of pesticides on the basis of their toxicological profile (EFSA PPR Panel, 2013). In 2020, EFSA issued the first two reports on retrospective CRA of pesticides: one on the acute effects on the nervous system (EFSA, 2020a) and another on the chronic effects on the thyroid gland (EFSA, 2020b). These were subsequently followed by a retrospective CRA on chronic acetyl cholinesterase inhibition in 2021 (EFSA, 2021), a retrospective CRA regarding craniofacial alterations in 2022 (EFSA, 2022) and more recently the 2024 update on the CRA on thyroid (EFSA, 2024).

In 2021, a long-term EFSA-SANTE Action Plan on CRA for pesticides residues (EFSA-SANTE, 2021) was developed. This plan stipulates that by 2030, all toxicological effects of pesticides of relevance for CRA will have been identified and the CAGs in the respective organs and systems will have been established. This plan relies on two main pillars: prioritisation of ASs/metabolites and organs/systems and cooperation with Member States' competent organisations.

1.1 | Background and Terms of Reference as provided by EFSA

As part of the long-term EFSA-SANTE action plan of 2021, EFSA decided to perform a CRA for the cumulative effects on the liver. This was motivated by the incidence and severity of liver effects observed in toxicological studies (Technical University of Denmark, DTU, 2012) and the outcome of the prioritisation exercise of organ/systems requiring CRA to be performed by EFSA (EFSA, 2024).

The entire CRA process is indicatively planned over 27 months. It consists of a sequence of steps, which are described in Figure 1. These 14 steps cover the standard scheme of risk assessment (hazard assessment, exposure assessment and risk characterisation) with periods of consultations (public consultation, consultation with PPR Panel and/or selected experts).

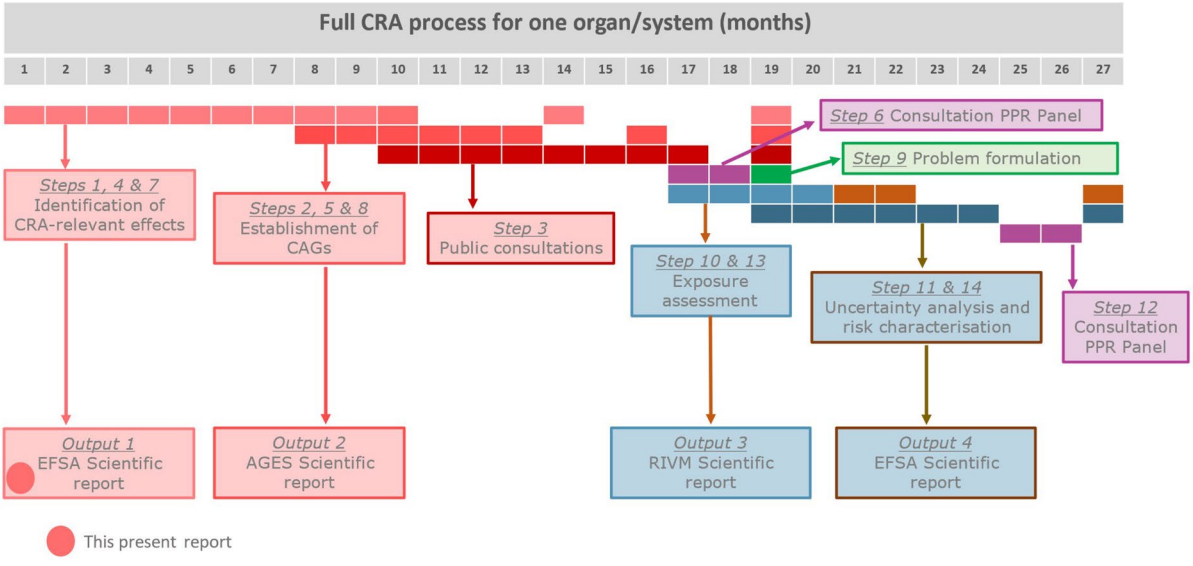

FIGURE 1 The 14 steps of the CRA of pesticide residues. The content of each step is described in detail in Appendix A

The CRA process is performed via the cooperation with Member States. Output 2 will be prepared by the Austrian Agency for Health and Food Safety (AGES) under the FPA GP/EFSA/PREV/2021/01. It is reported in the following outputs:

[1]Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1.

Output 1 (the present report): EFSA scientific report on specific effects on liver relevant for performing a dietary CRA of pesticide residues (referred to as 'specific effects' in the rest of the present report). The preparation of this report is supported by the EFSA Working Group (WG) on CRA.

Output 2: Scientific report of AGES on the hazard characterisation (covering the data collection) and establishment of CAGs of pesticides for specific effects on liver.

Output 3: EFSA scientific report on the cumulative dietary exposure assessment of pesticides that have effects on the liver using SAS® software.

Output 4: EFSA scientific report on the uncertainty analysis and the cumulative dietary risk characterisation of pesticides that have effects on the liver.

Public consultations are foreseen on draft outputs 1 and 2 and the submitted comments will be addressed in an annex of the respective scientific reports.

1.2 | Purpose and content of the present report

The present report (output 1) covers the identification of the liver-specific effects of relevance for the CRA of pesticide residues.

Furthermore, in preparation to the establishment of CAGs, the report also defines the following parameters:

- the indicators relevant to these specific effects;
- the conditions (hazard identification criteria) triggering the inclusion of pesticides (ASs and metabolites) into the respective CAGs;
- the principles for the hazard characterisation of the ASs and metabolites included in the CAGs for the respective specific effects;
- the LoE for the assessment of the probability that any pesticide included in a CAG is causing the respective specific effect (hereafter referred to as CAG-membership probability).

2 | BACKGROUND INFORMATION FOR SETTING THE SPECIFIC EFFECTS ON LIVER RELEVANT FOR PERFORMING A DIETARY CUMULATIVE RISK ASSESSMENT OF PESTICIDE RESIDUES

2.1 | Overview of liver anatomy and physiology

The liver is the largest internal organ in the mammalian body and it is located in the cranial/upper abdomen. It is divided into lobes that differ amongst species in number and shape and is covered by a thin connective tissue capsule externally lined by mesothelial cells. The liver has a dual blood supply, one from the hepatic portal vein (approximately 75%) and the other from the hepatic artery (approximately 25%). The hepatic portal vein drains the spleen, gastrointestinal tract, and pancreas, whereas the hepatic artery supplies oxygenated blood (Cattley & Cullen, 2018; Thoolen et al., 2010; Treuting et al., 2017). Within the liver, blood vessels subdivide into small capillaries, called sinusoids, leading to a lobule that drains towards the central hepatic vein. The lobule is the morphological unit of the liver and has a hexagonal structure that is composed of plates of hepatocytes separated by sinusoids.

The classical lobule is based on blood flow and it is a hexagonal structure with portal tracts at the periphery and central vein in the centre; it is divided into centrilobular, midzonal and periportal segments. The microscopic architecture of the liver consists of the portal lobule and the acinus (Ishibashi et al., 2009; Malarkey et al., 2005; Thoolen et al., 2010; Treuting et al., 2017) (Figure 2). The triangular portal lobule is based on bile flow and it is centred on the portal tract. The liver acinus is the functional subunit of the liver and is divided into zone I (periportal), zone II (transitional; midzonal), and zone III (centrilobular), based on hepatocellular enzymatic function and blood oxygenation. Functionally, zone I hepatocytes are specialised for oxidative liver functions such as gluconeogenesis, β-oxidation of fatty acids and cholesterol (CHOL) synthesis. Zone II hepatocytes are an important source of new hepatocytes during homeostasis and regeneration, while zone III cells are more important for glycolysis, lipogenesis and cytochrome P450 (CYP450)–based drug detoxification (Thoolen et al., 2010, Treuting et al., 2017). Notably, certain types of hepatic injury preferentially affect hepatocytes in particular zones. These differing sensitivities result from variation in hepatocyte oxygenation (highest in zone I, lowest in zone III) and metabolic activities from the periphery to the centre of the lobule (Crawford & Liu, 2021).

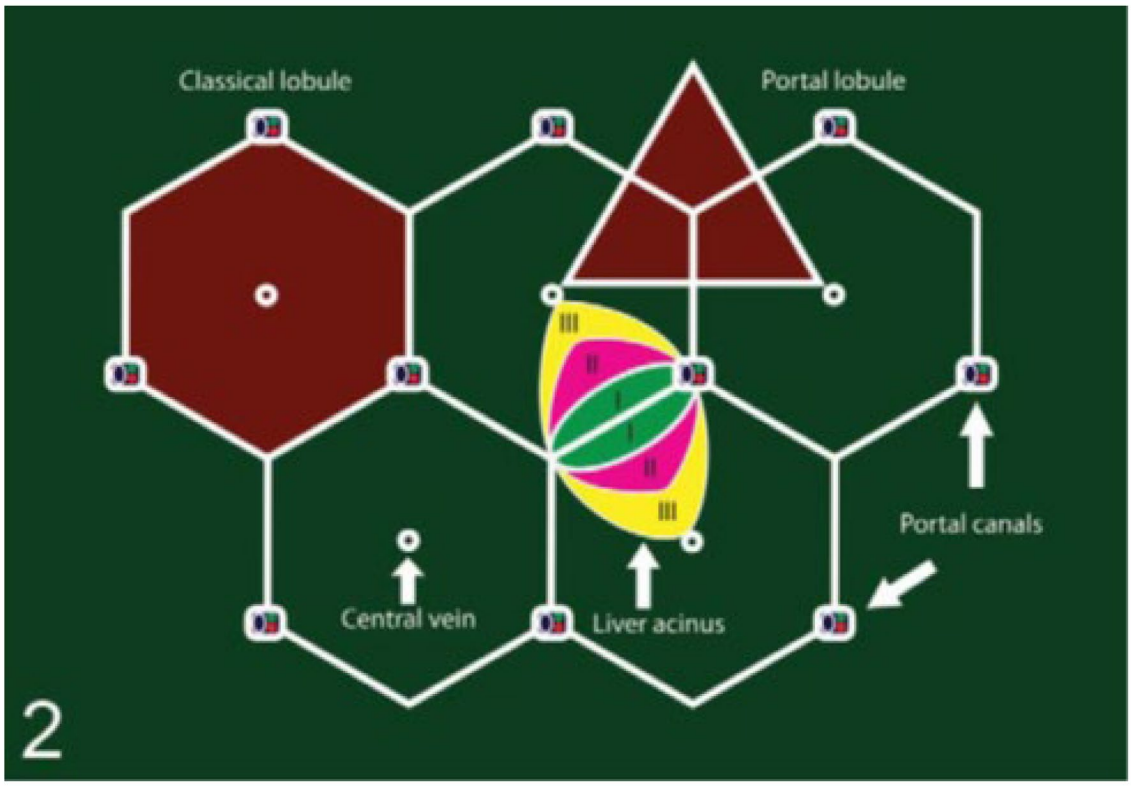

FIGURE 2 Hepatic microscopic structure, schematic representation of the classical lobule, portal lobule and acinus (Image source: From Thoolen et al., 2010, copyright Sage).

Mixed arterial and venous blood moves from portal regions to the central vein through sinusoids, whereas bile secreted into canaliculi is directed in the opposite direction to portal regions by specialised hepatocellular microvilli. Hepatic cords are single-cell-thick columns of hepatocytes extending from the portal region to central vein, separated by the sinusoids. Between the sinusoids and the hepatocytes there is the pericapillary space of Disse, where blood plasma is in direct contact with the hepatocytes, permitting free exchange of macromolecules. In addition to hepatocytes, the liver is composed of a variety of other cell types, including biliary epithelial cells, endothelial cells, Kupffer cells (resident macrophages) and Ito cells (or stellate cells, which stores lipids and vitamin A) (Thoolen et al., 2010; Treuting et al., 2017).

Physiologic functions of the liver are complex and diverse, including endocrine (secretion of some hormones such as Insulin-like growth factors, angiotensinogen and thrombopoietin) and exocrine activities (bile secretion), metabolism, detoxification, and haematopoiesis in early embryonic and foetal development. The liver is essential for the control of several metabolic functions, including glucose homeostasis, gluconeogenesis and glycogenolysis, fatty acid and cholesterol synthesis, processing of dietary fats to lipoproteins, and urea metabolism. Besides glycogen, it can store iron, copper and some vitamins like vitamin A, D, K and B12. Finally, it produces an extensive array of plasma proteins, including albumin (ALB), binding proteins, apolipoproteins, fibrinogens and other coagulation factors (Morini et al., 2020).

A pivotal hepatic function in toxicology is biotransformation of xenobiotics (Thoolen et al., 2010). In general, xenobiotic biotransformation serves to increase the water solubility of a lipophilic chemical and thereby enhances its excretion via bile or urine and limits its toxicity. However, in some cases, biotransformation leads to the formation of a variety of reactive intermediates that can be cytotoxic or mutagenic. Xenobiotic metabolism by hepatocytes can occur by phase I and/or phase II reactions, which mainly take place in the smooth endoplasmic reticulum of hepatocytes. Phase I reactions include primarily oxidation, reduction and hydrolysis involving primarily the cytochrome P450 (CYP450) family of enzymes. Phase II reactions conjugate the functional groups present in xenobiotics or metabolites generated from phase I reactions with glucuronate, glutathione or sulfate, resulting in more hydrophilic molecules that can be readily secreted into blood or bile (Kalra et al., 2023).

The gallbladder is a hollow, blindly ending organ that is part of the extrahepatic biliary system. In mice, dogs and primates, the gallbladder is located adjacent to the median lobe of the liver, and it functions to store and modify bile between meals, concentrating bile via bile salt-dependent mechanisms between meals and adding bicarbonate and other secretions during digestion. Rats lack a gallbladder, thus continuously secreting unconcentrated bile. Bile is important in the digestive process as the bile acids found in bile promote emulsification of lipid constituents of the diet (Cattley & Cullen, 2018).

2.2 | Elements of chemically-induced liver toxicity

Liver is particularly susceptible to chemically-induced injury due to its anatomical location (first site of contact for orally ingested xenobiotics after intestinal absorption) and its prominent role in metabolism and elimination of xenobiotics (Cullen, 2005).

Liver injury may result from one of the following events: (1) direct toxicity to hepatocytes and biliary epithelial cells; (2) conversion of a xenobiotic to a reactive species; or (3) immune mechanisms, usually by a drug or a metabolite acting as a hapten to convert a cellular protein into an immunogen (Crawford & Liu, 2021). The distinction between a 'direct toxic damage', which is dose-dependent, predictable and experimentally reproducible, and an 'idiosyncratic damage' (metabolic and immune-mediated), which is not dose-dependent, not predictable and not experimentally reproducible, is generally accepted (Grattagliano et al., 2002; Grattagliano et al., 2009). Genetic differences in the hepatic metabolism of xenobiotics play a major role in individual susceptibility to hepatotoxins (Crawford & Liu, 2021). Liver injury induced by chemical exposure can be reversible or irreversible depending on the nature, duration and intensity of the insult.

A major challenge in understanding mechanisms of liver injury is that several events upstream or downstream of the initial injury may be involved (Russmann et al., 2009). In a simplistic view, the initial injury can cause direct cell stress, direct mitochondrial inhibition or specific immune reactions (Russmann et al., 2009). Several basic mechanisms of chemically-induced liver injury have been identified, including disruption of calcium homeostasis and cell membrane injury; canalicular and cholestatic injury; metabolic bioactivation to reactive species by CYP450 enzymes; induction of autoimmunity; activation of apoptosis; mitochondrial injury; inhibition of β-oxidation, respiration or both, leading to oxidative stress; membrane lipid peroxidation; protein synthesis inhibition; cytoskeletal actin filament aggregation (Cullen, 2005; Hardisty & Brix, 2005; Lee, 2003). It is important to note that these mechanisms are not mutually exclusive as they can occur simultaneously or be interlinked, and it is often not clear which mechanism is of primary importance or which mechanisms are exactly responsible for the pathogenesis of cell injury by an individual drug or toxicant (Hardisty & Brix, 2005).

The gallbladder is rarely described as a toxicologically relevant target organ in the species that do have it. In the dog, cystic mucinous hyperplasia has been described following the administration of progestins and considered not relevant to humans since no risk of gallbladder disease has been associated to the widespread use of progestins in humans (Geil & Lamar, 1977). Inflammatory conditions of the gallbladder (cholecystitis) have been observed as a consequence of treatment with cholesterol-lowering agents in dogs (Gerson et al., 1991), while calculi (gallstones) are rarely reported in animals in contrast to humans. Proliferative lesions of the gallbladder epithelium such as adenomas and carcinomas are also rarely observed in toxicological studies and generally are spontaneous background conditions (Cattley & Cullen, 2018).

2.2.1 | Toxicological endpoints of liver toxicity in regulatory settings

The toxicological endpoints presented here are limited to the ones measured/observed in toxicological studies performed in laboratory animal species in agreement with data requirements set for pesticides.

2.2.1.1 | *Clinical pathology parameters, microsomal enzymes and gross pathology*

Some recommendations are available from the American Society for Veterinary Clinical Pathology (ASVCP) (Boone et al., 2005) for the selection of clinical pathology parameters and interpretation of liver-specific clinical pathology data for the assessment of hepatic injury in animal species.

Measurement of the activity of aminotransferases, also termed as transaminases, i.e. alanine aminotransferase (ALT) and aspartate aminotransferase (AST), cytosolic enzymes present in hepatocytes, is recommended for the assessment of hepatocellular injury in rats, dogs and non-human primates (NHPs) in toxicological studies. An increase in the activity of serum ALT, is commonly used as an indicator of hepatocellular injury. It is released in serum as a consequence of loss of hepatocyte membrane integrity or in case of hepatocellular hypertrophy (e.g. from sodium phenobarbitone) in the absence of evident hepatobiliary toxicity. However, differences in circulating half-life and dynamic range of ALT can not only influence the utility of ALT activity measurement across species as an indicator of hepatocellular injury, but can also make the interpretation of results in toxicological studies rather difficult (Ramaiah et al., 2017). In addition, ALT is present in substantial amount in other organs/tissues (kidney, etc.) and increased serum ALT can also be affected by extrahepatic factors such as muscle injury. ALT activity increase is considered a more specific and sensitive indicator of hepatocellular injury than AST in rats, dogs and NHPs; when serum levels are increased for both aminotransferase enzymes due to hepatic injury, the magnitude of ALT increase is usually greater than that of AST, in part due to the longer half-life of ALT and to the greater proportion of AST that is bound to mitochondria (Boone et al., 2005). Glutamate dehydrogenase (GLDH) is another liver-specific hepatocellular injury marker that is commonly evaluated in toxicological studies. In rat, GLDH has a longer half-life and larger dynamic range than ALT (O'Brien et al., 2002), making it a more sensitive indicator than ALT for detection of liver injury.

Although ALT is the most relevant clinical pathology parameter for the identification of potential chemically-induced hepatocellular injury in both toxicological studies and human patients, the best method for identification of potential chemically-induced hepatic injury in toxicological studies remains to be the integrated evaluation of clinical pathology parameters with results of histological evaluation and other study data (e.g. ADME, microsomal enzymes data, etc.) by using a weight of evidence approach (Boone et al., 2005; Hall et al., 2012; Ramaiah et al., 2017).

As regards to bilirubin, increases in serum levels are generally the result of bile retention subsequent to impairment of intrahepatic or extrahepatic bile flow (cholestasis), or originating from accelerated erythrocyte destruction or altered bilirubin metabolism (Boone et al., 2005). To clearly demonstrate chemically-induced cholestasis, increases in serum bilirubin supported by other evidence, including elevations in other biomarkers (e.g. alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT)), and most importantly, histopathological evidence of bile accumulation, need to be shown (Foster et al., 2020).

ALP and GGT are considered hepatobiliary markers but are also present in other tissues in the body. ALP shows highest expression levels in the liver but is present also in the bone, intestine, kidney and placenta; GGT is primarily present in the liver but also in the kidney. Differences amongst species are also reported and this should be taken into consideration in the interpretation of study findings (Boone et al., 2005; Ramaiah et al., 2017). An elevation in ALP and bilirubin in disproportion to ALT and AST would characterise a cholestatic pattern (Lala et al., 2023).

Total protein, albumin, triglycerides, cholesterol, glucose, urea, coagulation factors (activated partial thromboplastin time and prothrombin time) are considered as supplemental indicators of the hepatic synthetic functions. In chemical-induced hepatic injury, evaluation of these parameters may be instrumental in the identification of deleterious effects on glucose metabolism and hepatic synthesis of proteins, lipids and coagulation factors (Meyer & Harvey, 2004).

The clinical pathology parameters relevant for the examination of liver toxicity in the species used in toxicological studies are listed in Table 1.

Liver microsomal enzymes play an important role in metabolism of chemicals; however, they are not routinely investigated in studies performed according to commonly used OECD Test Guidelines. They are rather mostly investigated in liver homogenates in mechanistic studies, where more in-depth understanding of chemical metabolism, often in context of species-specific differences or mode of action (MoA), is needed. Some examples of microsomal enzymes that might be measured in toxicological studies are reported in Table 1.

Gross pathology findings (i.e. changes in colour, shape, size observed at macroscopic examination, see Table 1) are collected and analysed in the majority of the studies following OECD Test Guidelines. Changes in these endpoints are generally not specific and are used to corroborate study interpretation when integrated with other findings (clinical pathology, microsomal enzymes, liver weight and histopathology).

TABLE 1 Examples of clinical pathology parameters, microsomal enzymes and gross pathology considered as toxicological endpoints of liver toxicity.

	Parameters
Clinical pathology	• Alanine aminotransferase (ALT) • Aspartate aminotransferase (AST) • Glutamate dehydrogenase (GLDH) • Gamma-glutamyltransferase (GGT) • Alkaline phosphatase (ALP) • Total bilirubin (TBIL) • Unconjugated bilirubin (UBILI) • Total bile acids (TBA) • Lactate dehydrogenase (LDH) • Albumin (ALB) • Cholesterol (CHOL) • Triglycerides (TG) • Coagulation time: Activated partial thromboplastin clotting time (APTT) and Prothrombin time, (PT) • Sorbitol dehydrogenase (SDH) • 5′-nucleotidase (5′-NT) • Ornithine carbamyltransferase (OCT) • Glucose (GLU)
Microsomal liver enzymes	• CYP450 • Alkoxyresorufin-O-dealkylases: 7-ethoxy-resorufin O-deethylation (EROD), 7-methoxyresorufin O-demethylation (MROD), 7-benzyloxyresorufin (BROD) and pentoxy-resorufin O-deethylation (PROD) • p-nitrophenol-hydroxylase (PNPH), • Coumarin-7-hydroxylase • Uridine 5′-diphospo-glucuronosyltransferase (UDP-UGT)
Liver gross pathology	• Modification in size • Modification in shape • Modification in colour • Irregular surface • Masses

2.2.1.2 | *Changes in relative liver weight*

Increased liver weight is frequently observed in toxicological studies, particularly those conducted in rodents. According to JMPR 2015,[2] increases in relative (to body weight) liver weight in rats or mice of < 15% without further histopathological effects observed should not be considered adverse as such degree of increase has been seen in controls in numerous studies and is considered part of normal biological variation. On the other hand, a ≥ 15% increase in liver weight (relative to body weight) as compared to concurrent controls is per se an indicator of liver hypertrophy (see Section 4.4.2 for further details), in line with JMPR 2015 and reflecting the common basis for decision on adversity in the EFSA peer review of active substances.

2.2.1.3 | *Histopathology and special investigations*

Histopathology examination is considered the most relevant source of evidence to evaluate liver toxicity, since it can provide information on the type of toxicological insult, it is sensitive and routinely carried out in most regulatory toxicological studies for pesticide assessments. Dedicated terminology is available in toxicological species (INHAND, International Harmonisation of Nomenclature and Diagnostic Criteria; Mann et al., 2012), facilitating the harmonised description and interpretation of findings.

Distinct techniques, such as special histochemistry and immunohistochemistry, in situ hybridisation, proliferation markers and ultrastructural investigations (transmission and scanning electron microscopy), can be used to substantiate diagnosis (e.g. electron microscopy to diagnose phospholipidosis) and are generally used in mechanistic studies.

2.2.2 | The interpretation of liver findings in regulatory toxicological studies and their relevance to humans

The extrapolation of liver toxicity from laboratory animal studies to humans should take into account the anatomical (e.g. gallbladder is not present in rats), physiological (extramedullary haematopoiesis frequent in rodents, less in humans) and metabolic differences. Toxicological investigations generally provide a comprehensive overview of the effects of a chemical in the liver; however, it must be noted that liver toxicity can manifest as an idiosyncratic condition, difficult to extrapolate or used to predict human liver toxicity. Furthermore, in the case of non-genotoxic hepatocellular carcinogenesis in rodents, the modes of action involving hepatocellular proliferation following (or secondary to) the induction of nuclear receptors such as CAR/PXR (constitutive androstane receptor/pregnane-X receptor) and PPARα (peroxisome proliferator activator receptor alpha) have been described in the literature and demonstrated to be not human relevant (Felter et al., 2018; IARC, 1995). In addition, some effects such as spontaneous occurrence of foci of cellular alteration and increased nuclear ploidy are commonly observed in both rats and mice during the aging process. However, they may be further exacerbated by treatment, resulting in increased multiplicity or decreased latency; they are usually considered adverse when they are above a threshold level or when there is an apparent treatment-related increase or decrease in their occurrence (National Toxicology Program, NTP - Nonneoplastic Lesion Atlas, 2023).[3]

In the area of drug evaluation, it is well recognised that the liver is one of the organs most susceptible to drug toxicity, and in the clinic, drug-induced liver injury (DILI) has accounted for more than 50% of acute liver failure cases. However, current preclinical testing paradigms based on a combination of various in vitro and in vivo models are poorly predictive, at a quantitative and mechanistic level, of showing the potential for a new drug candidate to cause DILI in humans, in particular those drugs that show poorly defined dose–response relationships and/or human-specific mechanisms of toxicity (Weaver et al., 2020). Models aiming at investigating the mechanism of toxicity are of utmost importance and are under exploration (Weaver et al., 2020).

3 | METHODOLOGIES AND DATA FOR THE IDENTIFICATION OF THE LIVER-SPECIFIC EFFECTS AND PRIORITISATION OF PESTICIDES

3.1 | Methodology

3.1.1 | Criteria for grouping pesticides into cumulative assessment groups (CAGs)

Amongst all possible effects of chemicals on liver, those relevant for grouping pesticides into CAGs can be identified by using hazard-driven criteria as advised by the guidance of the EFSA Scientific Committee on scientific criteria for grouping chemicals into assessment groups for human risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2021).

[2]Guidance Document for WHO Monographers and Reviewers. Pesticide Residues in Food. WHO Core Assessment Group on Pesticide Residues. WHO, Geneva, 2015. Available online: https://iris.who.int/bitstream/handle/10665/144511/WHO_HSE_FOS_2015.1_eng.pdf?sequence=1.

[3]NTP Nonneoplastic lesion Atlas, 2023. Available online: https://ntp.niehs.nih.gov/atlas/nnl

The framework proposed by the EFSA Scientific Committee, as depicted in Figure 3, is based on the use of a hierarchical approach to evaluate the body of information available for each chemical.

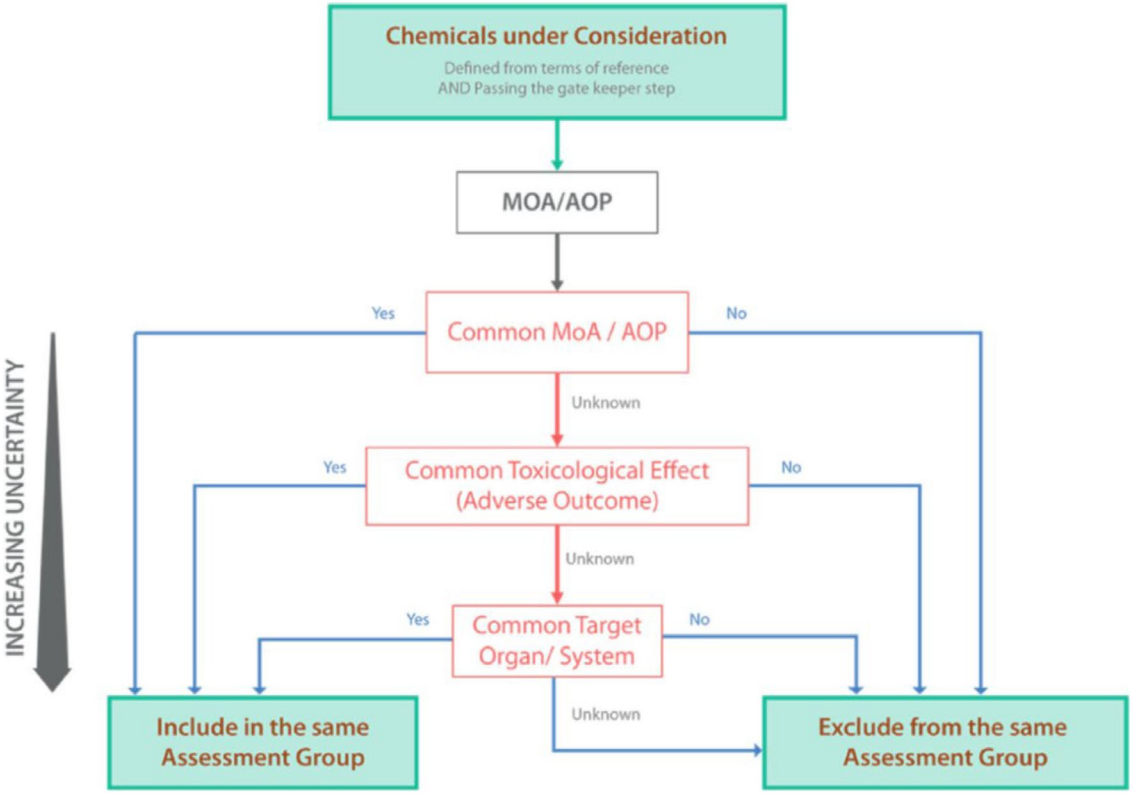

FIGURE 3 Top-down hierarchical process for grouping chemicals into assessment groups using hazard-driven criteria (from EFSA Scientific Committee, 2021).

3.1.1.1 | *Using common MoA/AOP – Relevance of AOPs to identify liver-specific effects*

According to the EFSA Scientific Committee's framework, the gold standard for grouping chemicals is considered to be the common MoA and/or the adverse outcome pathways (AOPs). In case of incomplete mechanistic information, the grouping may be based on the commonality of adverse outcomes (AOs). This approach is also of interest in case of AOP networks converging to a same AO even if they have different initiating events and pathways.

AOPs can inform chemical grouping and subsequent data gap filling by read across or trend analysis. Chemicals that are shown to share the same AOP based on results of assays or predictions of the molecular initiating events (MIEs) or key events (KEs) can be grouped together, thereby improving the robustness of the data gap filling approach for the AO, compared to grouping chemicals solely based on their structural similarity. AOPs thus provide an opportunity to group chemicals based on their intrinsic chemical properties as well as their biological activity at different levels of biological organisation. Whilst a complete knowledge of the AOP from the MIE to the final AO is not considered critical for the purposes of grouping chemicals sharing a common MIE or KE, it is necessary to have the linkages between the MIE or KEs and the AO (or key event relationships/KERs) established in order to justify the data gap filling (such as read across) performed (OECD, 2017).[4]

The grouping based on common target organ/system toxicity implies a large degree of uncertainty when the dose addition model is used for quantifying the combined effects of groups of chemicals assembled in this way due to inclusion of potentially less relevant substances. Increasing specificity and lowering this uncertainty are achieved when mechanistic information on toxicity (MoA or AOP) is considered and applying a weight of evidence (WoE) approach.

3.1.1.2 | *Using common toxicological effects to identify liver-specific effects*

Considering the possible limitations of the currently available AOPs (e.g. limited number of OECD-endorsed AOPs focused on liver effects, limited mechanistic data for pesticides on liver effects), the ASs and metabolites can instead be grouped in the respective CAGs based on the common AOs captured as apical endpoints in regulatory toxicity studies, in which these endpoints can be reasonably assumed to result from a MIE or/and KEs originated from the liver.

[4]Guidance document for the use of adverse outcome pathways in developing integrated approaches to testing and assessment (IATA), Series on Testing & Assessment No. 260, Environment, Health and Safety, Environment Directorate, OECD.

3.1.2 | Definitions

In the context of this report, the following general definitions are proposed:

Toxicological effects	**Toxicological effects** are adverse effects induced by xenobiotic substances. They are generally identified and characterised in in vivo toxicological studies performed according to OECD test guidelines.
Toxicological endpoints	**Toxicological endpoints** are endpoints measured/observed in toxicological studies performed in laboratory animal species in agreement with data requirements set for pesticides (i.e. clinical and functional signs, organ weights, clinical pathology parameters, gross pathology or histopathology findings, etc.).
Specific effect	A **specific effect** is a well-characterised and unambiguous toxicological adverse effect relevant for grouping pesticides into cumulative assessment groups (CAGs) in the context of cumulative risk assessment (CRA; EFSA PPR Panel, 2013). When the specific effect is assumed to be associated to one or several AOPs, it can be referred to as 'adverse outcome' (AO; EFSA, 2021) (see Figure 3 under 3.1.1). The specific effect is identified by expert judgement on the basis of its intrinsic relevance for CRA. It should be noted that 'specific effects' do not necessarily consist of a single toxicological effect observed or measured in regulatory toxicology studies but can also consist of a set of effects representative of a progressive toxicological continuum (e.g. preneoplastic and neoplastic changes including foci of cellular alteration, adenoma, carcinoma) or a functional/biological dysfunction (e.g. hypothyroidism, or functional alterations of the motor division of the nervous system, or liver preneoplastic and neoplastic changes). Criteria for the selection of specific effects relevant for CRA, including their identification and characterisation, were described in the EFSA Scientific Opinion of the PPR Panel published in 2013 (EFSA PPR Panel, 2013). Specific effects are identified based on expert judgement, aiming to exclude local or non-adverse effects, effects not relevant to humans and non-specific effects (e.g. age-related or occurring at or above the maximum tolerated dose). Specific effects are unambiguous and well-defined in terms of site of occurrence and nature. It is reasonable to assume that in case of co-exposure to pesticides triggering the same specific effect, the dose addition (DA) method is valid for the estimation of the risk from the exposure to multiple pesticides. The hypothesis behind the DA method is that all chemicals in a mixture act as if they were simply dilutions of one another. Thus, the true slopes of the dose–response curves for the individual chemicals are identical, and the responses elicited by the mixtures can be predicted by summing the individual doses after adjusting for differences in potency (EFSA Scientific Committee, 2019; Hertzberg et al., 2013; Loewe & Muischneck, 1926; US EPA;, 2000).
Indicator	An **indicator** describes a change in a toxicological endpoint considered relevant for the identification of a specific effect (EFSA PPR Panel, 2013). A single or a set of indicators can be used to identify a specific effect. In some circumstances, when the specific effect defines a very discrete toxicological insult, specific effects and indicators may be identical. For instance, 'acetylcholinesterase inhibition' is the single indicator of the specific effect 'acetylcholinesterase inhibition'. Based on canonical knowledge and expert judgement, indicators are defined as **primary indicators**, informing about the direct chemical interaction(s) of the substance with a biological target. Primary indicators are considered the most suitable for the identification of a specific effect (EFSA PPR Panel, 2013). **Secondary indicators** inform about the consequence or progression or a regenerative/reparative response following the primary toxic insult. They are not necessarily representative of one specific effect only (i.e. they are not unambiguous); nevertheless, they can corroborate the evidence that a substance is actually causing the specific effect.
Ancillary endpoints	**Ancillary endpoints** are toxicological endpoints considered as not sufficiently informative to indicate a specific effect since they are non-specific and/or not sensitive. However, they could contribute to corroborating the evidence that a substance is actually causing the specific effect.

Further details are provided in Figure 4, where the roles of primary indicators, secondary indicators and ancillary endpoints for the identification of the specific effects in liver are illustrated.

3.2 | Prioritisation of pesticides

In order to make the best use of resources available for CRA, EFSA developed and implemented a pesticide prioritisation methodology (EFSA, 2024). This methodology was based on short- and long-term exposure assessments for each individual pesticide quantified in at least one food sample of the official monitoring programme of EU Member States over a 3-year monitoring cycle from 2019 to 2021. These assessments were performed by probabilistic modelling in 30 population groups, covering different age groups (adults, children and toddlers) in 17 countries. The threshold to prioritise a pesticide was an acute or chronic hazard quotient (HQ), i.e. the ratio of the estimated short- or long-term exposure to the acute reference dose (ARfD) or acceptable daily intake (ADI), respectively, equal or exceeding 0.1 (10% of the reference value) at P99.9 of the exposure distribution in at least one of the 30 population groups under consideration. This resulted in a priority list of 67 pesticide residues (ASs and metabolites), of which 21 reached only the threshold in short term exposure assessments, 15 only in long-term exposure assessments, and 31 in both short- and long-term exposure assessments.

4 | ASSESSMENT – IDENTIFICATION OF THE LIVER-SPECIFIC EFFECTS AND THEIR RESPECTIVE INDICATORS

4.1 | Using MoA/AOP-relevance of AOPs to identify liver-specific effects

In the context of the current exercise on liver toxicity, 31 AOPs were identified in the AOP-Wiki as of 6 January 2024 by using generic terms (e.g. liver and hepatic). The identified AOPs were then screened and evaluated for relevance for this present exercise. The starting point for the evaluation of the 31 AOPs was their endorsement status by the OECD/Working Party on Hazard Assessment/Working Group of the National Coordinators of the Test Guidelines Programme (WPHA/WNT). Next, from the OECD-endorsed AOPs, the potential linkage of the identified indicators for liver effects to the KE was assessed. Based on these two fundamental criteria, three AOPs were considered of relevance for this present project to identify liver-specific (adverse) effects, namely: AOP 220/Cyp2E1 Activation Leading to Liver Cancer (https://aopwiki.org/aops/220); AOP 38/Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38); and AOP 131/Aryl hydrocarbon receptor activation leading to uroporphyria (https://aopwiki.org/aops/131).

Nevertheless, as a general observation, it is worth mentioning that the MIEs and KEs in several liver-related AOPs involve receptor activation, such as Aryl hydrocarbon Receptor (AhR), Liver X Receptor (LXR), PPARα, oestrogen and androstane receptors (AOPs: 37/PPPRa, 41/AHR, 107/CAR, 362/LXR, 401/ER[GPER], 383/Ang II, 107/androstane). Other MIEs are related to effects on proteins, such as alkylation, misfolded proteins, receptor coupling (AOPs: 38, 285, 260, 401) and involvement of mitochondrial dysfunction (144, 273, 362/[immune-mediated]). The formation of reactive oxygen species (ROS), reactive electrophiles and oxidative stress appears quite often as a relatively unspecific downstream event, rather close to the AO (AOPs: 27, 46, 213, 273, 260, 220, 383) and the formation of proinflammatory mediators (AOPs: 287 and 173).

As indicated above, the available liver-related AOPs that have been endorsed by the OECD are limited, and the respective mechanistic data related to the effects of pesticides on liver and gallbladder are also limited or not available at all in regulatory assessment reports. Due to the limited information available, the commonality of MoA and/or AOPs could not be used as a grouping criterion and for the establishment of CAGs.

4.2 | Using common toxicological effects to identify liver-specific effects

As described in Section 4.1, the commonality of MoA and/or AOPs could not be used as a grouping criterion and for the establishment of CAG due to the scarcity of data on mode/mechanism of action for most ASs/metabolites in regulatory toxicological studies. Instead, the grouping proposal was based on the common AOs on the liver or gallbladder. It can be reasonably assumed that these AOs resulted from KEs that took place in the liver. The liver-specific effects (i.e. common toxicological effects, adverse outcomes) of relevance for performing CRA on liver have been defined according to the criteria provided in the PPR Scientific Opinion (EFSA, 2013) (i.e. well-characterised, unambiguous effects) by using scientific knowledge and expert judgement (see the definition of specific effect under Section 3.1.2).

In summary, liver (and gallbladder) specific effects were identified and these were primary effects of toxicity (e.g. hepatocellular degeneration and cell death) and proliferative changes.

4.3 | Preparatory work for data collection on the prioritised pesticides

As part of the preparatory work for data collection, an Excel database template was developed and its applicability was tested on three randomly-selected pesticides with effects on liver as reported in the Draft Assessment Reports or Renewal Assessment Reports (DARs/RARs, respectively). These substances were not necessarily included in the main data collection, but they were considered for the initial implementation, evaluation and refinement of the template.

This preliminary data collection was performed by retrieving information on histopathological findings and on changes in liver weight and clinical pathology parameters from DARs/RARs and populating these in the Excel database template. Findings were subject to an in-depth analysis considering liver anatomy and physiology, as well as chemically-induced liver toxicity, and the nomenclature used to report liver histopathological changes was revised and enhanced with synonyms in line with INHAND/GoRENI.[5] An assessment of the relevance of changes in liver weight and in clinical pathology parameters related to liver dysfunction/damage was also performed, using the information retrieved from the open literature. Other toxicity endpoints routinely collected in toxicological studies (including gross pathology findings and microsomal enzymes, see Section 2.2.1.1) were also considered to complement information on respective liver toxicity.

[5]International Harmonization of Nomenclature and Diagnostic Criteria for Lesions (INHAND). Global Open Registry Nomenclature Information System (goRENI).

4.4 | Results

4.4.1 | Approach used for the identification of liver-specific effects

In toxicological studies, chemically-induced liver toxicity is generally identified by histopathology as confirmed in the preparatory work described in Section 4.3. Histological lesions accurately inform on the type of damage and its pathogenesis, and were therefore considered most adequate as indicators of liver (and gallbladder) specific effects. In addition to histopathology, a ≥ 15% increase in relative (to body weight) liver weight as compared to concurrent controls is per se an indicator of a specific effect, i.e. liver hypertrophy (see Section 4.4.2 for further details), in line with JMPR, 2015.[6] Thus, reflecting also the common practice for decision on adversity in the EFSA peer review, a ≥ 15% increase in relative (to body weight) liver weight was also considered a primary indicator of liver effects.

Liver histological lesions observed in toxicological studies can represent the (direct) consequence of the toxic effect of a chemical or follow up conditions (see Section 2.2); consistently, based on scientific knowledge and experts' judgement, and following the approach by Foster et al., 2020, histological lesions have been grouped into primary or secondary (see definitions under Section 3.1.2).

It is conceivable that chemicals causing the same primary lesion act on the same (or similar) biological target. Therefore, these chemicals could likely contribute to the cumulative effect following the dose addition model, and such primary lesions were therefore suitable to identify specific effects (described by primary indicators). Secondary histological lesions (described by secondary indicators) that may be indicative of but are not sufficient for the identification of a primary effect on the liver (or gallbladder) are used to support the evidence provided by the primary indicators (see Figure 4).

Proliferative findings (preneoplastic and neoplastic changes) constituted a specific liver effect in this exercise, irrespective of the molecular initiating event(s) and MoA since they are part of a continuum of effects in liver.

The other parameters listed in Table 1 are considered insufficient for the definition of specific effects but can serve as ancillary endpoints. As such, they constitute supportive information for study interpretation and will be considered later in the process under the lines of evidence for CAG-membership probabilities (see lines of evidence and elicitation of their weight under Section 7.3).

The approach used for the identification of specific effects is summarised in Figure 4 below.

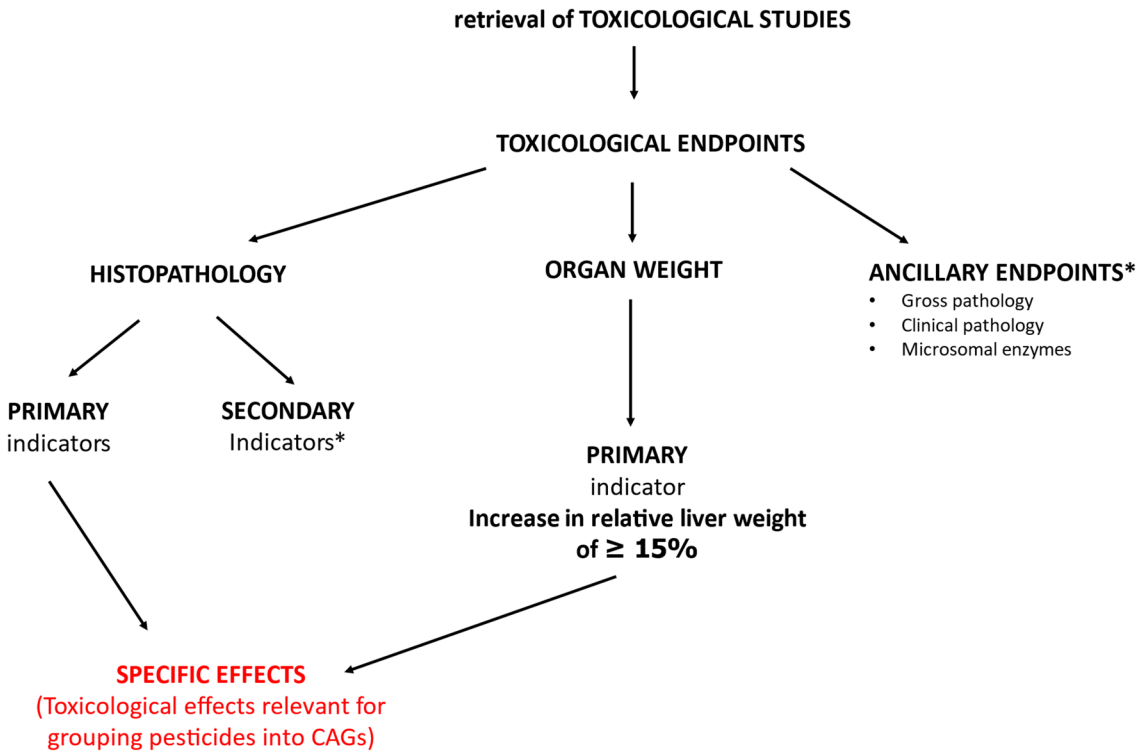

FIGURE 4 Figure showing the role of primary indicators for the identification of the specific effects in liver.
*Secondary indicators and ancillary endpoints are used to support the evidence provided by the primary indicators (as line of evidence for CAG-membership probability).

[6]WHO. (2015). Pesticide residues in food: WHO Core Assessment Group on Pesticide Residues. Guidance document for WHO monographers and reviewers WHO/HSE/GOS/2015.1, 1–106. http://www.who.int/foodsafety/publications/jmpr_guidance_document_1.pdf.

In detail, for histological lesions a step-by-step approach was followed to identify the specific effects and their indicators:

1. Revision of the nomenclature of the liver histological lesions according to GoRENI/INHAND[7] in order to support a precise definition of the specific effects and respective indicators. Synonyms suggested by INHAND or other sources (e.g. NTP, DAR/RAR) were also collected.
2. Identification and exclusion of not relevant histological lesions such as the following:

   - congenital lesions (e.g. hepatodiaphragmatic nodules);
   - background/spontaneous lesions (e.g. histopathological findings also observed in concurrent controls such as focal fatty change – tension lipidosis near the attachment of the falciform ligament);
   - human non-relevant tumours (e.g. tumours observed in rodents and demonstrated to be the consequence of hepatocellular proliferation following induction of nuclear receptor-mediated mechanisms such as CAR/PXR and PPARα).

3. Categorisation of chemical-induced histological lesions into primary and secondary indicators based on scientific knowledge and/or expert judgement and according to the structure involved (hepatocytes, biliary tract and gallbladder, where applicable).

Findings not relevant for CRA

In line with the EFSA PPR Panel, 2013, the following findings were not considered specific for liver toxicity and not relevant to support the identification and characterisation of specific effects:

- findings occurring above the maximum tolerated dose (MTD);
- effects secondary to extrahepatic conditions (such as hemosiderin deposition, extramedullary haematopoiesis)

Ancillary endpoints were described by gross pathology findings, clinical pathology parameters and microsomal enzymes. They were used to interpret liver findings applying a weight of evidence approach; however, they were not considered suitable candidates per se to identify a specific effect in liver.

4.4.2 | Liver-specific effects

Liver-specific effects were identified on the basis of lesions unique to hepatocytes and/or bile ducts cells and gallbladder in agreement with INHAND nomenclature.

Liver-specific effects were identified as follows (see also Table 2):

- Hypertrophy due to enzymatic induction, liver (chronic)
- Fatty change and/or phospholipidosis, hepatocellular (chronic)
- Degeneration/cell death, hepatocellular (acute or chronic)
- Porphyria, hepatocellular, biliary duct (chronic)
- Cholestasis, hepatocellular, biliary duct (chronic)
- Preneoplastic and neoplastic changes, hepatocellular (chronic)
- Neoplastic changes, biliary duct (chronic)

In addition, special considerations were made on the nature of each specific effect: acute, i.e. typically occurring after a single exposure; or chronic, i.e. after repeated exposure over a long period of time. The distinction between acute and chronic effects is needed for the next steps of the risk assessment (see outputs 3 and 4 under Section 1.1).

Hypertrophy is a frequent response of the liver following the administration of chemicals in toxicity studies; diagnostic histopathological features include the presence of enlarged hepatocytes with homogeneous or granular cytoplasm. The distribution can show a zonal pattern (centrilobular, periportal, midzonal) or be diffuse, and it involves most or all lobules. In prominent cases, it might be associated with loss of hepatocellular plate architecture, sinusoidal compression and concurrent degeneration and/or single cell necrosis. It is generally accompanied by increased liver weight and sometimes with increased size/enlargement of the organ at macroscopic observation. This effect commonly occurs following exposure to enzyme-inducing xenobiotics. The enlargement of the hepatocyte cytoplasm can be secondary to increase in the cytosolic protein or number of organelles (e.g. smooth endoplasmic reticulum (SER), peroxisomes, mitochondria). Although specific histological features could be identified (e.g. bright red, finely granular eosinophilic cytoplasm is generally indicative of peroxisome or mitochondrial proliferation, whereas a more homogenous cytoplasm indicates SER proliferation), electron

[7]goRENi is the standard reference for nomenclature and diagnostic criteria in toxicologic pathology and at the same time the Internet discussion platform for the global initiative INHAND (the International Harmonization of Nomenclature and Diagnostic criteria); INHAND is the result of collaboration between the Societies of Toxicologic Pathology from Europe (ESTP), Great Britain (BSTP), Japan (JSTP) and North America (STP) to develop internationally accepted nomenclature for proliferative and non-proliferative lesions in laboratory animals (Mann et al., 2012).

microscopy might be necessary to determine the exact nature of hepatocyte hypertrophy. Since hepatocyte hypertrophy can be microscopically detected once cytoplasmic expansion is sufficiently extensive, changes in liver weight can be used as a more sensitive indicator in case of minor changes. As indicated in Section 2.2.1.2, the increase in liver weight ≥ 15% as compared to concurrent controls is per se considered an indicator for liver hypertrophy.

Chemicals can act as inducers of various enzymes, and histology/weight changes might not be sufficient to discriminate which enzymatic system is involved. In a conservative manner, dose addition is assumed in the cumulative exposure calculation to chemicals causing liver hypertrophy as this effect is associated to well-established liver-mediated MoAs. The fact that these MoAs may differ from one chemical to the other will be considered in the assessment of uncertainties during the risk characterisation (uncertainty about the dose addition model). It should be mentioned that this effect is of **chronic** nature.

Fatty change/Phospholipidosis

Fatty change describes the accumulation of lipids (generally neutral fatty acids) in hepatocellular cytoplasm and can occur as focal/multifocal effect. Histopathologically it is generally described as hepatocellular cytoplasmic vacuolation; it can be micro- or macrovesicular in type although mixed forms can also be observed. Special stains can be used to confirm that hepatocellular cytoplasmic vacuolation is related to lipid deposition (e.g. Oil red O, Sudan black, osmium tetroxide). Fatty change can occur as a secondary physiological effect in case of poor condition, where the metabolism has shifted to the use of stored lipid as a primary energy source, which is rather a non-specific finding. In contrast, moderate to severe fatty change, in the absence of other significant toxicities and of reduced food intake, is likely to indicate a primary effect with dose addition expected for chemicals causing this effect.

Phospholipidosis consists of the accumulation of phospholipids in lysosomes. It can be consequent to interference of normal turnover of cellular membranes and/or to inhibition of lysosomal enzymes, both leading to a common downstream morphological effect observed as fine cytoplasmic vacuolation of hepatocytes (and other liver cells), for which dose addition is likely.

In routine toxicity studies, it might be difficult to differentiate between vacuolation due to phospholipidosis and fatty change. Special techniques (immunohistochemistry or transmission electron microscopy) are needed in order to confirm the phosholipidosis diagnosis which are not routinely performed in regulatory toxicity studies. Thus, a single specific effect named 'fatty changes/phospholipidosis' is proposed for this project. Fatty change/phospholipidosis can be accompanied by inflammatory cell infiltrate and interstitial fibrosis in chronic toxicology studies.

Like hypertrophy, chemical-induced fatty change/phospholipidosis are considered of **chronic** nature.

Degeneration/cell death, hepatocellular includes hepatocellular cytoplasmic alterations, degeneration (such as hydropic change) and/or necrosis (in various forms, such as single cell necrosis, focal/multifocal, zonal, centrilobular; midzonal; periportal or diffuse). It can be accompanied by inflammatory cell infiltrate and interstitial fibrosis (observed in chronic toxicity studies). Various mechanisms can induce such changes, and it might not always be possible to identify them with sufficient certainty. In the absence of specific information (e.g. mechanistic studies) dose addition is assumed for chemicals causing such effect. This effect can be of **acute or chronic** nature.

Porphyria is related to a decreased activity in uroporphyrinogen decarboxylase, the hepatocellular cytoplasmic enzyme that catalyses the decarboxylation of uroporphyrinogen to form coproporphyrinogen and participates in the haeme biosynthetic pathway. Its decreased activity can be caused by chemicals (e.g. hexachlorobenzene, Louw et al., 1977; ethanol, De Molina et al., 2002) and it results in accumulation of haeme precursors (porphyrin) in the liver (and other organs/tissues). Histologically, dark brown crystals are seen within the hepatocytes, Kupffer cells, bile canaliculi and the lumen of the bile ducts; they show a characteristic birefringence under polarised light (Maltese cross pattern) (Chang et al., 2022). It is highly specific, and therefore dose addition is likely for chemicals causing such effect. Chemical-induced hepatic porphyria is of **chronic** nature.

Cholestasis is known to occur after the administration of various chemicals and can be an idiosyncratic condition. It is histologically characterised by pigment deposition (bile material) in the hepatocytes as well as Kupffer cells and bile plugs in canaliculi. It can be associated with hyperplasia of biliary duct and fibrosis. Clinical pathology findings, such as increased ALP, bilirubin, GGT and bile acids, can support the diagnosis of cholestasis, as ancillary findings. Dose addition is likely for chemicals causing such downstream effect even though the MIE could be different amongst chemicals. This effect is of **chronic** nature.

Preneoplastic and neoplastic changes are only considered in cumulative risk assessments if they are human relevant (see Section 2.2.2) and not caused by genotoxic mechanisms. It is conceivable that the dose addition model is not applicable to non-threshold genotoxic mechanisms for chemicals reacting directly with the DNA (e.g. clastogens or gene mutagens). Foci of hepatocellular alteration and adenoma/carcinoma are preneoplastic and neoplastic hepatocellular changes, respectively; cholangioma and cholangiocarcinoma represent neoplastic changes of biliary duct. These effects are of **chronic** nature.

***Gallbladder*-specific effects are identified as follows (see also Table 2):**

- **Erosion/ulceration** is intended as damage (necrosis) of the gallbladder mucosa. It can be variably associated with inflammation and hyperplasia of the mucosa itself, and other gallbladder components (submucosa, serosa). Dose addition is likely for chemicals causing such effect. This effect can be of **acute** or **chronic** nature.

– **Calculi** or gallstones are concretion(s) in the gallbladder, single or multiple, composed of a mixture of cholesterol, calcium salts, haemoglobin and occasionally as a pure stone of just one of these; calculi can be observed macroscopically and can cause inflammation and hyperplasia of the gallbladder as secondary effect. This effect is of **chronic** nature.
– **Neoplastic changes** include adenomas and carcinomas of the gallbladder not caused by genotoxic mechanisms. It is conceivable that the dose addition model is not applicable to non-threshold genotoxic mechanisms for chemicals reacting directly with the DNA (e.g. clastogens or gene mutagens). These effects can derive from sustained degenerative/regenerative effects on the gallbladder mucosa and are of **chronic** nature.

4.4.3 | Indicators of liver-specific effects

Primary and secondary indicators for each liver-specific effect are listed in the table below.

TABLE 2 A list of liver-specific effects and their respective primary and secondary indicators.

	Specific effect	Primary indicators	Secondary indicators
Non neoplastic	**Hypertrophy due to enzymatic induction, liver**	• Hypertrophy, hepatocellular • Liver weight increase ≥ 15% relative to body weight as compared to concurrent controls	
	Fatty change and/or phospholipidosis, hepatocellular	• Fatty change, hepatocellular • Phospholipidosis, hepatocellular	• Inflammatory cell infiltrate, liver • Fibrosis
	Degeneration/cell death, hepatocellular	• Cytoplasmic alteration, hepatocellular • Degeneration, hepatocellular • Necrosis, hepatocellular	• Inflammatory cell infiltrate, liver • Fibrosis
	Porphyria, hepatocellular, biliary duct	• Pigment, porphyria, hepatocellular, biliary duct	
	Cholestasis, hepatocellular, biliary duct	• Pigment, bile hepatocellular, biliary duct	• Hyperplasia, biliary duct • Fibrosis
Preneoplastic/neoplastic	**Preneoplastic and Neoplastic changes, hepatocellular**	• Foci of hepatocellular alteration • Adenoma, hepatocellular • Carcinoma, hepatocellular	–
	Neoplastic changes, biliary duct	• Cholangioma • Cholangiocarcinoma	–

An exhaustive list of synonyms of the indicators listed in Table 2 will be compiled by AGES during data collection and establishment of CAGs, based on descriptions of endpoints in DARs/RARs.

As indicated in Table 2, the majority of indicators of the specific effects are histopathological findings with the only exception being liver weight changes. Increase in relative (to body weight) liver weight ≥ 15% without further effects observed at (histo)pathology, as described in Section 4.4.1, is considered an indicator of liver hypertrophy.

Only primary indicators will be used for CAG allocation. Secondary indicators will only be used as supportive evidence of the CAG-membership probability and not to allocate the substance to a CAG.

Indicators for gallbladder-specific effects are listed in the Table 3.

TABLE 3 A list of gallbladder-specific effects and their indicators.

	Specific effect	Primary indicators	Secondary indicators
Non neoplastic	**Erosion/ulceration, gallbladder**	• Erosion, gallbladder in the absence of calculi • Ulceration, gallbladder in the absence of calculi	• Inflammation, gallbladder • Hyperplasia, gallbladder
	Calculi, gallbladder	• Calculi, gallbladder	• Inflammation, gallbladder • Hyperplasia, gallbladder
Neoplastic	**Neoplastic changes, gallbladder**	• Adenoma, gallbladder • Carcinoma, gallbladder	-

4.4.4 | Results – Liver ancillary endpoints

The ancillary endpoints considered not sufficiently informative to indicate a specific effect in the liver are depicted by gross pathology findings, clinical pathology parameters and microsomal liver enzymes.

As indicated in Section 2.2.1.1, several of the clinical pathology parameters listed in Table 1 are routinely collected as a part of the standard examinations in regulatory toxicity studies and are considered to be non-specific if evaluated in isolation. Therefore, they cannot serve as indicators of liver-specific effects but can only support and/or integrate histopathology. Clinical pathology parameters are considered ancillary endpoints that can contribute to corroborating the evidence that a chemical is actually causing the specific effect.

The same considerations apply to the induction of microsomal enzymes and liver gross pathology in rodents.

Further considerations to changes in ancillary endpoints are given under the lines of evidence for CAG-membership probability of risk drivers[8] only.

5 | INSTRUCTIONS FOR HAZARD IDENTIFICATION CRITERIA TO BE USED FOR THE INCLUSION OF PESTICIDES INTO CAGs

5.1 | Scope of data collection

The final list of prioritised ASs and metabolites will be defined by EFSA according to the methodology described in Section 3.2 and will be included in the report on data collection and establishment of CAGs to be provided by AGES.

5.2 | Instructions for data collection

For the list of ASs and metabolites selected, all reported primary and/or secondary indicators for each specific effect defined in Section 4.4.3 will be collected along with further information needed to implement efficiently the principles of the hazard characterisation (see Section 6) and the elicitation of CAG-membership probabilities (see Section 7).

The main sources of data will be draft or revised assessment reports (DARs and RARs, respectively) of active substances generated by Rapporteur Member States (RMSs) in the context of Regulation (EC) No 1107/2009 and the respective EFSA conclusions. The original study reports might be consulted for further clarification on a case-by-case basis. Additional data sources (e.g. Joint Meeting on Pesticide Residues (JMPR) evaluations) will be considered and potential differences in the evaluation of studies or additional studies from those in DARs and RARs and EFSA conclusions will be captured in the data collection spreadsheet. Information on ancillary endpoints that can be considered under the different lines of evidence for CAG-membership probability will only be collected for risk drivers.

The toxicological studies to be considered are all in vivo studies by the oral route that include histopathological examination and liver weight measurements, with the exception of rodent studies with up to five animals/sex/dose group, which are not considered robust enough for toxicological characterisation. This rule does not apply to metabolites with the consideration that oral 28-day repeated dose studies, albeit having lower confidence for evaluation than other repeated dose studies (e.g. 90-day), are very often the only toxicity data available for metabolites and are used for setting of reference values. Specifically for the reproductive toxicity studies, histopathological examination of the parental animals only will be considered.

The data will be collected in the searchable Excel database developed by AGES, who is in charge of the data collection under the FPA GP/EFSA/PREV/2023/03. The outcome of the data collection will be published in a scientific report (see Output 2 in Section 1.1).

5.3 | Criteria governing the inclusion of pesticides in a CAG

Only studies by the oral route will be considered, with the exception of rodent studies with up to five animals/sex/dose group, which are not considered robust enough for toxicological characterisation. This rule does not apply to metabolites with the consideration that oral 28-day repeated dose studies, albeit having lower confidence for evaluation than other repeated dose studies (e.g. 90-day), are very often the only toxicity data available and used for setting reference values.

As a general rule, the inclusion of a pesticide in a CAG will be determined by the observation of one or more primary indicator(s) of the respective specific effect (see Section 4.4.3). Such observations should be treatment-related, statistically significant and/or biologically relevant. An increase in incidence and/or severity of the observation, in alignment with the interpretation given in the DAR/RAR by using historical control data (HCD) when appropriate and available, even if observed in only one sex at a given time point, including interim sacrifice, in at least one in vivo toxicological study assessed as « acceptable » and/or «

[8]Risk drivers are active substances or metabolites that contribute at least 5% of the cumulative exposure exceeding the 99th percentile estimate for a specific commodity under given modelling conditions.

supportive »[9] in the DAR, RAR or equivalent document will have to be considered for the inclusion of the substances in the relevant CAG, with the following exceptions:

- effect occurring at doses causing decreases in body weight gain ≥ 10% associated with changes in feed intake, or clinical toxicity/behavioural changes, or mortality;
- effect not relevant for human health risk assessment, e.g. neoplastic changes demonstrated to be the consequence of MoAs that are considered human non-relevant, i.e. secondary to CAR/PXR and PPARα induction in rodents.

Due to the possible observation of multiple indicators relevant for more than one specific effect, an AS or a metabolite can be included in one or more CAGs.

CAGs will be established in view of cumulative risk assessments of chronic exposure following the general rule mentioned above in this section. The principles applicable to the characterisation of the substances included in this 'chronic' CAG are described in the following section.

Considering that the design of the required toxicological studies investigating the effects on liver are not suitable to identify the possible effects of the amount that can be ingested in a period of 24 h or less without appreciable health risk, it is considered not appropriate to establish acute CAGs for liver effects. Therefore, cumulative risk assessments from acute exposure leading to liver-specific effects will not be performed. This is in line with previous decision taken for acute CAGs established for kidney effects (see Report entitled 'Specific effects on kidneys relevant for performing a dietary cumulative risk assessment of pesticide residues').[10]

6 | INSTRUCTIONS FOR HAZARD CHARACTERISATION OF PESTICIDES INCLUDED IN CAGs IN VIEW OF CRA OF CHRONIC EXPOSURE

This section illustrates the envisioned methodology for characterising the ASs/metabolites to be included in the CAGs in view of risk assessments of chronic exposure to pesticide residues.

Each AS/metabolite included in each CAG will be characterised by the identification of a NOAEL and a LOAEL for the specific effect of interest. This may be different from the study's overall NOAEL/LOAEL if the liver-specific effect is not the most critical effect of the study. More specifically, for each substance, a NOAEL/LOAEL will be set for each indicator of the liver-specific effect. Then, amongst all the studies available from all different species, the study showing the lowest LOAEL with its respective NOAEL will be used for hazard characterisation as it reflects the most sensitive endpoint (indicator) of the most sensitive species for that liver-specific effect.

In case a study failed to identify a NOAEL and only provided a LOAEL for an indicator(s) of interest, a NOAEL will be derived from this LOAEL by applying an extra uncertainty factor (UF). Although the EFSA guidance on default values to be used in the absence of measured data (EFSA Scientific Committee, 2012) recommends defining the size of such extra UF on a case-by-case basis, the concerned studies will not be reassessed, and, as a systematic approach, a default UF of 10 will be used. This conservative UF is then taken into account in the uncertainty analysis.

All the primary indicators of chronic nature are considered equally relevant for the setting of the NOAEL/LOAEL of the respective specific effect. Secondary indicators and ancillary findings will only be used for assessing CAG-membership probability (see CAG-membership probability under Section 7).

Although the use of sub-acute studies is allowed for CAG allocation (hazard identification), it is generally not recommended for hazard characterisation when these studies serve as dose range finding (DRF) or mechanistic studies being not suitable to define the NOAEL and LOAEL because of limited power, i.e. low number of animals tested. The use of enhanced sub-acute studies in rodents (e.g. 28-day studies) can be considered for hazard characterisation in the case they are of sufficient power (i.e. with at least six animals/sex/group) to enable robust statistical analysis. Any exemption from this will be justified in the respective report.

6.1 | BMD analysis

Once the risk drivers are identified from the cumulative exposure calculations (see Step 10 under Appendix A), benchmark dose (BMD) analysis will be performed if data allow.

[9]To the extent that the eventual reported limitations do not impair the evaluation of the specific effect under consideration.

[10]https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2025.9406.

7 | INSTRUCTIONS FOR CAG-MEMBERSHIP PROBABILITY

7.1 | Introduction

The amount, reliability, relevance, specificity and consistency of evidence for causing the specific effects listed in Section 4.4.2 vary amongst ASs. This makes it uncertain as to which ASs/metabolites should be included in a given CAG, with some ASs/metabolites being more likely to belong to the CAG than others. This can be quantified by assessing the probability that a substance actually causes the specific effect.

In this report, this probability is referred to as CAG-membership probability. This probability can be estimated by a weight of evidence assessment consisting in a stepwise process with the following sequence of tasks:

1. Defining in precise terms the assessment question applicable to selected ASs/metabolites included in the CAG.
2. Identifying the lines of evidence (LoE) that are important for the assessment question. In the assessment of CAG-membership probabilities, the LoE typically refer to the indicators of the specific effect under consideration but are not necessarily restricted to these indicators. Depending on the specific effect, additional factors contributing to the evidence can be defined.
3. Rating qualitatively the weight of each line of evidence: the LoE are assessed with respect to their reliability and relevance to the assessment question. This assessment is conducted by expert discussion and results in qualifying each line of evidence for the weight of its contribution to the probability of the AS/metabolite causing the effect.
4. Reviewing the information available and listing the available LoE for the selected ASs/metabolites included in the CAG.
5. Assessing for the selected ASs/metabolites the probability under consideration using the 'approximate probability scale' from EFSA's uncertainty guidance (EFSA Scientific Committee, 2018).

The present report covers the assessment of the weight of the LoE, while the assessment of the CAG-membership probabilities will be addressed in a future report on the uncertainty analysis and the risk characterisation (Output 4 in Section 1.1). If necessary, additional or refined lines of evidence will be considered under Output 4, accordingly.

7.2 | Assessment question

The assessment question applicable to each AS/metabolite included in a CAG was agreed as follows:

'What is the probability that substance [AS/metabolite X] can cause, as a primary toxicological insult, [specific effect]?'

7.3 | Lines of evidence and elicitation of their weight

For each specific effect, the LoE applicable to the assessment question were defined and agreed (see Table 4).

TABLE 4 Strength agreed for each line of evidence.

LIVER

Specific effect	Lines of evidence	Strength (agreed score)	Reasoning
Hypertrophy due to enzymatic induction, liver	Observation of the two primary indicators	High	Observation of the two primary indicators highly contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and ancillary findings – Increase in microsomal enzymes (from the DAR/RAR): (1) experimental evidence of liver enzyme induction; (2) ADME data (TD and TK); (3) mechanistic studies	High	Observation of increase in microsomal enzymes highly contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and ancillary findings - gross pathology (e.g. enlarged liver)	Low	Observation of gross pathology findings (e.g. enlarged liver), since not specific, adds limited further evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and ancillary findings – clinical pathology: increase in ALT and/or AST and/or GGT in the absence of necrosis	Low	Increase in ALT and/or AST and/or GGT in the absence of necrosis provides limited additional evidence to liver hypertrophy observed at histopathological examination, being these parameters not specific for liver hypertrophy.

TABLE 4 (Continued)

Specific effect	Lines of evidence	Strength (agreed score)	Reasoning
	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of primary indicator(s) of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observations of primary indicator(s) of the effect in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for liver and there is evidence that the classification is driven by liver hypertrophy, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA: mode of action relevant for liver such as liver hypertrophy, induction of liver enzymes (CYPs) through the activation of nuclear receptors, e.g. AhR, etc.	High	Whenever available, factual information on the MoA for liver hypertrophy highly contributes to the evidence that an AS/metabolite is actually causing the effect.
Fatty change and/or phospholipidosis, hepatocellular	Observation of the two primary indicators	High	Observation of the two primary indicators highly contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and one or more secondary indicators – histopathological findings	Low	Observation of one or more secondary histopathological findings provides limited contribution to the evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and ancillary findings - gross pathology (i.e. changes in colour and size) and/or liver weight increase	Low	Changes in colour and size of the liver, since unspecific, are of limited relevance to the evidence of fatty changes and/or phospholipidosis.
	Observation of a primary indicator and ancillary findings – clinical pathology: increase in CHOL and/or TG	Medium	Increases in CHOL and/or TG are partly contributing to the evidence that an AS/metabolite is causing the effect.
	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of primary indicator(s) of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of primary indicator(s) in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for liver and there is evidence that the classification is driven by fatty change and/or phospholipidosis, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA: Induction of liver enzymes (CYPs) through the activation of receptors, e.g. altered lipid metabolism, altered GLU metabolism, etc.	High	Whenever available, factual information on the MoA for fatty change and/or phospholipidosis highly contributes to the evidence that an AS/metabolite is actually causing the effect. It is well known that the induction of liver enzymes through constitutive androstane receptor (CAR), pregnane-X-receptor (PXR), altered liver and GLU metabolism can result in fatty changes and/or phospholipidosis.

(Continues)

TABLE 4 (Continued)

Specific effect	Lines of evidence	Strength (agreed score)	Reasoning
Degeneration/ cell death, hepatocellular	Observation of more than one primary indicator	High	Observation of more than one primary indicator contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and one or more secondary indicators - histopathological findings	Low	Observation of one or more secondary histopathological findings provides limited contribution to the evidence that an AS/metabolite is actually causing the effect.
	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of primary indicators of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observations of primary indicators of the effect in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and ancillary findings – clinical pathology: biologically significant increase in ALT (> than two-fold) and/or GLDH	High	Changes in ALT and/or GLDH are highly contributing to the evidence that an AS/metabolite is causing degeneration/cell death in the liver, since quite specific for this kind of effect (Boone et al., 2005, EMA, 2010).
	Observation of a primary indicator and ancillary findings – clinical pathology: biologically significant increase in ALP and/or AST in the absence of other target organ/ systemic toxicity	Low to medium	Whenever there is the possibility that changes in ALP and/or AST are due to effects occurring in other organs (i.e. bone for ALP, muscle for AST, etc.) their changes provide limited contribution to the evidence that an AS/metabolite is actually causing the effect; when changes in ALP and/or AST are unlikely due to effects in other organs, this partially contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of a primary indicator and ancillary findings – clinical pathology: biologically significant increase in markers indicating liver dysfunction (total proteins, ALB, coagulation factors, CHOL, TG, GLU)	Low	Increases in markers indicating liver dysfunction (total proteins, ALB, coagulation factors, CHOL TG, GLU) provide limited contribution to the evidence that an AS/metabolite is actually causing the effect (degeneration/cell death in the liver) (Boone et al., 2005).
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for liver and there is evidence that the classification is driven by degeneration/cell death, hepatocellular, this represents the strongest evidence that an AS/ metabolite is actually causing the effect.
	Known MoA: mode of action for hepatocellular degeneration/cell death	High	Whenever available, factual information on the MoA for hepatocellular degeneration/cell death highly contributes to the evidence that an AS/metabolite is actually causing the effect.
Porphyria, hepatocellular, biliary duct	Evidence of dose–response relationship for the primary indicator	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of the primary indicator of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observations of the primary indicator in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for liver and there is evidence that the classification is driven by porphyria, this represent the strongest evidence that an AS/metabolite is actually causing the effect.

TABLE 4 (Continued)

Specific effect	Lines of evidence	Strength (agreed score)	Reasoning
	Known MoA (information available from literature) relevant for porphyria (e.g. disturbance in enzymes involved in haeme metabolism)	High	Whenever available, factual information on the MoA for porphyria (e.g. disturbance in enzymes involved in haeme metabolism) highly contributes to the evidence that an AS/metabolite is actually causing the effect.
Cholestasis, hepatocellular, biliary duct	Observation of a primary indicator and more than one secondary indicator	Low	Observation of more than one secondary indicator provides limited contribution to the evidence that an AS/metabolite is actually causing the effect.
	Evidence of dose–response relationship for the primary indicator	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of primary indicator of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observations of primary indicator in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of ancillary findings - clinical pathology: biologically significant increase in at least two of the following parameters: total bilirubin, GGT, ALP, bile acids	High	Biologically significant increase in at least two of the following parameters: total bilirubin, GGT, ALP, 5′-NT, bile acids, highly contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for liver and there is evidence that the classification is driven by cholestasis, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA (information available from literature) relevant for cholestasis (e.g. inhibition of bile acid transport, etc.)	High	Whenever available, factual information on the MoA for cholestasis (e.g. inhibition of bile acid transport) highly contributes to the evidence that an AS/metabolite is actually causing the effect.
Preneoplastic and neoplastic changes, hepatocellular	Observation of at least two primary indicators	High	Observation of at least two indicators highly contributes to the evidence that an AS/metabolite is actually causing the effect.
	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of the primary indicators of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observations of the primary indicator/s in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for liver and there is evidence that the classification is driven by preneoplastic and neoplastic changes, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA (information available from literature) relevant for preneoplastic or neoplastic changes (e.g. reduced apoptosis, etc.)	High	Whenever available, factual information on the MoA for preneoplastic and/or neoplastic changes (e.g. reduced apoptosis, etc.) highly contributes to the evidence that an AS/metabolite is actually causing the effect.
	Absence of evidence of known MoA of human non-relevance for tumour formation	High	Whenever a known MoA is not available and demonstrating the human non-relevance for some tumours, this highly contribute to the evidence that an AS/metabolite is actually causing the effect.

(Continues)

TABLE 4 (Continued)

Specific effect	Lines of evidence	Strength (agreed score)	Reasoning
Neoplastic changes, biliary ducts	Observation of the two primary indicators	Medium	Observation of the two primary indicators partly contributes to the evidence that an AS/metabolite is actually causing the effect.
	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of primary indicators of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observations of primary indicators in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for liver and there is evidence that the classification is driven by neoplastic changes in the biliary ducts, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA (information available from literature) relevant for neoplastic changes in the biliary ducts (e.g. genotoxic mechanisms, biliary hyperplasia observed in short term studies, etc.)	High	Whenever available, factual information on the MoA for neoplastic changes in the biliary duct (e.g. biliary hyperplasia observed in short term studies, etc.) highly contributes to the evidence that an AS/metabolite is actually causing the effect.

GALLBLADDER

Specific effect	Lines of evidence	Strength (agreed score)	Reasoning
Erosion/ulceration	Observation of a primary indicator and at least one secondary indicator	Low	Observation of at least one secondary indicator provides limited contribution to the evidence that an AS/metabolite is actually causing the effect.
	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of primary indicators of the effect in more than one species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of primary indicators in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for gallbladder and there is evidence that the classification is driven by erosion/ulceration, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA: mode of action relevant for erosion, ulceration in the gallbladder	High	Whenever available, factual information on the MoA for erosion/ulceration in the gallbladder highly contributes to the evidence that an AS/metabolite is actually causing the effect.
Calculi	Observation of a primary indicator and at least one secondary indicator	Low	Observation of at least one secondary indicator provides limited contribution to the evidence that an AS/metabolite is actually causing the effect.
	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.

(Continued)

Specific effect	Lines of evidence	Strength (agreed score)	Reasoning
	Observation of primary indicators of the effect in more than 1 species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of primary indicator/s in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for gallbladder and there is evidence that the classification is driven by calculi, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA: mode of action relevant for calculi/crystals in the gallbladder	High	Whenever available, factual information on the MoA for calculi in the gallbladder highly contributes to the evidence that an AS/metabolite is actually causing the effect.
Neoplastic changes	Evidence of dose–response relationship for any of the primary indicator(s)	High	When demonstrated, a dose–response relationship establishes causality link between the exposure to the chemical and the effect.
	Observation of primary indicators of the effect in more than 1 species	High	Considering the differences amongst species related to their physiology, repetition of observations in more than one species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Observation of primary indicator/s in two independent studies in the same species	High	Repetition of observations in two independent studies in the same species contributes to the evidence that an AS/metabolite is actually causing the effect.
	Harmonised classification and labelling (STOT SE and/or STOT RE)	High	Whenever a substance has a harmonised classification and labelling (STOT SE and/or STOT RE) for gallbladder and there is evidence that the classification is driven by neoplastic changes in the gallbladder, this represents the strongest evidence that an AS/metabolite is actually causing the effect.
	Known MoA: mode of action relevant for neoplastic changes in the gallbladder	High	Whenever available, factual information on the MoA for neoplastic changes in the gallbladder highly contributes to the evidence that an AS/metabolite is actually causing the effect.

8 | CONCLUSIONS

The present report defines the specific effects on liver and gallbladder eligible for CRA; it is therefore dedicated to hazard identification.

Eight specific effects on liver and three effects for the gallbladder were identified in view of the establishment of respective CAGs. The specific effects selected for the liver are: (1) hypertrophy due to enzymatic induction, liver; (2) fatty change and/or phospholipidosis, hepatocellular; (3) degeneration/cell death, hepatocellular; (4) porphyria, hepatocellular, biliary duct; (5) cholestasis, hepatocellular, biliary duct; (6) preneoplastic and neoplastic changes, hepatocellular; (7) neoplastic changes, biliary duct. The specific effects identified for the gallbladder are: (1) erosion/ulceration, gallbladder (2) calculi, gallbladder and (3) neoplastic changes, gallbladder. Primary and secondary indicators of each specific effect and their acute and/or chronic nature were also defined.

Criteria for inclusion of ASs/metabolites into CAGs were established and a hazard characterisation methodology was elaborated.

Finally, this report illustrates the LoE for assessing the CAG-membership probability to determine the likelihood that the ASs/metabolites included in the CAGs are actually causing the effect of interest.

The present report was finalised after due consideration by the WG of the comments received through the public consultation (https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0lTk000000PFyMIAW/pc0895). The original comments submitted during the public consultation and the way they were addressed by the WG can be found in the Public Consultation Report as Annex of the present output under supporting information.

9 | RECOMMENDATIONS

- In the absence of fully defined and validated AOPs, the liver-specific effects proposed were based exclusively on adverse outcomes (histopathological findings and relative to body liver weight increase by ≥ 15% compared to control) observed in regulatory toxicity studies. However, considering the recent indications provided by the EFSA Scientific Committee, it is recommended to further develop liver-specific AOPs by focusing on the adverse outcomes that are described in the pesticide dataset.
- To encourage further development of liver-specific AOPs, and the use of new approach methodologies (NAMs) (in vitro, in silico, ex vivo, *in chemico*) (Miccoli et al., 2022) to support mechanistic understanding of the observed effects.
- If the outcome of CRAs conducted with the CAGs established on the basis of the present report exceeds regulatory thresholds of acceptance, empirical research is recommended on how ASs driving the risk combine their effects at the anticipated dietary exposure levels, and on the extent to which this combination of effects deviates from dose addition assumption.

ABBREVIATIONS

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
AGES	Austrian Agency for Health and Food Safety
AhR	aryl hydrocarbon receptor
ALB	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AOs	adverse outcomes
AOPs	adverse outcome pathways
APTT	activated partial thromboplastin time
ARfD	acute reference dose
AS	active substance
AST	aspartate aminotransferase
ASVCP	American Society for Veterinary Clinical Pathology
BMD	Benchmark Dose
BROD	7-benzyloxyresorufin
BSTP	Great Britain Society of Toxicologic Pathology
CAG	Cumulative Assessment Group
CAR	Constitutive Androstane Receptor
CHOL	cholesterol
CRA	cumulative risk assessment
CYP	cytochrome
CYP450	cytochrome P-450
DA	dose addition
DAR	Draft Assessment Report
DILI	drug-induced liver injury
DRF	dose range finding
DTU	Technical University of Denmark
ECHA	European Chemical Agency
EPA	Environmental Protection Agency
EROD	7-ethoxy-resorufin O-deethylation
ESTP	European Society of Toxicologic Pathology
FPA	Framework Partnership Agreement
GGT	gamma glutamyl transferase
GLDH	glutamate dehydrogenase
GLU	glucose
goRENI	Standard reference for nomenclature and diagnostic criteria in toxicologic pathology
HCD	Historical Control Data
HQ	hazard quotient
IARC	International Agency for Research on Cancer
INHAND	International Harmonisation of Nomenclature and Diagnostic Criteria
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
JSTP	Japanese Society of Toxicologic Pathology
KEs	Key Events
KERs	key event relationships
LDH	lactate dehydrogenase
LOAEL	lowest observed adverse effect level

LoE	lines of evidence
LXR	liver X receptor
MIE	molecular initiating event
MoA	mode of action
MOET	total margin of exposure
MRLs	maximum residue levels
MROD	7-methoxyresorufin O-demethylation
MTD	maximum tolerated dose
NAMs	new approach methodologies
NHPs	non-human primates
NOAEL	no observed adverse effect level
NTP	National Toxicology Program
OCT	ornithine carbamyltransferase
OECD	Organisation for Economic Cooperation and Development
PNPH	p-nitrophenol-hydroxylase
PPARα	peroxisome proliferator-activated receptor alpha
PPPs	Plant Protection Products
PPR	Plant Protection Products and their Residues
PROD	pentoxy-resorufin O-deethylation
PT	prothrombin time
PXR	pregnane-X-receptor
RAR	renewal assessment report
RMS	Rapporteur Member State
ROS	reactive oxygen species
SDH	sorbitol dehydrogenase
SER	smooth endoplasmic reticulum
STOT RE	specific target organ toxicity repeated exposure
STOT SE	specific target organ toxicity single exposure
STP	Society of Toxicologic Pathology
TBA	total bile acids
TBIL	total bilirubin
TD	toxicodynamic
TK	toxicokinetic
TG	triglycerides
UBILI	unconjugated bilirubin
UF	uncertainty factor
UDP-UGT	uridine 5′-diphospho-glucuronosyltransferase
WG	Working Group
WoE	weight of evidence
WPHA/WNT	Working Party on Hazard Assessment/Working Group of the National Coordinators of the Test Guidelines Programme
5′-NT	5′-nucleotidase

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

The 14 steps of the CRA of pesticide residues.

Step	Month	Content description
1	1–10	Hazard identification: Definition of the specific effects of relevance for CRA The task consists in the identification and unambiguous definition of the specific effects of relevance for CRA for the organ/system under consideration in parallel with the hazard-driven criteria described in the guidance document of the EFSA Scientific Committee on scientific criteria for grouping chemicals into assessment groups for human risk assessment of combined exposure to multiple chemicals.[11] For each specific effect, respective relevant indicators (i.e. toxicological endpoints identifiable in toxicological studies reflecting the specific effect) are defined. To prepare Step 2, the conditions triggering the inclusion of active substances in each CAG to be established and the principles of their characterisation for the respective specific effects are also defined. Where relevant, the lines of evidence (LoE) to be considered in the assessment of the CAG-membership probability (i.e. the probability that a substance included in a CAG is actually causing the respective specific effect) are also defined. A first draft scientific report on the identification of the specific effects for the organ/system under consideration will be prepared by month 10 of the project timeline.
2	8–13	Hazard characterisation: Establishment of CAGs For each of the specific effects defined in Step 1, a CAG will be established. The first task consists in collecting all the indicators of the specific effects concerning the active substances prioritised by the prioritisation method (see Section 1.2.4) and causing effects on the organ/system under consideration as well as any further information needed to efficiently implement the principles of the hazard characterisation defined in Step 1 (e.g. details on the study design needed to allow the combination of studies for the characterisation of substances) or the uncertainty analysis in Step 11 (e.g. data allowing the assessment of the dose–response relationship). These data are collected from Draft Assessment Reports, Renewal Assessment Reports and their addenda generated by Rapporteur Member States in the context of Regulation (EC) No 1107/2009 as well as from other sources of similar nature (e.g. JMPR evaluations) in a predefined format/template. A robust quality check procedure of the data collection needs to be put in place. Second, the collected data are assessed to decide which active substance is to be included in the CAG(s), using the conditions of inclusion defined in Step 1. Finally, all the substances included in the CAGs are characterised for the respective specific effects, using the characterisation principles defined in Step 1. A first draft scientific report on the establishment of CAGs for the organ/system under consideration will be prepared by month 13.
3	10–19	Public consultations Two public consultations are conducted. The first public consultation is launched at month 10 and concerns the draft scientific report on the identification of the specific effects. The second public consultation is launched at month 14 and concerns the draft scientific report on the establishment of the CAGs. Both public consultations are initiated by EFSA and take place on the EFSA website within a 2-month period. Technical reports covering the two public consultations are prepared during month 17. These technical reports describe the results of the public consultations and provide answers to each submitted comment. They are published as annexes to the respective scientific reports.
4	14	Hazard identification (update) A second draft scientific report on the identification of the specific effects for the organ/system under consideration is prepared, based on the outcome of the public consultation, for submission to the PPR panel.
5	16	Hazard characterisation (update) A second draft scientific report on the establishment of CAGs for the organ/system under consideration is prepared, based on the outcome of the public consultation, for submission to the PPR panel.
6	17–18	PPR panel consultation or consultation with selected experts The PPR panel is consulted for the endorsement of the second draft scientific report on the identification of the specific effects for the organ/system under consideration and the second draft scientific report on the establishment of CAGs for the organ/system under consideration. Alternatively, the draft outputs may be submitted to external review.
7	19	Hazard identification (finalisation) The final scientific report on the identification of the specific effects for the organ/system under consideration (**Output 1**) is prepared, based on the outcome of the consultation of the PPR panel and submitted to the internal EFSA approval during month 19.
8	19	Hazard characterisation (finalisation) The final scientific report on the establishment of CAGs for the organ/system under consideration (**Output 2**) is prepared, based on the outcome of the consultation of the PPR panel submitted to the internal EFSA approval during month 19.

[11]EFSA Scientific Committee, More, S. J., Bampidis, V., Benford, D., Bragard, C., Hernandez-Jerez, A., Bennekou, S. H., Halldorsson, T. I., Koutsoumanis, K. P., Lambré, C., Machera, K., Naegeli, H., Nielsen, S. S., Schlatter, J. R., Schrenk, D., Silano, V., Turck, D., Younes, M., Benfenati, E., Crépet, A., Te Biesebeek, J. D., Testai, E., Dujardin, B., Dorne, J. L. C. M., & Hogstrand, C. (2021). Guidance Document on Scientific criteria for grouping chemicals into assessment groups for human risk assessment of combined exposure to multiple chemicals. EFSA Journal, 19(12), 7033. https://doi.org/10.2903/j.efsa.2021.7033.

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Step	Month	Content description
9	19	Problem formulation After establishment of the CAGs for the organ/system under consideration, the assessment questions (i.e. the precise health effects, for which a cumulative risk assessment needs to be performed) to be addressed in the following steps of the process (exposure assessment, uncertainty analysis and risk characterisation) are defined. The number of assessment questions per organ/system is not necessarily the same as the number of identified specific effects. If this does not impair the protectiveness of the CRA for the organ/system under consideration, the number of assessment questions can be smaller than the number of CAGs. The scope (e.g. commodities, consumer populations) and type of assessment (e.g. acute, chronic) related to each assessment question are also defined.
10	17–22	Cumulative exposure assessments Cumulative exposure assessments are performed by probabilistic modelling. These assessments meet the following conditions: • They are conducted with the SAS software. • Indicatively, they are performed for the up to about 30 populations of adults, children and toddlers in different EU Member States. • Indicatively, they use monitoring data from the latest available 3-year cycle of the Member States monitoring programmes on up to about 40 raw primary commodities, some of their processed products and on commodities intended for infants and children. • Inner loop execution is performed in accordance with the harmonised technical approach on the parameters governing retrospective cumulative exposure assessment of the European Commission,[12] for the Tier II scenario. • Sampling uncertainties affecting the occurrence and consumption data are quantified by outer loop execution where the inner loop execution is repeated 100 times. Prior to each execution, new consumption and occurrence data sets of the same size are generated from random sampling with replacement from the original data sets. • Sensitivity analyses are performed to explore the impact of sources of uncertainty on the results. At least the following sensitivity analyses are performed: a. Sensitivity with left-censored data imputed at 1/2 LOQ on commodities for which the use of the active substance is authorised b. Sensitivity analysis with all left-censored data imputed at zero c. Sensitivity analysis assuming that residues will not be present in any processed food d. Sensitivity analysis excluding samples obtained through a selective sampling strategy e. Sensitivity analysis assuming that samples are not subject to unit-to-unit variability (acute effects only) f. Sensitivity analysis assuming that no residues are present in drinking water g. Sensitivity analysis excluding non-compliant samples (i.e. samples in which residues of at least one pesticide exceeds the MRL multiplied by 2) Additional sensitivity analyses might be performed following the analysis of the detailed results of the calculations and/or according to needs identified during the uncertainty analysis process (Step 11 below). The results of cumulative exposure calculations are expressed as total margin of exposure (MOET). 95% confidence intervals (incl. central estimate) are reported for P50, P90, P95, P99 and P99.9 of the exposure distribution at least. Violin plots of the confidence intervals at the threshold for regulatory consideration (i.e. P99.9) are presented for the selected population groups. Detailed information on the input data and output data are formatted and reported in a way similar to the recent EFSA scientific reports on CRA regarding chronic AChE inhibition[13] and craniofacial alterations.[14] A draft scientific report on the cumulative exposure assessment is prepared by month 22. For each CAG, this report includes one Excel workbook containing the input data for the exposure assessment (see https://zenodo.org/record/7143238#.Y1FeXHZBzD4 for guidance on the content) and one excel workbook containing the output data concerning the Tier II exposure assessment (see https://zenodo.org/record/7143238#.Y1FeXHZBzD4 for guidance on the content)
11	19–24	Uncertainty analysis and risk characterisation An uncertainty analysis is conducted according to the stepwise process described in the recent EFSA scientific reports on CRA regarding chronic AChE inhibition and craniofacial alterations. It includes the following steps: • Step 11.1: Identification of sources of uncertainty and collection of information supporting the assessment of their impact. • Step 11.2: Evaluation of the individual impact of all sources of uncertainty. • Step 11.3: Evaluation of the combined impact of uncertainties relating to exposure on one hand and relating to toxicology on the other hand. • Step 11.4: 1-D Monte Carlo simulations to combine distributions quantifying uncertainties related to exposure and toxicology with the uncertainty distribution for the MOET at the 99.9th percentile of exposure generated by the exposure model. • Step 11.5: Accounting for dependencies and differences between populations.

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[12] https://ec.europa.eu/food/plant/pesticides/max_residue_levels/cumulative_risk/technical-annex_en.

[13] EFSA (European Food Safety Authority), Anastassiadou, M., Choi, J., Coja, T., Dujardin, B., Hart, A., Hernandez-Jerrez, A. F., Jarrah, S., Lostia, A., Machera, K., Mangas, I., Mienne, A., Schepens, M., Widenfalk, A., & Mohimont, L. (2020). Cumulative dietary risk assessment of chronic acetylcholinesterase inhibition by residues of pesticides. EFSA Journal, 19(2), 6392. https://10.2903/j.efsa.2021.6392.

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Step	Month	Content description
		This uncertainty analysis aims at quantifying the probability of the MOET at 99.9th percentile of the exposure distribution being less than 100 for all populations under consideration, which is the final expression of the cumulative risks. It is supported by expert knowledge elicitation, following the EFSA guidance.[15] At least 6 independent experts are involved in each area of scientific expertise necessary for the elicitation process (toxicology and dietary exposure). The process is supported by technical notes which document the impact of uncertainties and by ad-hoc sensitivity analyses. These can include recalculation of the cumulative exposure after Benchmark Dose (BMD) modelling for the substances which contribute the most to the cumulative risk. When this is required, the principles described in the guidance of the EFSA Scientific Committee on the use of the BMD approach in risk assessment,[16] including its updates, are applied and the corresponding EFSA platform for BMD analysis is used. Step 11.5 is also facilitated by simulations testing the impact of dependencies and the effect of downshift/upshift of the MOET distribution on the probability of the MOET at 99.9th percentile being below 100. Hearings of stakeholders in possession of particular data or knowledge informing the uncertainty analysis can be organised. A draft scientific report on the uncertainty analysis and the risk characterisation is prepared by month 24.
12	25–26	PPR Panel consultation or consultation with selected experts The PPR panel is consulted on the draft scientific report on the cumulative exposure assessment and on the draft scientific report on the uncertainty analysis and the risk characterisation. Alternatively, the draft outputs may be submitted to external review.
13	27	Cumulative exposure assessment (finalisation) The final scientific report on the cumulative exposure assessment (**Output 3**) is prepared based on the outcome of the consultation of the PPR panel.
14	27	Uncertainty analysis and risk characterisation (finalisation) The final scientific report on the uncertainty analysis and the risk characterisation (**Output 4**) is prepared based on the outcome of the consultation of the PPR panel.

As indicated in the above table, for each organ/system, the outcome of the entire process will be reported by the following outputs:

- **Output 1**: Scientific report identifying the specific effects of relevance for CRA and defining the relevant indicators, criteria for inclusion in CAGs, hazard characterisation rules and LoE for eventual assessment of the CAG-membership probability
- **Output 2**: Scientific report establishing the CAG, covering the data collection, the identification of substances causing the effect and their characterisation by a point of departure
- **Output 3**: Scientific report on the cumulative exposure assessment for the (critical) specific effects defined in output 1
- **Output 4**: Scientific report on the uncertainty analysis and the risk characterisation for the specific effects addressed in output 3

[15]EFSA (European Food Safety Authority). (2014). Guidance on Expert Knowledge Elicitation in Food and Feed Safety Risk Assessment. EFSA Journal, 12(6), 3734. https://10.2903/j.efsa.2014.3734.

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ANNEX A

Public Consultation Report

Annex A is available under the Supporting Information section on the online version of the scientific output.