Approved: 28 February 2024

DOI: 10.2903/j.efsa.2024.8672

SCIENTIFIC REPORT

efsa JOURNAL

Specific effects on the thyroid relevant for performing a dietary cumulative risk assessment of pesticide residues: 2024 update

European Food Safety Authority (EFSA) | Federica Crivellente | Antonio F. Hernández-Jerez | Anna Lanzoni | Francesca Metruccio | Luc Mohimont | Dimitra Nikolopoulou | Anna F. Castoldi

Correspondence:
pesticides.mrl@efsa.europa.eu

Abstract

EFSA updated its previous work on the establishment of specific effects that are considered relevant for grouping pesticide residues targeting the thyroid and for performing the retrospective assessment of dietary cumulative risk (CRA). The two specific effects already selected in 2019 leading to the two cumulative assessment groups (CAGs) 'hypothyroidism' and 'C-cell hypertrophy, hyperplasia and neoplasia' were reconfirmed. Compared to 2019, the list of indicators that can be used to identify these specific effects was refined to only include histopathological changes. In a second phase of the work, data will be extracted on indicators of the specific effects from the dossiers on active substances (a.s.) used as plant protection products. The criteria for including a.s. into CAGs were also updated, together with the hazard characterisation methodology and the lines of evidence for assessing CAG-membership probabilities. The tasks related to the data extraction and the establishment of the CAGs on hypothyroidism and on C-cell hypertrophy, hyperplasia and neoplasia are beyond the scope of this report. This part of the CRA process has been outsourced and will be the subject of a separate report.

KEYWORDS

cumulative risk assessment, cumulative toxicity, endocrine, hypothyroidism, pesticide residues, thyroid

CONTENTS

SUMMARY

EFSA was requested to update its 2019–2020 retrospective cumulative risk assessment (CRA) for pesticide residues targeting the thyroid, in line with the agreed 2021 EFSA-SANTE Action Plan on Cumulative Risk Assessment for pesticide residues.

The specific aim of this EFSA internal mandate was to review the specific effects on the thyroid relevant for grouping pesticide residues into cumulative assessment groups (CAGs). Additionally, EFSA, with the support of a working group (WG), revised the indicators of each specific effect (i.e. the toxicological endpoints measurable in regulatory studies), triggering the inclusion of active substances (a.s.) and metabolites into the respective CAG. The WG also refined the methodology for the hazard characterisation of a.s./metabolites included in the CAGs for their respective specific effects, as well as the lines of evidence (LoEs) to assess the probability that any substance included in a CAG actually causes the respective specific effect (referred to as CAG-membership probability in the rest of this report). Finally, the WG also developed a data collection template for extracting the relevant data for the allocation of the prioritised substances into the appropriate CAGs.

The WG reconfirmed the same two specific effects (corresponding to two CAGs) already identified in 2019, that is 'hypothyroidism' and 'C-cell (parafollicular cells) hypertrophy, hyperplasia and neoplasia'. Hypothyroidism is defined in this context as a disruption of the hypothalamic–pituitary–thyroid (HPT) axis resulting in follicular cell hypertrophy, hyperplasia and neoplasia. C-cell hypertrophy, hyperplasia and neoplasia represent a continuum of the same pathophysiological process starting from sustained C-cell stimulation and therefore constitute one single specific effect in the context of CRA.

Concerning the CAG on hypothyroidism, the list of indicators has been reconfirmed as in 2019 in terms of histopathological findings, which comprise follicular cell hypertrophy, follicular cell hyperplasia, follicular cell adenomas and follicular cell carcinomas, but not in terms of changes in serum hormone levels [decreased triiodothyronine (T3) and tetraiodothyronine (T4) and increased thyroid-stimulating hormone (TSH) levels] or in thyroid gland weight (increased absolute or relative weight). Hormonal and thyroid weight changes are rather considered by the WG as LoEs in the assessment of the specific effect. In the context of regulatory risk assessment, which is mainly based on animal studies, hypothyroidism is essentially inferred from histopathology changes, and supported by hormonal changes and other information, where available. For this reason, information on these parameters will only be collected for pesticide residues identified as risk drivers based on hazard and exposure considerations.

Likewise, concerning the CAG on C-cell hypertrophy, hyperplasia and neoplasia, histopathology is considered as the most appropriate source of evidence to define the indicators. Thus, C-cell hypertrophy, C-cell hyperplasia, C-cell adenomas and C-cell carcinomas were reconfirmed as indicators of this specific effect. Based on the experience gained by EFSA in 2020 in relation to CRA characterisation, the WG considered that a CRA for the CAG on C-cell hypertrophy, hyperplasia and neoplasia is no longer needed. Indeed, in 2020, the cumulative risk for the selected population groups of C-cell hypertrophy, hyperplasia and neoplasia from dietary exposure to pesticide residues was estimated to be lower than (and fully covered by) the cumulative risk of hypothyroidism. Still, the data collection and set-up of the CAG on C-cell hypertrophy, hyperplasia and neoplasia up to the hazard characterisation step will be carried out for the newly prioritised pesticide residues.

The WG reviewed the criteria for inclusion of a.s./metabolites into CAGs, with the main one being the finding of at least one statistically significant and/or biologically relevant change in an indicator of a specific effect in a regulatory toxicology study.

Regarding the criteria for hazard characterisation, the WG proposed that the potency of each a.s. is established by means of an overall No Observed Adverse Effect Level (NOAEL) and an overall Lowest Observed Adverse Effect Level (LOAEL) for the respective specific effect, considering the totality of the available oral studies showing indicators of that specific effect.

Finally, the WG agreed on the LoEs (i.e. known or presumed mode of action (MoA), evidence of damage progression, consistency of indicator changes within the same species and/or across species, decreased T3 and/or T4, increased TSH in serum) as well as on their relative weight (high, medium and low) for consideration in CAG-membership probability, that is to support the assessment of the probability that an a.s./metabolite has been correctly included in the CAG for hypothyroidism. Since past experience showed that the outcome of a CRA is in most cases driven by just a limited number of a.s., CAG-membership probability will be determined for risk drivers only.

A list of 21 prioritised a.s. and six metabolites was drawn up by EFSA based on a hazard quotient HQ value (i.e. ratio between chronic exposure estimated from monitoring studies and the NOAEL set for the chronic critical effect) higher than 10% and existing evidence for their potential disrupting effects on the HPT axis from an ad hoc database prepared by the Danish Technical University (DTU) in 2023 (unpublished).

The implementation of the above decisions about specific effects, indicators, principles of hazard characterisation and elicitation of CAG-membership probabilities is outside the scope of this report. The subsequent steps of this work, related to data extraction followed by the establishment of the CAGs on hypothyroidism and on C-cell hypertrophy, hyperplasia and neoplasia, have been outsourced to the International Centre for Pesticides and Health Risk Prevention (ICPS, Milan, Italy) under a framework partnership agreement. Data relevant to the prioritised substances will be collected in the template (developed by the WG and annexed to this report) using final Draft Assessment Reports (DARs), Renewal Assessment Reports (RARs) and other regulatory documents available as a source. ICPS will publish the outcome of this work and the composition of the two CAGs in a separate report.

1 | INTRODUCTION

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

In 2007, EFSA and the Panel on plant protection products (PPPs) and their residues (PPR Panel) started the development of the necessary methodologies to carry out CRA for pesticide residues. This methodological development included a tiered approach for the assessment of cumulative risks of pesticide 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 based on their toxicological profile (EFSA PPR Panel, 2013, 2014).

Such work complies with Regulation (EC) No 396/2005 on maximum residue levels (MRLs) in or on food and feed of plant and animal origin,[1] and Regulation (EC) No 1107/2009 concerning the placing on the market of PPPs,[2] which require a thorough consideration of the cumulative and synergistic effects of residues of PPPs. However, in 2013, the PPR Panel concluded that synergisms are less likely to occur at low doses/concentrations corresponding to the dietary exposure to mixtures of pesticides and are thus less relevant for the CRA of pesticide residues in food (EFSA PPR Panel, 2013). In addition, the limited available empirical evidence suggests that synergisms at dietary exposure levels are rather rare, and experimentally occurred at unrealistically high concentrations (Hernández et al., 2017).

In 2020, EFSA issued the first two reports on retrospective CRA of pesticides that have acute effects on the nervous system and chronic effects on the thyroid gland (EFSA, 2020a, 2020b). These were followed in 2021 by a retrospective CRA regarding chronic acetylcholinesterase inhibition (EFSA, 2021), and in 2022 by a retrospective CRA regarding craniofacial alterations (EFSA, 2022).

In 2021, EFSA and SANTE agreed on a long-term Action Plan on CRA for pesticide residues, which will involve close cooperation with Member States (MSs) and competent organisations. The aim of this action plan was to complete by 2030 the identification of all toxicological effects of pesticides of relevance for CRA and the establishment of CAGs in the respective organs/systems (EFSA-SANTE, 2021). To speed up this complex and resource-consuming process, this plan entails the prioritisation of the most critical a.s. and organs/systems (i.e. those presenting the highest risk in terms of dietary exposure) that require a refined CRA and the elaboration of new CAGs. Furthermore, the prioritisation will be repeated every 3 years to select the most critical organ systems to be (re-)considered for CRA, to update the CAGs with the substances emerging from the prioritisation exercise, and to review the toxicological characterisation of pesticides when new information is available to EFSA. The present report just falls in the context of the update of the retrospective CRA of pesticides that have chronic effects on the thyroid gland 3 years after its publication (EFSA, 2020b). Specifically, between 2019 and 2020, EFSA issued four reports covering all the steps of risk assessment, one dealing with cumulative hazard assessment (EFSA, 2019a), two addressing cumulative dietary exposure (EFSA, 2019b; Van Klaveren et al., 2019) and the last one reporting the outcome of the cumulative risk characterisation and of the uncertainty analysis (UA) (EFSA, 2020b). This report will specifically deal with the update of the hazard assessment part of the CRA process.

1.1 | Background and Terms of Reference as provided by EFSA

As part of the 2021 long-term EFSA-SANTE action plan, the Pesticides Peer Review Unit and the Plant Health & Pesticides Residues Unit are requested by EFSA to update the retrospective CRA for the effects of pesticide residues on the thyroid. This internal mandate is consistent with the EFSA duties resulting from Article 32 of Regulation (EC) No 396/2005, which provides that EFSA draws up annual reports on pesticide residues considering the results of official control of pesticide residues in food commodities carried out by MSs and including an analysis of the acute and chronic risks to the health of consumers.

This assessment will be performed under a cooperation between EFSA and the International Centre for Pesticides and Health Risk Prevention (ICPS, Milan, Italy), which was formalised by the specific agreement GP/EFSA/PREV/2021/01 SA-12-2023-ICPS. The latter includes the following main tasks and deliverables:

Collection of data on all the indicators of the specific effects concerning the a.s. and metabolites identified by the prioritisation method and causing effects on the thyroid.

- Task 1: Establishment of CAGs: identification of the substances to be included and characterisation of these substances by an overall No Observed Adverse Effect Level (NOAEL) and an overall Lowest observed adverse effect level (LOAEL) for the respective specific effect.
- Task 2: Preparation of an external scientific report on the establishment of CAGs for the thyroid.
- Task 3: Preparation of technical notes documenting the uncertainties affecting the establishment of CAGs.
- Task 4: Active participation in the uncertainty analysis.

[1]Regulation (EC) No 396/2005 of the Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. OJ L 70, 16.03.2005, pp. 1–16.

[2]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.

1.2 | Purpose, content and methodology of the present report

The present report deals with the revision of the hazard assessment part of the CRA process carried out in 2019, being therefore an update of the 'Scientific report on the establishment of cumulative assessment groups of pesticides for their effects on the thyroid' (EFSA, 2019a).

As compared to EFSA (2019a), however, the scope of this report is more limited, as it provides the 'instructions' to set up the CAGs and how to characterise the substances included in these CAGs. However, the actual establishment of the CAGs, from the data extraction onwards, has been outsourced to ICPS (see Section 1.1) and will be published elsewhere. The UA is also beyond the scope of this document.

A new EFSA working group (WG) has been set up for the preparation of this report. As regards the methodology used, the WG took stock of the 2019a report, confirmed and/or revised the decisions taken at that time, and agreed to integrate the text with some new sections as described further below.

More specifically, the WG was tasked to:

- to review the previous work done and conclude on whether the same two specific effects on the thyroid relevant for setting up CAGs and for performing the retrospective dietary CRA for pesticide residues could be reconfirmed or new ones should be considered;
- revise the indicators relevant to support the specific effects;
- refine the conditions (hazard identification criteria) triggering the inclusion of a.s. and/or metabolites into the respective CAGs;
- lay down the scientific principles for the hazard characterisation of the a.s. and/or metabolites included in the CAGs for the respective specific effects;
- review the lines of evidence (LoEs) for the CAG hypothyroidism as well as their relative weight for the assessment of the probability that any substance included in a CAG is causing the respective specific effect (referred to as CAG-membership probability);
- a data collection template to be used by the contractor for collecting data from prioritised substances (see Annex A).

In comparison with the 2019a report, this report includes:

- a new section, that is Section 2, illustrating the anatomy and physiology of the thyroid gland for a better understanding of the following sections;
- clearer definitions of specific effects and indicators thereof (see Section 5.1.1);
- a new section on adverse outcome pathways (AOPs) as part of the mode of action (MoA) analysis of indicators of the thyroid-specific effects under assessment (see Section 5.1.2);
- the list of prioritised a.s. and metabolites targeting the thyroid that EFSA will consider for CAG inclusion (see Section 5.2.1).

2 | THYROID ANATOMY AND PHYSIOLOGY

The content of this section is based on Hernández et al., 2020; Nielsen et al., 2012; Jing & Zhang, 2022.

The thyroid gland is located within the visceral compartment of the neck (along with the trachea, oesophagus and pharynx). It consists of two lobes, right and left, on each side of the trachea, joined together in humans by an intermediate structure called the isthmus. The gland consists of many typical units called 'thyroid follicles' and small clusters of C-cells (formerly known as parafollicular cells) in between the follicles (Figure 1). The thyroid follicles consist of thyroid follicular cells (cuboidal epithelium in Figure 1) surrounding a lumen filled with a material called colloid. The colloid is mainly composed of thyroglobulin, an iodinated glycoprotein, used as a substrate for the synthesis of thyroid hormones (THs) and as an iodine storage in the body.

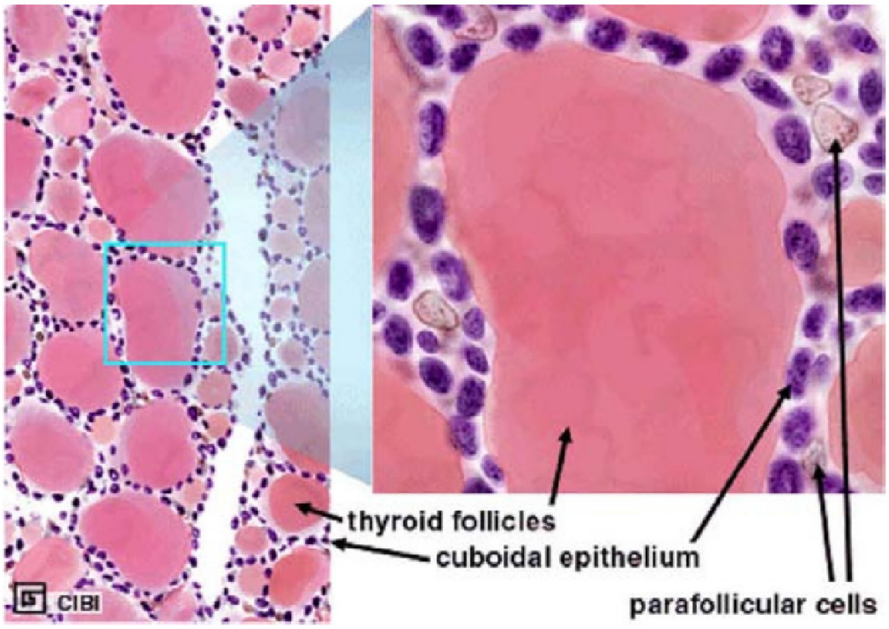

FIGURE 1 Histological section of a normal thyroid gland (from Endocrinology Website, online, www.drharper.ca).

The thyroid gland is part of the endocrine system and produces hormones that affect the function of almost every organ system in the body. The thyroid gland contains two types of cells that can be affected by chemicals:

- C-cells produce the hormone calcitonin, which takes part in the regulation of calcium levels in the blood. Only a few chemicals have been found to disturb the structure or function of the C-cells.
- The thyroid follicular cells produce iodine-containing hormones called iodothyronines. The circulating forms of THs are thyroxine (or tetraiodothyronine, T4) and, to a minor extent (about 10%), triiodothyronine (T3), which is the active form. T3 and T4 play essential roles in the normal development of, for example, brain, testis, lungs and heart in embryos and maturing animals. In adults, the main functions regulated by T3 and T4 include cardiac output, basal metabolic rate, resting respiratory rate and minute ventilation, reproductive health (e.g. ovulatory cycle and spermatogenesis) and other endocrine organ functions (e.g. pituitary), as well as renal clearance of many substances. Chemicals that affect the thyroid might change the structure or function of the follicular cells or change the concentration or the peripheral action of T3 and T4.

The function of the thyroid gland is primarily regulated via a feedback mechanism. The hypothalamus secretes thyrotropin-releasing hormone (TRH), which stimulates the pituitary gland to secrete thyroid-stimulating hormone (TSH) in response to decreased circulating levels of T3 and T4. TSH is essential for the thyroid gland to synthesise and secrete T3 and T4.

TSH is a major regulator of the expression and activity of sodium iodide symporter (NIS), a plasma membrane protein that plays an essential role in the uptake and accumulation of iodide in the thyroid. Iodide is actively transported into the thyroid follicular cells, where it is oxidised by thyroid peroxidase (TPO) into iodine and released into the colloid. The thyroid follicular cells, upon stimulation by TSH, synthesise thyroglobulin, which is released into the colloid-filled follicular lumen. Iodine binds to tyrosine residues within thyroglobulin, producing intramolecular monoiodothyronine and diiodothyronine (MIT and DIT, respectively), a process known as organification also catalysed by TPO. TPO then further catalyses the coupling of MIT and DIT in the same thyroglobulin molecule to produce T3 and T4. Following stimulation by TSH, the thyroglobulin with T3 and T4 is reabsorbed into the follicular cells and proteolytically cleaved to free T3 and T4, which ultimately are released into the blood circulation.

In the human blood, the majority of T4 and T3 (nearly 99.97% and 99.70%, respectively) are bound to various serum proteins, of which thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin are the most important. TBG accounts for 75%, TTR for 15% and albumin for 10% of total THs in the plasma. TBG is the main plasma T4-binding protein in humans, whereas TTR is the main plasma T4-binding protein in rats. In addition, the binding affinity of human TBG for T4 is approximately 1000 times greater than that of TTR. The effect of the binding proteins is to serve as a reservoir to buffer free T4 and T3 concentrations against transient fluctuations and ensure that the hormones are continuously and immediately available to the target organs. In general, T3 is bound less tightly to the proteins than T4, resulting in a shorter plasma half-life of T3. T3 is therefore more susceptible to alterations in hormone production than T4. Since the long-term free T3 and T4 levels are determined by the HPT axis, abnormality in the abundance or in the binding affinity of binding proteins can only result in changes in the concentrations of bound TH levels in the blood.

The membranes of target cells for T3 and T4 contain specific transporters, such as specific isoforms of monocarboxylate transporter (MCT) and organic anion transporting polypeptides (OATPs). These transport proteins are responsible for THs to reach their nuclear and mitochondrial receptors, where they exert their biological activity. Most biological actions of

iodothyronines seem to be mediated through receptors for T3 in the target cells. T3 activates the energy metabolism when it binds to receptors on the inner mitochondrial membrane. T3 stimulates the synthesis of membrane proteins, enzymes and hormones when it binds to nuclear receptors.

T4 is only produced in the thyroid gland. T3 is the physiologically active form of the iodothyronines, as it shows the highest affinity for TH receptors. In humans, about 80% of circulating T3 comes from deiodination of T4. T4 is converted to T3 predominantly by type I iodothyronine deiodinase (DIO1), which is largely found in the kidney, liver and thyroid. Conversely, type II iodothyronine deiodinase (DIO2) is present primarily in the skeletal muscle, central nervous system (CNS) and pituitary. Type III iodothyronine deiodinase (DIO3), which is present in the brain, skin, placenta and fetal tissues, inactivates T4 and T3, leading to the formation of reverse T3 (rT3) and T2, respectively. The expression of these enzymes changes during the lifetime of an individual in relation to the different needs of each organ and to ageing.

There are important inter-species differences in the metabolic clearance of THs. In rats, T3 and T4 are primarily excreted in the bile after conjugation in the liver to glucuronic acid (mainly T4) or sulfate (mainly T3). These reactions are catalysed by different isoforms of uridine 5′-diphospho (UDP)-glucuronosyltransferase (UGT) and sulfotransferases (SULT). Sulfate conjugation of T3 also enhances the conversion of T4 to T3 by DIO1. In contrast, in humans, the predominant route of metabolism for THs is inactivation by deiodination (Huisinga et al., 2020). In humans, about one-third of T4 (prohormone) is converted to T3 and about one-third to rT3. The rest of T4 is metabolised by different pathways, in particular glucuronidation and sulfation. T3 is further metabolised largely by inner ring deiodination and rT3 largely by outer ring deiodination, yielding in both cases the metabolite 3,3′-diiodothyronine (T2) (Peeters & Visser, 2017).

UGT and SULT activities can be induced by several endogenous and exogenous compounds, such as drugs and pesticides, by various mechanisms that is via nuclear receptors, which also induce various cytochrome P450 enzymes in experimental animals and humans (see Section 3). Induction of these Phase II enzymes enhances the conjugation of T3 and T4.

Bacteria in the intestinal lumen hydrolyse biliary excreted conjugated T3 and T4, such that the hydrolysed hormones are partially reabsorbed.

3 | MECHANISMS UNDERLYING DISRUPTIVE EFFECTS OF CHEMICALS ON THE HPT AXIS

Many chemical substances can disrupt the HPT axis by disturbing several pathways of TH regulation and signalling. The biological targets and/or molecular mechanisms involved are diverse, and the most prominent based on the current knowledge include the following (Bianco & Kim, 2006; Chemicals Regulation Directorate, Health & Safety Executive, UK, 2013; Crofton, 2008; Dong & Wade, 2017; Groeneweg et al., 2017; Lerro et al., 2018; Miller et al., 2009; Mughal et al., 2018):

a. Blockage of or competition for the iodide uptake into follicular cells of the thyroid gland via NIS, which is the initial step of TH biosynthesis. Several anions are blockers or competitive inhibitors of NIS, such as perchlorate and thiocyanate, which prevent iodide uptake by the thyroid gland.
b. Inhibition of TPO, the follicular cell enzyme involved in the catalysis of the first two steps in TH synthesis (see Section 2). As a result of TPO inhibition, TH production is decreased. TPO can be inhibited by thionamides (e.g. propylene thiourea – PTU and ethylene thiourea – ETU), anilines (aminoheterocyclic compounds) and substituted phenols (e.g. chloro- and nitrophenols, the main degradation products of chlorinated phenoxyacids and some organophosphorus pesticides).
c. Interference with TH-binding proteins, which are transport proteins that facilitate the distribution of the TH through the blood and further delivery to their target tissues. There are three principal plasma TH-binding proteins: TTR (originally called prealbumin), TBG and albumin (see Section 2). Some halogenated aromatic compounds may undergo hydroxylation in their aromatic rings, yielding hydroxylated metabolites that structurally resemble THs and show affinity for TTR binding. These compounds can thus competitively displace THs from TTR, thus increasing their circulating levels.
d. Liver enzyme induction, resulting in increased TH metabolic clearance and disruption of the HPT axis. Hepatic glucuronidation is the rate-limiting step for biliary excretion of T4, whereas hepatic sulfation is the rate-limiting step for biliary excretion of T3. Activation of nuclear receptors/xenosensors (such as AhR – aryl hydrocarbon receptor, CAR – constitutive androstane receptor, PXR – pregnane X receptor and PPARα – peroxisome proliferator activated receptor alpha) induces the expression of phase II liver enzymes (i.e. UGT and SULT). These enzymes increase the rate of hepatic catabolism and excretion of THs, leading to increased TSH secretion from the pituitary and further compensatory hyperplasia in the thyroid. According to this MoA, hypothyroidism might be considered as secondary to increased metabolic clearance and excretion of THs. Hepatocellular hypertrophy, as an indicator of an adaptive liver response, may also be observed. While the induction of UGT has been associated with thyroid tumours in rodents, in humans, the increase in metabolism would initially be compensated for by the reservoir of THs bound to TBG. Hence, rodent thyroid tumours induced by chronic induction of UGT are considered of little or no relevance to humans with respect to carcinogenesis.
e. Reduced uptake of THs into target cells by interference with T4- and T3-membrane-bound transporters, such as MCT8, MCT10 and OATP 1C1. In particular, MCT8 and OATP1C1 are important for the regulation of local TH activity in the brain and, thus, for brain development. However, so far, there is no evidence that any pesticide inhibits these membrane transporters.
f. Inhibition of iodothyronine deiodinases (DIOs) in peripheral tissues. Once inside the cell, T4 can be activated via conversion to T3 by the DIOs, such that the cytoplasmic pool of T3 includes both T3 from the plasma and T3 generated by DIOs.

The different DIO isoforms catalyse the conversion (deiodination) of intracellular T4 (inactive prohormone) into T3, the most metabolically active TH. Then, T3 is transferred to the nucleus and binds to its receptors, initiating downstream gene expression responses. Hence, DIOs play a pivotal role in regulating the circulation of peripheral TH levels and keeping the ratios of T4 and T3. Where DIO1 and DIO2 are inhibited, T4 levels increase and T3 is reduced. Conversely, DIO3 (normally expressed in the CNS, the placenta and fetal tissues) is the principal inactivating enzyme that degrades T4 to rT3, thus playing a role in the inactivation/turnover of T4. Reduced T3 formation in peripheral tissues following DIO1 and DIO2 inhibition is followed by an increased TSH production to compensate for a decrease in serum T3. PTU and chemicals structurally similar to THs (e.g. hydroxylated halogenated compounds or metabolites) have been shown to be potent inhibitors of DIOs. This MoA is relevant for humans because, although excess T4 is buffered by TBG, there may be effects on the negative feedback system in the pituitary that will cause thyroid stimulation.

g. Direct binding of TH to nuclear receptors (TRα1, TRβ1 and TRβ2) in peripheral tissues resulting in the induction or suppression of the transcription of TH-responsive genes that regulate metabolic and developmental functions. These genes are involved in a variety of regulatory functions in development and metabolism. Halogen-substituted phenolic moieties in pesticides can mimic natural THs and interact with multiple targets.
h. Altered gene expression (RNA transcription levels) of genes involved in the HPT axis homeostasis, including those encoding TRH receptor-2 (TRHR2), TSH receptor (TSHR), NIS, TPO, TTR, DIO1 and DIO2 and TRα and TRβ in different tissues. However, a consistent gene expression fingerprint has not been reported so far. Amitrole, flutolanil and ioxynil are examples of chemicals acting via this MoA.

These mechanisms are shown graphically in Figure 2.

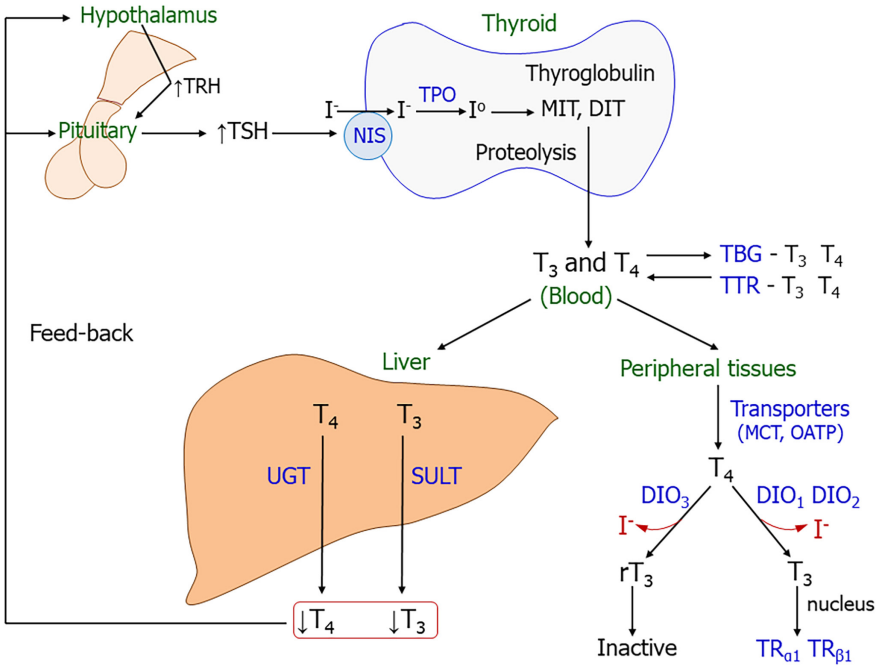

FIGURE 2 Mechanisms of toxic action of pesticidal active substances disrupting the thyroid hormone homeostasis (taken from EFSA, 2019a). Potential targets are shown in blue. DIO, deiodinases; DIT, diiodothyronine; MCT, monocarboxylate transporter; MIT, monoiodothyronine; NIS, sodium/iodide symporter; OATP, organic anion transporting polypeptide; SULT, sulfotransferases; TBG, thyroid-binding globulin; TPO, thyroperoxidase; TRa1 TRb1, thyroid receptors a and b; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; TTR, transthyretin; UGT, uridine 5′-diphospho-glucuronosyltransferases.

4 | PREVIOUS ASSESSMENT

4.1 | The 2019 EFSA scientific report «establishment of CAGs of pesticides for their effects on the thyroid»

This section summarises the key features of the report and the main decisions taken in 2019 when the CAGs of pesticides related to the thyroid were first established. At that time, two specific effects (corresponding to two CAGs) were identified as relevant for the thyroid CRA (Table 1):

CAG related to **Hypothyroidism**: defined there as 'an altered function of the thyroid gland resulting in follicular cell hypertrophy, hyperplasia and neoplasia';

CAG related to **C-cell hypertrophy, hyperplasia and neoplasia**.

Histopathological findings were taken as the main criteria to define these effects (Table 1). The rationale for selecting these specific effects and excluding others has been extensively explained in the 2019 report and is further described in Section 5.

TABLE 1 Specific effects and indicators agreed in 2019 for CAGs of pesticides for their effects on the thyroid CRA (see EFSA, 2019a).

Specific effect (CAG)	Hypothyroidism	C-cell hypertrophy, hyperplasia and neoplasia
Indicators	• Decreased circulating T3 level, decreased circulating T4 level • Increased circulating TSH levels • Increased relative thyroid weight • Follicular cell hypertrophy • Follicular cell hyperplasia • Follicular cell neoplasia: follicular cell adenoma, follicular cell carcinoma • Evidence of a MoA in direct relation to hypothyroidism	• C-cell hypertrophy • C-cell hyperplasia • C-cell tumours: C-cell adenoma, C-cell carcinoma • Evidence of a MoA in direct relation with C-cell hypertrophy, hyperplasia and neoplasia

The approach to the identification of the substances causing the specific effects (and triggering inclusion in the respective CAG) was conservative. Specifically, to trigger inclusion of a substance into a CAG, it was sufficient that at least one indicator was observed at a statistically significant and/or biologically relevant level in at least one toxicological study assessed as 'acceptable' in the Draft Assessment Reports (DARs), Renewal Assessment Reports (RARs) or equivalent document (unless the observation was clearly non-specific, for example age-related or occurring at or above the maximum tolerated dose (MTD), or the dose–response relationship showed that the observation was not treatment related).

Only 17 a.s. met the criteria for inclusion in CAG for C-cell hypertrophy, hyperplasia and neoplasia, whereas 128 a.s. were members of CAG for hypothyroidism. Twelve out of the 17 a.s. included in CAG for C-cell hypertrophy, hyperplasia and neoplasia were also included in the CAG for hypothyroidism. As regards potency, only two a.s. (Thyram and 2,4-DB) present in the CAG for C-cell hypertrophy, hyperplasia and neoplasia had a NOAEL below 2 mg/kg body weight (bw) per day, in contrast to 25 a.s. and metabolites present in the CAG for hypothyroidism (with a NOAEL < 2 mg/kg bw per day).

Principles for the characterisation of the substances included in the CAGs

The potency of each a.s. included in the CAGs was defined on the basis of the NOAEL for the most sensitive indicator across studies, species and sexes. Changes in hormone levels were not taken as a basis for the setting of NOAELs, mostly due to the inconsistent availability of data, especially from old dossiers. Only studies rated as 'acceptable' in the DARs were considered as a source of data. In studies where only Lowest Observed Adverse Effect Levels (LOAELs) but no NOAELs were identified, a default and conservative uncertainty factor (UF) of 10 was applied to the LOAEL to derive a conservative NOAEL. In some cases, for a given substance, it was possible to derive a LOAEL by combining studies within the same species. The EFSA conclusions on pesticides peer review, which necessarily contained some elements of expert judgements in relation to thyroid effects, were not challenged.

Selection of index compounds

In 2019, index compounds (ICs) were selected to express the potency of all members of a CAG in terms of relative potency factors (RPF). However, this approach was superseded in 2020 by the application of the concept of combined margin of exposure (MOET, also known as total margin of exposure) to express cumulative risks based on a mathematical demonstration described in the 2020 report (EFSA, 2020b).

4.2 | The 2020 EFSA scientific report «risk characterisation of CAGs of pesticides for their effects on the thyroid»

In 2020, a full CRA was carried out for both CAGs. Cumulative exposure assessments had been performed through probabilistic modelling using monitoring data from MSs official pesticide monitoring programmes in 2014, 2015 and 2016 and individual consumption data from 10 populations of consumers from different countries and different age groups.

As stated in this report: 'Considering all uncertainties identified by experts, for **hypothyroidism**, it was concluded that, with varying degrees of certainty, cumulative exposure does not reach the threshold for regulatory consideration for all the population groups considered. This certainty exceeds 99% for all four adult populations, 95% for two children's populations, 90% for one children population and one toddler population and 85% for the remaining two toddler populations. For **C-cell hypertrophy, hyperplasia and neoplasia**, the same conclusion was drawn with a certainty exceeding 99% for all 10 populations. These populations can be considered as representative of the European populations with the highest vulnerability in terms of potential exposure.' Based on these results, it was noted that a CRA for the C-cell hypertrophy, hyperplasia and neoplasia CAG would not be needed. In fact, the MOET at the 99.9th percentile of exposure was sufficiently

high (largely > 100 in all cases), and the conduct of a CRA for the CAG for hypothyroidism was considered to cover the combined effects of pesticides associated with C-cell hypertrophy, hyperplasia and neoplasia.

5 | UPDATED ASSESSMENT

5.1 | Hazard identification methodology: Specific effects and indicators relevant for the CRA of pesticides targeting the thyroid

5.1.1 | Definitions

In the context of this report, a *specific effect* is defined as the precise toxicological insult that can result from a combined action of pesticides and is relevant for grouping pesticides into CAGs for CRA (EFSA PPR Panel, 2013). The specific effects were identified by applying the criteria set by the EFSA PPR Panel (2013), that is adversity of the effect, systemic and unambiguous nature (primary effect, MoAs/AOPs), and relevance to humans. The specific effect is identified by expert judgement based on relevant toxicity endpoint(s), that is indicator(s). It should be noted that 'specific effects' are not necessarily described by a single toxicity endpoint but rather by a set of endpoints that make up an effect of greater biological complexity, for example hypothyroidism.

An *indicator* of a specific effect is a toxicological endpoint identifiable in toxicological studies reflecting the specific effect and useful for characterising the toxicological potency of the pesticide causing that effect. A single or a set of indicators can be used to identify a specific effect.

5.1.2 | MoAs and AOPs

Among all the possible effects of pesticides on the thyroid, those relevant for grouping pesticides into CAGs were identified using hazard-driven criteria as advised in 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). The framework proposed by the EFSA Scientific Committee is based on the use of a hierarchical approach to evaluate the body of information available for each a.s., as described in Figure 3.

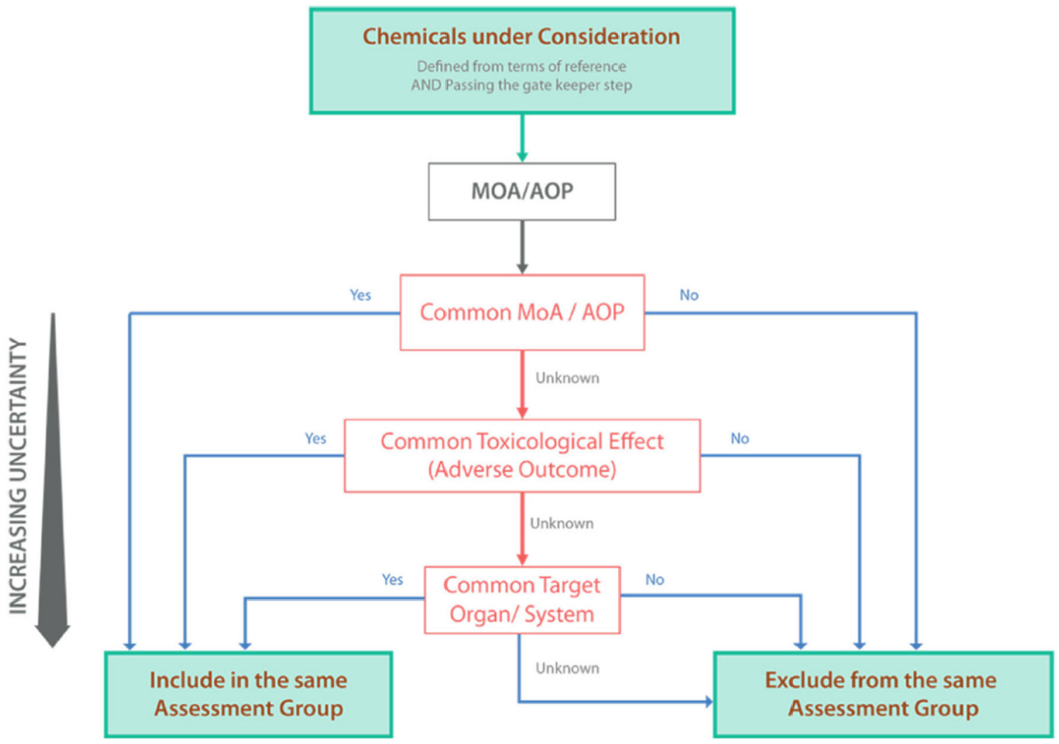

FIGURE 3 Top-down hierarchical process for grouping chemicals into assessment groups using hazard-driven criteria (from EFSA Scientific Committee, 2021).

More specifically, the golden standard for grouping of chemicals is considered to be a common MoA and/or AOPs. In the case of incomplete mechanistic information, the grouping may be based on the commonality of adverse outcomes (AOs). This approach is also of interest in the case of AOP networks converging to the same AO, even if they have different

molecular initiating events (MIEs) and toxicity pathways. The grouping based on common target organ/system toxicity rather than on MoA or AO implies a larger uncertainty in predicting possible combination effects. However, the degree of uncertainty in CRA would even increase when limiting CAGs to known common MoA, thereby excluding pesticides for which information on MoA is not available. This source of uncertainty can be addressed in the UA later in the CRA process.

In the case of thyroid toxicity, 35 AOPs were identified in the Collaborative Adverse Outcome Pathway Wiki (AOPwiki) as of 16 July 2023 (AOPwiki, Version 2.6, released on 29 April 2023). For the identification of AOPs, the broad term 'thyroid' was used as the sole keyword for searching since early searches on 'C-cell' and 'parafollicular' did not give any results, whereas searches on 'hypothyroidism' resulted in the retrieval of only a limited number of AOPs where the term hypothyroidism was included in the AOP abstract. The retrieved AOPs were screened for human relevance based on their title and/or AO. Thus, the eight AOPs on altered amphibian metamorphosis (AOP175, AOP176, AOP188, AOP189, AOP190, AOP191, AOP366, AOP367), the seven AOPs on increased mortality and decreased population growth rate in fish via swim bladder inflation (AOP155, AOP156, AOP157, AOP158, AOP159), altered retinal layer structure (AOP363) or decreased eye size (Microphthalmos) (AOP399), as well as the AOP271 on inhibition of TPO leading to impaired fertility in fish were not considered further. The human relevance of available AOPs may be revised in the future based on the outcome of ongoing research on the evidence-based applicability of AOPs for HPT axis disruption to other taxonomic groups and cross-species extrapolations (Haigis et al., 2023). In addition, information on MIE, key events (KEs) and AO is not provided for AOP484 (Bisphenol A analogues induce cellular dysfunction in human trophoblast cells in a thyroid hormone receptor-dependent manner: in silico and in vitro analyses), which is therefore also not included in the list of relevant AOPs for this report.

The remaining 18 AOPs provide mechanistic information on:

- follicular cell adenomas/carcinomas (AO741)
- developmental neurotoxicity (DNT; AO341: Impaired learning and memory; AO402: Decreased cognitive function; AO2095: Periventricular heterotopia formation; AO1348: increased seizure)
- hearing loss (AO319: Loss of cochlear function)
- kidney toxicity (AO814)
- metabolic disruption (AO2119: increased insulin resistance)
- reproductive toxicity (AO406: impaired fertility, AO405: irregularities, ovarian cycle, AO406: malformation, Male reproductive tract).

The selection of AOPs relevant for this report was based on the identification of indicators of the specific effects on 'hypothyroidism' and 'C-cell hypertrophy, hyperplasia and neoplasia'. No AOPs were identified for C-cell hypertrophy, hyperplasia and neoplasia. Three AOPs relevant for hypothyroidism were selected, that is AOP110, AOP119 and AOP162, and they are mapped as an AOP network in Figure 4, although none of them has been endorsed by the Organisation for Economic Co-operation and Development (OECD) yet. All three indicators for hypothyroidism are included in these AOPs as KE739 (thyroid follicular cell hypertrophy), KE740 (thyroid follicular cell hyperplasia) and AO741 (follicular cell adenomas/carcinomas). Other downstream events of altered HPT axis will be considered in other dedicated CAGs (e.g. DNT, kidney toxicity, reproductive toxicity etc.).

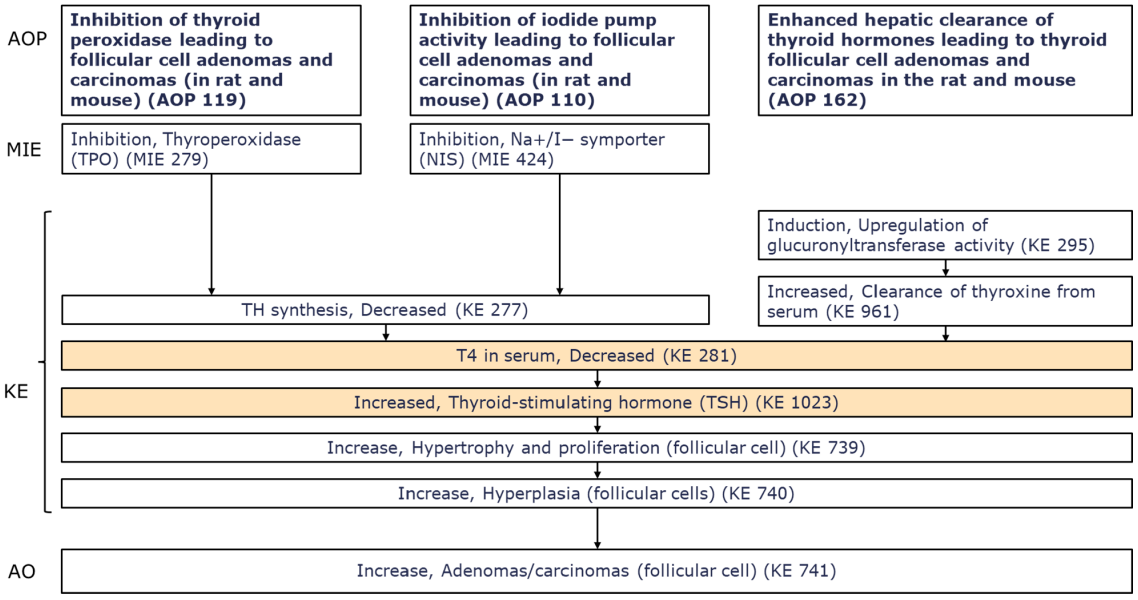

FIGURE 4 Mapping available adverse outcome pathways (AOPs) for hypothyroidism as a primary effect. Colour code: common key events prior to thyroid follicular cell histopathology (orange box).

The AOPs established on thyroid follicular cell adenomas/carcinomas involve either (i) direct interference with TH synthesis through inhibition of the NIS and/or TPO, or (ii) increased T4 metabolic clearance through phase II liver enzyme induction. Decreased serum T4 with subsequent increased TSH levels are identified as common KEs prior to thyroid follicular cell histopathology, expressed as increased hypertrophy and proliferation, progressing to hyperplasia and neoplasia.

Additional mechanistic information on MIEs and/or upstream KEs leading to decreased serum T4 (KE281) through decreased TH synthesis (KE277) or increased metabolic clearance of T4 from serum (KE961) may be obtained from other AOPs, as illustrated in Figure 5.

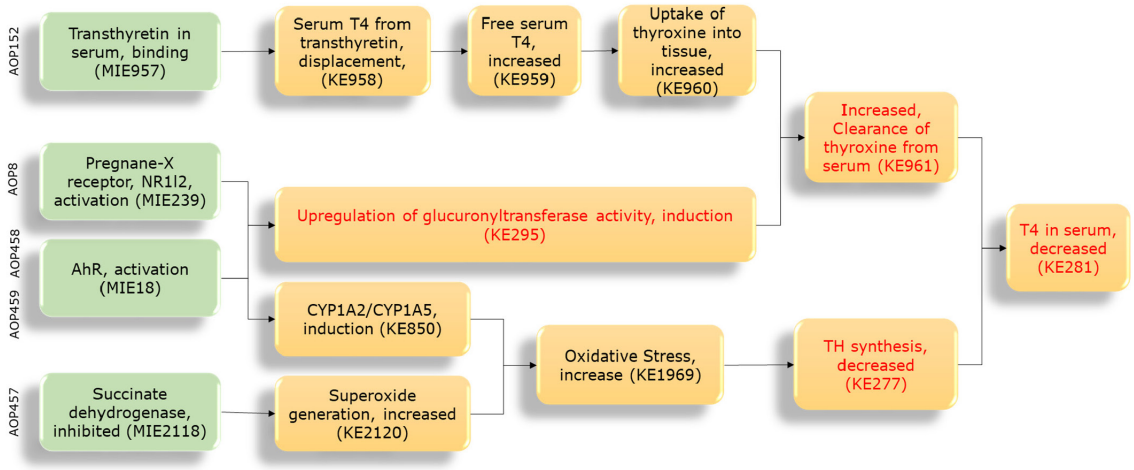

FIGURE 5 Additional mechanistic information on MIEs and/or upstream KEs leading to decreased serum T4. Colour code: MIE (green box), KE (orange box), KE also present in AOPs for hypothyroidism (red font).

Although none of the AOPs included in Figure 5 are endorsed by the OECD and the AOs of these AOPs are beyond the scope of this report, it is considered that the MIEs and early KEs identified may provide further insight into the molecular and cellular mechanisms of hypothyroidism and should not be ignored (see Section 7 'Recommendations').

The AOP393 on thyroid disorder via thyroid receptor beta (TRβ) transactivation is under development. There is currently limited AOP data on the role of inhibition or activation of gene transcription through thyroid receptor (TRα, TRβ) binding/transactivation and on changes in tissue-specific T4 and/or T3 through interference with peripheral TH metabolism (DIO1, DIO2, DIO3 inhibition) (Noyes et al., 2019).

Further development of AOPs, the construction of more complex and comprehensive networks from individual AOPs, as well as improving quantitative understanding of KE relationships, may in the future provide additional information on the molecular mechanisms of hypothyroidism (Knapen et al., 2018; Villeneuve et al., 2018).

Currently, there are no validated/standardised in vitro assays to assess the identified MIEs and KEs leading to thyroid follicular cell histopathology (see Appendix A). Biological activity data from high-throughput screening has been generated by the EPA's Toxicity Forecaster (ToxCast) research effort, and it is included in the Comptox Chemicals Dashboard (US EPA, 2022). The ToxCast data in the Dashboard provide information about the assays used to evaluate the chemicals as well as the obtained results, including concentration-response plots, response potency and efficacy CompTox Chemicals Dashboard Factsheet). Thyroid pathway assays on specific chemicals may be available in the Dashboard, focusing on the assessment of TRα (ant)agonistic activity, the effect on TH-responsive gene and the effect on the expression of TRβ, TSHR (ant)agonistic activity, the effect on the expression of TPO, NIS, DIO1, DIO2, DIO3 etc. These data can be used to prioritise chemicals and to identify areas where further testing may be needed. Other literature data on many diverse types of assays designed to provide information on thyroid MoA may be considered, where available, as part of a weight of evidence (WoE) approach (ECHA and EFSA, 2018).

The availability of more in vitro/ex vivo methods covering the most relevant mechanisms for TH system disruption by chemicals, databases on thyroid pathway assays on specific chemicals, as well as the use of MoA data and AOPs in routine regulatory risk assessments, will facilitate the implementation of the golden standard for grouping of chemicals that is based on a common MIE and/or KEs and/or common toxicity pathways, even when MIEs are different.

5.1.3 | Specific effects on the thyroid and indicators

The WG reconfirmed as relevant for CRA the same two specific effects of chemical substances on the thyroid previously selected, namely hypothyroidism and C-cell (parafollicular cells) hypertrophy, hyperplasia and neoplasia (EFSA, 2019a) based on expert judgement, noting also the lack of sufficient mechanistic information.

Indicators of the two specific effects that had been identified in the 2019 report (EFSA, 2019a) were also revised to only include histopathological changes observed in animal studies (see the full list in Table 2) based on the rationale provided underneath.

TABLE 2 Specific effects of pesticide active substance on the thyroid and their indicators.

Specific effect (CAG1)	Indicators
Hypothyroidism *Defined here as a disruption of the HPT axis resulting in follicular cell hypertrophy, hyperplasia and neoplasia*	• Follicular cell hypertrophy • Follicular cell hyperplasia • Follicular cell tumours adenoma, carcinoma
Specific effect (CAG2)	**Indicators**
C-cell (*parafollicular cells*) hypertrophy, hyperplasia and neoplasia	• C-cell hypertrophy • C-cell hyperplasia • C-cell tumours: adenoma, carcinoma

Hypothyroidism

In the context of regulatory risk assessment, which is mainly based on animal studies, hypothyroidism, defined as decreased function of the thyroid gland informed by THs, is not observed as such but it is essentially inferred from histopathology changes, and 'supported' by hormonal changes and other information. Therefore, considering the definition of the indicator being a toxicological endpoint that triggers the inclusion of an a.s. into a CAG, histopathological changes (follicular cell hypertrophy, hyperplasia and neoplasia) are identified as indicators, whereas the following parameters, taken in isolation from animal studies, were not considered suitable as indicators of hypothyroidism but as LoEs in the assessment of the specific effect (see Section 5.4.3): changes in serum thyroid hormone levels (decreased T3 and T4), changes (increase) in circulating TSH levels and increased absolute or relative thyroid weight. For this reason, information on these parameters will only be collected for pesticide residues identified as risk drivers based on hazard and exposure considerations.

It is important to note that the neoplastic findings in animal studies are not directly translatable into the same effects in humans, as hypothyroidism does not eventually result in cancer in humans (see Section 5.1.4).

As histopathological changes indicative of hypothyroidism may be a consequence of repeated (subchronic or chronic) exposure to pesticide a.s., this specific effect is subject to long-term CRA.

C-cell hypertrophy, hyperplasia and neoplasia

In humans, it is important to distinguish physiological hyperplasia of C-cells from C-cell hyperplasia associated with neoplastic growth. Hypertrophy and hyperplasia of C-cells can occur physiologically as a homeostatic compensatory mechanism associated with hypercalcaemia and other conditions. However, C-cell hyperplasia in humans may also occur in association with medullary carcinoma and multiple endocrine neoplasia.

In the context of regulatory risk assessment, C-cell hypertrophy, hyperplasia and neoplasia can be observed in animal studies following repeated exposure to certain pesticides, and these effects are relevant for humans. Sustained C-cell stimulation leading to hyperplasia is expected to play a promoting role in further progression to neoplasia. Therefore, C-cell hypertrophy, hyperplasia and neoplasia are a continuum of the same pathophysiological process and can be considered as a single, specific effect in the context of CRA. As occurred with the CAG for hypothyroidism, histopathology is considered as the most appropriate source of evidence to define the indicators for the CAG for C-cell hypertrophy, hyperplasia and neoplasia. C-cells produce the hormone calcitonin, which is involved in calcium homeostasis and the regulation of bone formation, but information on serum levels of calcitonin is generally not available in regulatory toxicological studies. More in general, it is not possible to define a CAG of pesticides in explicit relation to alterations of calcium homeostasis. Calcium levels in the serum are not sufficiently specific, since they are subject to homeostatic regulation by the interplay of calcitonin and the parathyroid hormone (PTH), secreted by the parathyroid glands and representing the functional counterpart to calcitonin. Indeed, both PTH and calcitonin play important roles in calcium homeostasis through their actions on osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells), respectively (Carter & Schipani, 2006). Calcitriol (the active form of vitamin D) is the third actor in calcium homeostasis, acting by increasing calcium absorption in the gastrointestinal tract. PTH and calcitriol are not routinely measured in regulatory studies.

By nature, hypertrophy, hyperplasia and neoplasia of C-cells are triggered by repeated (subchronic or chronic) exposure to pesticide a.s. and are therefore subject to long-term CRA.

5.1.4 | Human relevance of animal studies

The European Chemicals Agency (ECHA)/EFSA Guidance for the identification of endocrine disruptors (ED) in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 reflects specifically in its Appendix A on the human relevance of effects on the thyroid (ECHA and EFSA, 2018).

Despite the fact that general function of TH is well-conserved in mammals, the HPT axis response to pesticides shows important inter-species differences in relation to TH metabolism and the response of the thyroid to chronic variations in TSH. Rats (particularly males) are the most sensitive rodent species (Rouquie et al., 2014) and also more sensitive than dogs and humans to TH perturbations. It is noted that fluctuations in TH levels may be less pronounced in humans (Nielsen et al., 2012), and that humans are quantitatively less susceptible to chemically induced follicular cell hyperplasia and

tumours than rats (Dellarco et al., 2006) because of a decreased sensitivity of the human HPT axis (Crofton, 2008). The difference in sensitivity largely relies on the different binding profiles and metabolic clearance rates of TH.

- In humans, circulating T4 is bound primarily to TBG, a high-affinity binding protein that is essentially absent in rodents (only representing 9% of the serum binding capacity as that in humans). Besides, the binding affinity of human TBG for T4 is approximately three orders of magnitude greater than that of TTR, as mentioned in Section 2. The percentage of unbound active T4 is therefore lower in species with high TBG concentrations, as occurs in humans.
- The half-life of T4 is much shorter in rats (12–24 h) than in humans (5–9 days) due to its rapid metabolism and excretion. The metabolic clearance of T4 in rats occurs mainly through hepatic glucuronidation, while in humans it occurs through deiodination of T4 and T3 (see Section 2). Furthermore, the induction of UGT in rat liver produced by numerous pesticides increases the rate of metabolic clearance of TH, leading to a decrease in serum T4 and an increase in serum TSH. In humans, the increased metabolism would initially be compensated for by the reservoir of TH bound to TBG.

Disruption of the HPT axis and subsequent decreased TH levels result in rapid (about 1 week) morphologic changes in the rat thyroid gland, consisting of follicular cell hypertrophy and hyperplasia. Persistently increased TSH is considered an important KE for follicular cell hypertrophy and hyperplasia and the eventual development of thyroid adenomas and carcinomas in the rat. Conversely, human thyroid cancer does not show progression from hyperplasia to adenomas and carcinomas. The type of carcinoma observed in humans is most often of the papillary type (85% of total), and it is more frequent in women than in men.

Although humans develop goitre (enlarged thyroid gland featuring histopathologically a diffuse thyroid follicular hyperplasia) in response to chronic increases in TSH concentrations, as occurs with iodide deficiency or exposure to goitrogens, it is not clear whether sustained increased serum TSH and goitre represent a preneoplastic condition in humans. There are no or minimal increases in cancer incidence in humans with goitre. It should be noted that patients with Hashimoto's disease, an autoimmune disorder that can cause hypothyroidism, may have an increased risk of thyroid cancer (most often papillary carcinoma), but also of cancer at other sites (breast, lung, digestive tract, urogenital system, blood cancers, and prolactinoma) as compared to people without Hashimoto thyroiditis, suggesting that chronic inflammation plays a role in cancer development (Hu et al., 2022).

Because there is no evidence of an increased incidence of thyroid tumours in humans from chemical exposures, thyroid tumours that appear in rodents (i.e. follicular cell adenoma or carcinoma) as a result of continuous thyroid stimulation by increased TSH levels are considered as not relevant to humans (Bartsch et al., 2018).

In 2005, the US EPA used a health-protective approach for covering the risk to susceptible individuals and considered thyroid follicular cell tumours in rodents to be of potential human relevance (US EPA, 2005). It was concluded that a reference dose level protective from HPT disruption in a sensitive species such as rat should also protect against potential follicular cell tumours in human populations. However, this approach is considered as highly conservative and not fully supported by the available evidence on the higher sensitivity of rodents as compared to humans to HPT disruption. It should be taken into account the different clinico-pathological phenotype of thyroid changes across species in response to chemicals (follicular cell hypertrophy and hyperplasia in rodents and diffuse goitre in humans), the different histological type of tumours more often observed (follicular adenoma/carcinoma in rats and papillary carcinoma in humans), the sex differences as males are more sensitive in rats while women are more sensitive in humans, and papillary thyroid cancer often co-occurs with Hashimoto's thyroiditis in humans (particularly women) but is rarely reported in rodents.

Based on what has been described above, a clear conclusion on the human relevance of disruption of the HPT axis and thyroid cancer in rats cannot be drawn. Although, on the basis of evidence from robust experimental observations and mechanistic data, the early sequence of KEs (i.e. NIS or TPO inhibition) leading to these adverse effects occurs in both species, the late KEs (downstream from raised serum TSH) and apical thyroid outcomes lack sufficient biological plausibility in humans due to the available evidence on inter-species differences described above. Accordingly, the MOET of 100 established by risk managers as a threshold for regulatory actions may be sufficiently conservative.

5.1.5 | Effects not selected as specific effects relevant for CAGs

Only effects related to the disruption of the HPT axis are considered as specific effects. Downstream events of changes in hormone levels (e.g. DNT, hearing loss, visual dysfunction and cardiovascular impairments) are outside the scope of this report. To protect the most sensitive population, in the close future, EFSA will carry out a dedicated CRA for DNT, taking into consideration both T-mediated effects and CNS-mediated effects.

The WG excluded other effects on the thyroid (see Table 3) as relevant for grouping pesticides for CRA, thus confirming the same decisions as in 2019 (the reader is referred to EFSA, 2019a, p. 12 for more details). An overview of the effects and the relative rationale for exclusion are included in Table 3.

TABLE 3 Effects on the thyroid not relevant to perform a CRA.

Effects	Rationale for exclusion
Alterations of TH levels as such	• Not consistently measured in regulatory toxicology studies • Validation of TH assays with rodent samples is critical for accurate and reproducible measurements • Following assay validation, a positive control study with a reference compound is recommended • Large variation of TH in rat control groups
Hyperthyroidism	• Lower prevalence than hypothyroidism in humans and less often associated with pesticide exposure • Less frequently reported in regulatory studies on experimental animals • Difficult interpretation of TH changes
Inflammation of the thyroid gland/lymphocytic thyroiditis	• Thyroiditis as such is not sufficiently specific to be the subject of a CRA. However, if a.s.-related inflammatory disorders lead to cell degeneration and hyperplasia of the thyroid, this will result in a disruption of the HPT axis, and the a.s. acting through this pathway will be included in the CAG for hypothyroidism
Treatment-related pigmentation of follicular cells	• Not considered to be adverse if observed in isolation
Additional histopathological changes listed in the DTU report (Nielsen et al., 2012), i.e. increased/decreased amount of colloids, small/large follicles, different shapes of follicular cells, increased vascularisation, increased vacuolisation, follicular cysts, follicular atrophy or necrosis of follicular cells	• These microscopic findings can accompany the indicator 'follicular cell hypertrophy' as supplementary descriptive information. In isolation, that is in the absence of follicular cell hypertrophy, do not necessarily reflect thyroid dysfunction rather are morphological variations of normal thyroids or artefacts (e.g tangential sectioning). Necrosis of follicular cells is not commonly described in toxicological species • These findings would become relevant in the case they result in a disruption of the HPT axis with observed signs of hypothyroidism, so they would then be covered by the CAG hypothyroidism
Thyroid amyloidosis in mouse	• Spontaneous systemic amyloidosis is common in mice, especially CD-1 and C57BL/6 strains

5.2 | Methodology for the inclusion of substances into CAGs

5.2.1 | List of prioritised substances

In order to minimise the resources needed for the establishment of CAGs and to focus on the a.s. predominating in food, EFSA developed and implemented *a prioritisation* methodology (EFSA, 2023). This methodology is based on short- and long-term exposure assessments for each individual AS quantified in at least one food sample of the official pesticide residues monitoring programmes of EU MSs over a 3-year monitoring cycle (2019–2021). These exposure 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 for CRA was an acute or chronic hazard quotient (HQ) – that is, the ratio for that chemical between its acute or chronic exposure levels and its acute reference dose (ARfD) or acceptable daily intake (ADI), respectively, – equal or exceeding 0.1 (10%) at P99.9 of the short- or long-term exposure distribution in at least one of the 30 population groups under consideration. This resulted in a list of 67 substances, of which 21 reached the threshold in short-term exposure assessments only, 15 in long-term exposure assessments only and 31 in both short- and long-term exposure assessments.

In terms of relevance to CRA, the specific effects of ASs on the two CAGs defined herein require repeated exposure to occur (more than 24 h that is considered the time span for acute effects). Therefore, only the 46 pesticides reaching the threshold in long-term exposure assessment are of interest for the present exercise.

Using an updated (as of 2023 and still unpublished) version of the DTU database (Nielsen et al., 2012), EFSA identified that 21 of the a.s. on the priority list and 6 of their metabolites were associated with endpoints of interest for their possible inclusion in CAGs for the thyroid. These a.s. are in alphabetical order: chlorates, chlordane, chlorpropham, p,p'-dicofol, ethoprophos, fenamiphos, fipronil, fluquiconazole, haloxyfop-P, heptachlor, maleic hydrazide, mancozeb, maneb, metiram, oxyfluorfen, propineb, pyrimethanil, tetraconazole, thiabendazole, thiram and ziram. The metabolites are: fipronil metabolite MB 45950, fipronil metabolite RPA 104615, ETU, ethylene bis isothiocyanate sulphide (EBIS), ethylene urea and propylene thiourea (PTU).

It was verified whether, for any of these a.s., a finalised ED assessment for the T-modality according to the EFSA/ECHA guidance for the identification of ED was available. This was not the case. If this had been the case, a prioritised a.s. meeting the ED criteria for the T-modality would have been directly included in the CAG for hypothyroidism. Conversely, if an a.s. had been concluded as not meeting the ED criteria for the T-modality, sufficient evidence in a complete data package would have been available to exclude a primary thyroid adversity of the a.s. (according to the terminology used by the EFSA/ECHA guidance). An eventual thyroid adversity would only be considered as secondary if, for example observed at doses exceeding the MTD or as a result of liver enzyme induction (observed in rodents) increasing the TH metabolic clearance (in addition, not relevant for humans; see Sections 2, 3 and 5.1.4). In other words, the HPT axis would not be a (primary) target organ system of the a.s. In such a case, the a.s. would not contribute by dose-addition to hypothyroidism,

as defined in this report, at low levels of dietary exposure, and consequently, it would not be considered for inclusion in the CAG related to hypothyroidism.

The 21 substances and six metabolites mentioned above will therefore form the scope of the data collection described in the next section of this report.

5.2.2 | Data collection methodology

Data sources

Relevant data from the available toxicological studies conducted with the 21 selected a.s. and the six selected metabolites will be collected by ICPS. These data refer to any reported indicator of the specific effects defined in Section 5.1.3, as well as any further information needed to efficiently implement the principles of the hazard characterisation (see Section 5.3) and of the elicitation of CAG-membership probabilities (see Section 5.4).

The main sources of data are the DARs and RARs generated by Rapporteur Member States (RMSs) in the context of Regulation (EC) No 1107/2009 and the respective EFSA conclusions. Additional sources of data (e.g. Joint Meeting on Pesticide Residues (JMPR) evaluations and biocides evaluation by ECHA) will always be checked as additional sources of data; potential differences will be flagged, and additional data will be collected on a case-by-case basis.

Relevant sources of data will also include the ED assessments conducted by EFSA on substances for which a decision on approval or renewal of approval was pending on 10 November 2018, according to Commission Implementing Regulation (EU) 2018/1659.

For those substances identified as risk drivers, the United States Environmental Protection Agency (US EPA) CompTox Chemicals Dashboard will be consulted for updated screening information on MoA (see Section 5.1.2) not already included in the regulatory documents above. For completeness, the ECHA's ED assessment list will also be checked.

Data collection for hazard identification and characterisation for establishing CAGs

Irrespective of the administration route and exposure duration, the data collection will cover all in vivo guideline studies rated in the DARs/RARs as 'acceptable' or 'supportive', as far as they assessed the thyroid gland histopathology (THs, i.e. T3, T4 and TSH, and thyroid weight will only be collected for risk drivers in a second step of the data collection). The acute toxicity (LD50) studies and the neurotoxicity studies will not be considered since the specific effects require a repeated stimulus to develop, and this type of study also lacks HPT axis-related measurements (see Section 5.1.3). Absorption, distribution, metabolism and excretion (ADME) studies and genotoxicity studies will not be considered either since they do not address T-related parameters.

More specifically, reference can be made to the ECHA/EFSA guidance where it is stated that 'To have the T-mediated adversity with regard to humans and mammals (as non-target organisms) sufficiently investigated, the HPT axis parameters foreseen to be investigated in the following studies OECD test guidelines (TG) 407 (28d oral toxicity, rat), 408 (90d oral toxicity, rat), 409 (90d oral toxicity, non-rodents; and/or the 1-year dog study, if available), 416 (2 generation reproduction toxicity) or 443 (Extended One-Generation Reproductive Toxicity Study – EOGRTS) if available and 451 (carcinogenicity), 452 (chronic toxicity), 453 (combined chronic toxicity/carcinogenicity) should have been measured and the results included in the dossier'. Historical control data will be used for the interpretation of neoplastic changes if these were used by the RMS/peer review.

Moreover, whenever available (not part of mandatory data requirements in pesticides), the studies following the OECD TG 414 (prenatal developmental toxicity), 421 (reproduction and developmental toxicity screening) and 422 (combined repeated toxicity + developmental toxicity screening) will be considered.

Other in vivo studies (e.g. mechanistic or non-guideline studies where effects on the HPT axis were assessed) will be collected, if available.

Specifically, for inclusion in a CAG (hazard identification), not only oral animal studies but also dermal and inhalation repeated toxicity studies will be considered. However, for hazard characterisation, only oral studies will be considered. Human medical and epidemiological data will not be used for triggering or not the inclusion of an a.s. into a CAG.

Limitations identified during the data collection should be flagged to enable an independent assessment of their impact on the validity of the thyroid effects. Also, studies rated as 'supportive' in the DAR/RAR will be considered as a source of data for both the hazard identification and characterisation steps, as far as the limitations do not affect the reliability of data on the thyroid. For example, missing data in one sex does not invalidate the study findings in the other sex that has been tested.

The data will be collected by ICPS in the Excel worksheet provided in Annex A of this report and will be published in a separate report. Detailed instructions on data collection are also included in the data collection template.

Data collection for CAG-membership probability (for risk drivers only; also see Section 5.4)

A broader data collection will be specifically performed for a.s. or metabolites identified as risk drivers to support the evaluation of the CAG-membership probability for each of these substances (see Section 5.4) and for the UA (see data

collection template in Annex A). For example, as regards in vivo guideline studies, for risk drivers, absolute and/or relative thyroid weight as well as circulating levels of T3, T4 and TSH will be collected if available.

All in vitro studies and other studies included in the DARs/RARs under section 6.8.3 (i.e. 'Other studies, Endocrine Disruption') and in the ED Excel sheet (appendix E of the RAR/DAR) will be included when they are considered relevant and reliable by the RMS/EFSA Peer Review. These studies may consist of additional data on adversity from the public literature, or data on activity (mechanistic data) from the public literature, databases (e.g. the US EPA Chemicals Dashboard) and/or studies specifically designed and conducted by the industry to further investigate the mechanism of action. For substances for which there is no information on the MoA, Toxcast data could assist in elucidating the action of the chemical on the thyroid. Since 2019, Toxcast data are routinely included in DARs/RARs, and therefore they can be included in the data collection. For older applications where mechanistic data are missing, a specific search of Toxcast could be decided at a later stage, should this become necessary (e.g. for risk drivers).

Concerning human medical and epidemiological data (if included in the RAR/DAR), these will be considered as supporting information and for the UA but will not be used for triggering the inclusion of an a.s. into a CAG. Information on human studies will only be needed for risk drivers and collected in the 'Remarks' column of the data collection sheet.

5.2.3 | Criteria for the inclusion of an a.s. into a CAG

The inclusion of a substance into a CAG is determined by the presence of at least one indicator of the respective specific effect. Such observations are expected to be statistically significant and/or biologically relevant and present in at least one sex and at least one in vivo toxicological study,[3] including interim sacrifice conducted by any administration route, assessed as 'acceptable' or as 'supportive[4]' in the final DAR, RAR or equivalent document, unless the observation is:

- the consequence of severe systemic toxicity and therefore clearly non-specific, for example age-related or occurring at or above the MTD, as concluded by the RMS/peer review. For example, in both humans and rodents, severe acute and chronic illnesses decrease TH serum concentrations without a concomitant rise in serum TSH, the so-called non-thyroidal illness syndrome (NTIS) (Fliers et al., 2015)
- non-treatment related, as concluded by the RMS/peer review
- not relevant for human risk assessment, as concluded by the RMS/peer review

The WG discussed about the information on systemic/target organ toxicity needed to assist in the decision to include or not a substance into a CAG. This information is important to assess whether changes in the indicator are secondary, non-specific consequences of systemic toxicity. To this purpose, information on relative liver-to-bw change (% change), liver hypertrophy and other effects will be collected at the dose at which a change in the indicator is observed.

In the previous EFSA Report (EFSA, 2019a), some substances were included in the CAG for hypothyroidism, even if the effect on the thyroid could be secondary to liver enzyme induction (resulting in lower circulating TH levels and subsequent compensatory mechanisms). At that time, this aspect was taken into account in the UA. Compared to 2019, now more conclusions of ED assessments are now available, and newer studies may support cases where liver enzyme induction is not relevant for humans (see Section 5.1.4). These conclusions must be considered in the CAG establishment. For example, substances that were concluded as non-ED for T-mediated parameters will be excluded from the CAG for hypothyroidism (and data will not be collected).

For substances for which the ED assessment could not exclude that the T effects were solely secondary to liver enzyme induction, it was agreed to include them both in liver and thyroid CAGs. This will be factored in during the UA. If the given substance results to be a risk driver in the T-CAG based on the exposure assessment, a sensitivity analysis could be performed to assess the impact of this specific substance on the cumulative risk and help quantify the uncertainty.

Based on the above considerations, an a.s. or a metabolite can be included in more than one CAG.

5.3 | Hazard characterisation methodology for the substances included in the CAGs

Each substance or metabolite included in the thyroid CAGs will be characterised by the assignment of an overall NOAEL and an overall LOAEL for the respective specific effect based on the totality of the available studies showing indicators of that specific effect. Only studies via the oral route will be used in the hazard characterisation step.

The potency of each a.s. included in the CAGs will be defined on the basis of an overall NOAEL based on the most sensitive indicator across studies, species and sexes. All indicators are equally valid for the setting of NOAELs. If applicable to data on indicators from risk drivers, a benchmark dose (BMD) modelling will be applied following the updated EFSA guidance on the BMD approach in risk assessment (EFSA Scientific Committee, 2022) to take into consideration the shape of the dose–response curve in addition to the critical effect dose. Furthermore, this would follow a recommendation of the CRA from 2020 to reduce the impact of the uncertainties identified (EFSA, 2020b).

[3]List of studies to be considered: see Section 5.2.2.

[4]As far as the limitation (to be recorded in the data collection) does not impact the validity of the T findings.

The data collection spreadsheets will be used to characterise each a.s. included in a CAG for the respective specific effect.

Only chronic NOAELs will be established for a.s. included in the thyroid CAGs. Establishing acute NOAELs for such chemicals is not justified because the two specific effects take much longer than 24 h (i.e. the time interval considered for acute effects) to develop. This approach is also substantiated based on experience from clinical practice. For example, due to the high pool of THs bound to TBG in the human blood, hypothyroidism will occur long after the removal of the thyroid gland or the administration of high doses of thyroid toxicants. After surgical removal of the thyroid, acute hypothyroidism can occur when L-thyroxine stops for 4–6 weeks (Dympep et al., 2014).

In studies where only LOAELs but no NOAELs were identified, a default and conservative NOAEL will be derived by applying an UF of 10.

In case two or more studies of equivalent quality are available and testing different dose ranges, they need to be considered collectively to derive combined NOAEL and LOAEL for the specific effect on the basis of the observed indicators. The combined NOAEL is the highest tested dose in the same species with no observed effect among all the acceptable/supportive studies that is below the lowest LOAEL. The combined LOAEL is the lowest of all observed LOAELs in those studies. Studies should be considered as being of equivalent quality and appropriate for a collective evaluation when they are performed in the same species and strain, and by the same administration route and they were judged to have the same level of acceptability (i.e. 'acceptable' or 'of limited acceptability') for the assessment of thyroid effects.

EFSA conclusions on the peer review of the pesticide risk assessment in the context of Regulation (EC) No 1107/2009 published by 31 October 2023, and dealing with a.s. included in the CAGs will be considered to retrieve any element of expert judgement regarding their effects on the thyroid.

For a.s. not reviewed by EFSA, if any, the scientific evaluations conducted by the body constituting the main source of the data collection will also be considered (e.g. JMPR evaluations).

Human studies involving deliberate exposure of individuals to chemicals will not be used for the establishment of CAGs, as the provisions of Commission Regulation (EU) No 283/2013 authorising their use (scientific validity, ethical generation and leading to lower regulatory limit values compared to animal studies) were never met.

5.4 | Methodology for assessing CAG-membership probability

5.4.1 | Introduction

The amount, reliability, relevance and consistency of evidence for causing the specific effects listed in Table 2 (Section 5.1.3) vary between a.s. This makes it uncertain which substances should be included in a given CAG, with some substances being more likely to belong to the CAG than others. This can be quantified by assessing the probability that any substance actually causes the specific effect.

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

(i) Defining in precise terms the assessment question applicable to each substance included in the CAG.
(ii) Identifying LoEs that are important for assessing whether the substance causes the effect. LoEs typically include, but are not necessarily restricted to, the indicators of the specific effect under consideration. Depending on the specific effect, additional factors contributing to the evidence can be defined.
(iii) Rating qualitatively the weight of each LoE. The LoEs 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 strength of its contribution to the probability of the substance causing the effect.
(iv) Reviewing the information available and listing the available LoEs for individual substances included in the CAG.
(v) Assessing for individual substances the probability under consideration using the 'approximate probability scale' from EFSA's uncertainty guidance.

The WoE assessment will be conducted by Expert Knowledge Elicitation (EKE). Two types of elicitation will be performed: the elicitation of the weight of each line of evidence (covering the points i to iii above) and the elicitation of the CAG-membership probability (covering the points iv and v above). In theory, the CAG-membership probability can be assessed for all the substances included in the CAG, as this was done when CAGs for the effects of pesticides on the thyroid were first established (EFSA, 2019a). However, this process is resource-consuming, and experience has shown that the outcome of a CRA is, in most cases, driven by a limited number of a.s. Therefore, it is possible to apply this procedure solely to the a.s. emerging as risk drivers from the cumulative exposure assessment. It should be noted that for risk drivers, additional data will be collected as compared to the other prioritised substances (see Section 5.2.2).

The present report addresses points i to iii of the process, while points iv and v will be addressed in a future publication on the UA and the risk characterisation.

5.4.2 | Assessment question

The assessment question applicable to each a.s. included in a CAG was agreed as follows:

What is the probability that the a.s. x can lead to, as a primary effect, any indicator directly related to the specific effect (CAG) 'Hypothyroidism'?

Based on the EFSA's previous experience in CAG establishment and cumulative risk characterisation for the CAG on C-cell hypertrophy, hyperplasia and neoplasia (EFSA, 2020a), neither an assessment question nor LoEs or any other follow-up were deemed necessary by the WG for this specific CAG. Indeed, the CRA for the CAG 'hypothyroidism' was considered to cover the combined effects of pesticides associated with C-cell hypertrophy, hyperplasia and neoplasia.

5.4.3 | Lines of evidence for hypothyroidism

For hypothyroidism, the LoEs defined in the 2019 report (EFSA, 2019a) and still applicable to the assessment question were discussed, reviewed and integrated by the WG. The WG also agreed by consensus on the relative weight (high, medium and low) assigned to each LoE. This weight will be used to adjust the MOET, assuming that the complete information about each LoE would be available.

As a result, the following LoEs and their relative weight have been agreed for the CAG 'hypothyroidism' (see Table 4).

TABLE 4 Lines of evidence and their relative weight.

CAG1 Hypothyroidism	
Line of evidence	**Weight of the line of evidence (high, medium, low)**
1. Known relevant MoA	High
2. Presumed relevant MoA	Medium
3. Evidence of progression of the damage (continuum of key effects in a temporal pattern, from hypertrophy to hyperplasia, adenoma and carcinoma) in the same study or independent studies	High
4. Consistent changes of an indicator in the same species across studies (Consistency within the same species)	High
5. Observation of indicators of the effect in at least 2 species (Consistency across species)	High
6. NOAEL for hypothyroidism is at the same level as or does not differ by a factor exceeding 2-fold from the NOAEL leading to the ADI	Medium
7. Increased serum TSH levels	High
8. Decreased serum T4 and/or T3 levels	Medium/High
9. Increased absolute and/or relative thyroid weight	Low

A very convincing LoE of hypothyroidism is the knowledge/demonstration of a relevant MoA for the a.s. or metabolite; therefore, a high weight is assigned to this LoE. Consistently, a lower weight is given if the mechanistic information is more limited, supporting a presumed relevant MoA.

A relevant LoE is the indication of a progression in time/severity/incidence of the effect, with this clearly showing the association of the effect with the treatment. Observation of indicators of effect in at least two species, including rodents and non-rodents, as this results from fully independent experimentations, is also considered as a strong line of evidence. The fact that the NOAEL for hypothyroidism is in the same range as the critical NOAEL (the reference point used to derive the ADI) brings some contribution to the evidence that an a.s. causes hypothyroidism as a primary effect. Changes in more than one indicator in the same species in two or more independent studies give high confidence in the fact that the effect is not a chance finding. Increased TSH levels (statistically and/or biologically significant) are considered as a stronger LoE, supporting a functional alteration of the thyroid.

Medium to high weight is assigned to decreased levels of serum T3 and T4. The general function of TH is well-conserved in mammals; however, the HPT axis response to pesticides shows important inter-species differences in relation to TH metabolism, with the rats displaying bigger fluctuations in TH levels than humans and this justifies the weight assigned to this LoE.

A weak LoE, because it is less specific, is the increase in absolute thyroid weight. Thyroid weight is often measured together with parathyroids, so the recorded change in thyroid weight, in the absence of concomitant histopathological correlates, could be misleading. Also, thyroid weight changes are not part of the continuum of KEs (interrelated to each other), but the consequence of other conditions like follicular hypertrophy, hyperplasia or tumours that, by themselves, are considered as indicators.

When hypothyroidism and liver hypertrophy are observed concomitantly in toxicological studies in rats, it is plausible that the hypothyroidism is not the result of a direct action on the HPT axis, but secondary to liver enzyme induction.

Observing indicator(s) of hypothyroidism in the absence of liver hypertrophy adds evidence that the substance causes hypothyroidism as a primary toxicity.

6 | CONCLUSIONS

EFSA reconfirmed the same two specific effects on the thyroid relevant for grouping a.s. into CAGs and performing a retrospective CRA from dietary exposure: these are the CAG for hypothyroidism and the CAG for C-cell hypertrophy, hyperplasia and neoplasia. Indicators of these two specific effects that had been proposed in the 2019a EFSA report were revised herein to only include histopathological changes. Specifically, follicular cell hypertrophy, hyperplasia, adenomas and carcinomas are considered as indicators of the CAG for hypothyroidism. C-cell hypertrophy, hyperplasia, adenomas and carcinomas are taken as indicators of the homonymous CAG. This report also illustrates the criteria for inclusion of a.s. into CAGs and considers as the main one the finding of at least one statistically significant and/or biologically relevant change in an indicator of a specific effect in a regulatory toxicology study. Regarding the methodology for hazard characterisation, the potency of each a.s. will be established by means of an overall NOAEL and LOAEL for the respective specific effect, considering all available oral studies showing indicators of that specific effect. Finally, the following LoEs were agreed upon to assess the CAG-membership probability of a.s. in the CAG 'hypothyroidism': known or presumed MoA, evidence of damage progression, consistency of indicator changes within the same species and/or across species, decreased T3 and/or T4 and increased TSH in serum. These LoEs were weighed according to three categories: high, medium and low. No LoEs were developed for the CAG on C-cell hypertrophy, hyperplasia and neoplasia, which is considered no longer needed based on EFSA's experience in 2020 on the CRA for this CAG. Indeed, the CRA for the CAG 'hypothyroidism' broadly covered the combined effects of the pesticides in the CAG on C-cell hypertrophy, hyperplasia and neoplasia.

The specific effects identified, their respective indicators, the criteria for inclusion of a.s. into CAGs, the hazard characterisation methodology, the LoEs and the data collection template will be used by a contractor (ICPS) for the subsequent step of data collection and establishment of CAGs with prioritised substances.

7 | RECOMMENDATIONS

In the absence of fully defined and validated AOPs, the thyroid-specific effects proposed are based exclusively on AOs (histopathological findings) observed in regulatory studies. However, considering the recent indications provided by the EFSA Scientific Committee, it is recommended to further develop AOPs by focusing on the AOs that are available in the a.s. dataset. The AOPs established on thyroid follicular cell adenomas/carcinomas (AOP 110, AOP 119 and AOP 162) involve either (i) direct interference with TH synthesis through inhibition of the NIS and/or TPO, or (ii) increased metabolic clearance from serum T4 through upregulation of UGT levels and subsequent enhanced activity. Decreased serum T4 with subsequent increased TSH levels are identified as common KEs prior to thyroid follicular cell histopathology, expressed as increased hypertrophy and proliferation, progressing to hyperplasia and neoplasia.

Therefore, to increase confidence in the WoE assessment of hypothyroidism in laboratory animals, it is necessary to include measurements of free TH and TSH levels in serum as shown in the updated OECD test guidelines for toxicity studies, or even if not foreseen in the experimental protocols used. As serum TH levels are subject to circadian variations (Georg et al., 2021), it is necessary to standardise sampling time. Concerning the methods used to measure THs, some are more accurate than others, which may lead to inconsistencies in hormone tests (Paczkowska et al., 2020). Liquid chromatography–tandem mass spectrometry is considered the gold standard for measuring serum THs due to its specificity, precision and necessary limits of quantification (Soldin & Soldin, 2011). However, the most common methods used for measuring free TH and TSH are chemiluminescent immunoassays. The results of both methods cannot be interchanged (D'Aurizio et al., 2023). It is therefore recommended to include measurements by the same methodology to compare the results and include sufficient details on the method used.

As part of investigating MoA in animals, it is recommended to conduct in vitro mechanistic studies to assess NIS and TPO inhibition. Selected test methods from the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) study (see Appendix A) as well as those included in the TSAR (Tracking System for Alternative methods towards Regulatory acceptance) website are recommended for this purpose (European Commission, 2023). In case the MoA of hypothyroidism in rodent studies is other than interference with TH synthesis via TPO or NIS inhibition, then additional information would be needed on phase II liver enzyme induction to assess the human relevance of the observed effect. Consideration of thyroid effects in non-rodents and inter-species differences in the disruption of the HPT axis would also facilitate the assessment of human relevance. This tiered approach for the assessment of human relevance of animal MoA for hypothyroidism assessment is in line with the World Health Organization/International Programme on Chemical Safety (WHO/IPCS) MoA/human relevance Framework (Meek et al., 2014).

Promising research on the development of new approach methodologies (NAMs) utilising *in embryo*/in vitro/in silico/*in chemico* testing strategies to characterise HPT axis disruption is currently ongoing in the frames of the Partnership for the Assessment of Risk from Chemicals (PARC) initiative (Ramhøj et al., 2023) and may in the future ease the identification of chemicals disrupting the HPT axis.

ABBREVIATIONS

a.s.	active substance(s)
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AhR	aryl hydrocarbon receptor
AO	adverse outcome
AOP	adverse outcome pathway
AOPwiki	Collaborative Adverse Outcome Pathway Wiki
BMD	benchmark dose
bw	body weight
CAG	cumulative assessment group
CAR	constitutive androstane receptor
CNS	central nervous system
CRA	cumulative risk assessment
DAR(s)	Draft Assessment Reports
DIO	deiodinases
DIT	diiodothyronine
DNT	developmental neurotoxicity
DTU	Danish Technical University
EBIS	ethylene bis isothiocyanate sulphide
ECHA	European Chemicals Agency
ED	endocrine disruptor
EKE	Expert Knowledge Elicitation
EOGRTS	extended one-generation reproductive toxicity study
ETU	ethylene thiourea
EURL ECVAM	EU Reference Laboratory for alternatives to animal testing
HPT	hypothalamic–pituitary–thyroid
HQ	hazard quotient
ICPS	International Centre for Pesticides and Health Risk Prevention
ICs	index compounds
IPCS	International Programme on Chemical Safety
JMPR	Joint Meeting on Pesticide Residues
KE(s)	Key event(s)
LD50	Lethal Dose 50
LOAEL	Lowest observed adverse effect level
LoE(s)	line(s) of evidence
MCT	monocarboxylate transporter
MIEs	molecular initiating events
MIT	monoiodothyronine
MoA	mode of action
MOET	cumulative (or total) margin of exposure
MRLs	maximum residue levels
MSs	Member States
MTD	maximum tolerated dose
NAM	new approach methodology
NIS	sodium/iodide symporter
NOAEL	No Observed Adverse Effect Level
NTIS	non-thyroidal illness syndrome
OATP	organic anion transporting polypeptide
OECD	Organisation for Economic Co-operation and Development
PARC	Partnership for the Assessment of Risk from Chemicals
PPARα	peroxisome proliferator activated receptor alpha
PPPs	plant protection products
PPR Panel	(EFSA) Panel on plant protection products and their residues
PTH	parathyroid hormone
PTU	propylene thiourea
PXR	Pregnane X Receptor
RARs	Renewal Assessment Reports
RMS	Rapporteur Member State
RPF	relative potency factor
rT3	reverse T3
SULT	sulfotransferases

T3	triiodothyronine
T4	thyroxine or tetraiodothyronine
TBG	thyroxine-binding globulin
T-ED	Endocrine disruption by the T-modality
TH	thyroid hormones
ToxCast	Toxicity forecaster
TPO	thyroperoxidase or thyroid peroxidase
TRH	thyrotropin-releasing hormone
TRHR2	TRH receptor-2
TRα1, TRβ1	thyroid receptors α and β
TSAR	Tracking System for Alternative methods towards Regulatory acceptance
TSH	thyroid-stimulating hormone
TSHR	TSH receptor
TTR	transthyretin
UA	uncertainty analysis
UF	uncertainty factor
UGT	uridine 5′-diphospho-glucuronosyltransferases or glucuronyltransferase
US EPA	United States Environmental Protection Agency
WG	working group
WHO	World Health Organization
WoE	weight of evidence

ACKNOWLEDGEMENTS

EFSA wishes to thank the following for the support provided to this scientific output: Katia Chukwubike and Monica Del Aguila for the definition of the template for the data collection, Monica Nepal for the editorial support to the report finalisation for publication and Germán Giner for the coordination of the project.

CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

EFSA

QUESTION NUMBER

EFSA-Q-2023-00573

REFERENCES

Bartsch, R., Brinkmann, B., Jahnke, G., Laube, B., Lohmann, R., Michaelsen, N. I., & Greim, H. (2018). Human relevance of follicular thyroid tumors in rodents caused by non-genotoxic substances. *Regulatory Toxicology and Pharmacology*, *98*, 99–208. https://doi.org/10.1016/j.yrtph.2018.07.025

Bernasconi, C., Langezaal, I., Bartnicka, J., Asturiol, D., Bowe, G., Coecke, N. S., Kienzler, A., Liska, R.,Milcamps, A., Munoz-Pineiro, M. A., Pistollato, M. F., & Whelan, M., (2023). Validation of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system, EUR 31456 EN, Publications Office of the European Union, Luxembourg. ISBN 978-92-68-01257-4, https://doi.org/10.2760/862948, JRC132532.

Bianco, A. C., & Kim, B. W. (2006). Deiodinases: Implications of the local control of thyroid hormone action. *Journal of Clinical Investigation*, *116*(10), 2571–2579. https://doi.org/10.1172/JCI29812

Carter, P. H., & Schipani, E. (2006). The roles of parathyroid hormone and calcitonin in bone remodeling: Prospects for novel therapeutics. *Endocrine, Metabolic & Immune Disorders Drug Targets*, *6*(1), 59–76. https://doi.org/10.2174/187153006776056666

Chemicals Regulation Directorate, Health & Safety Executive, UK. (2013). Investigation of the state of the art on identification of appropriate reference points for the derivation of health-based guidance values (ADI, AOEL and AAOEL) for pesticides and on the derivation of uncertainty factors to be used in human risk assessment. *EFSA Supporting Publications*, *10*(4), 169. https://doi.org/10.2903/sp.efsa.2013.EN-413

Crofton, K. M. (2008). Thyroid disrupting chemicals: Mechanisms and mixtures. *International Journal of Andrology*, *31*(2), 209–223. https://doi.org/10.1111/j.1365-2605.2007.00857.x

D'Aurizio, F., Kratzsch, J., Gruson, D., Petranović Ovčariček, P., & Giovanella, L. (2023). Free thyroxine measurement in clinical practice: How to optimize indications, analytical procedures, and interpretation criteria while waiting for global standardization. *Critical Reviews in Clinical Laboratory Sciences*, *60*(2), 101–140. https://doi.org/10.1080/10408363.2022.2121960

Dellarco, V. L., McGregor, D., Berry, S. C., Cohen, S. M., & Boobis, A. R. (2006). Thiazopyr and thyroid disruption: Case study within the context of the 2006 IPCS human relevance framework for analysis of a cancer mode of action. *Critical Reviews in Toxicology*, *36*(10), 793–801. https://doi.org/10.1080/10408440600975242

Dong, H., & Wade, M. G. (2017). Application of a nonradioactive assay for high throughput screening for inhibition of thyroid hormone uptake via the transmembrane transporter MCT8. *Toxicology In Vitro*, *40*, 234–242. https://doi.org/10.1016/j.tiv.2017.01.014

Dympep, B., Kakar, A. K., Tanwar, R., Shankar, R. L., Sen, I. B., & Belho, E. S. (2014). Postoperative hypothyroidism after thyroidectomy for nontoxic multinodular goiter: Can we prevent it by leaving more? *Thyroid Research and Practice*, *11*(2), 49–54. https://doi.org/10.4103/0973-0354.129724

ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC), Andersson, N., Arena, M., Auteri, D., Barmaz, S., Grignard, E., Kienzler, A., Lepper, P., Lostia, A. M., Munn, S., Parra Morte, J. M., Pellizzato, F., Tarazona, J., Terron, A., & Van der Linden, S. (2018). Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. *EFSA Journal*, *16*(6), 5311. https://doi.org/10.2903/j.efsa.2018.5311

EFSA (European Food Safety Authority), Crivellente, F., Hart, A., Hernández-Jerez, A. F., Hougaard Bennekou, S., Pedersen, R., Terron, A., Wolterink, G., & Mohimont, L. (2019a). Scientific report on the establishment of cumulative assessment groups of pesticides for their effects on the thyroid. *EFSA Journal*, *17*(9), 5801. https://doi.org/10.2903/j.efsa.2019.5801

EFSA (European Food Safety Authority), Dujardin, B., & Bocca, V. (2019b). Scientific report on the cumulative dietary exposure assessment of pesticides that have chronic effects on the thyroid using SAS® software. *EFSA Journal*, *17*(9), 5763. https://doi.org/10.2903/j.efsa.2019.5763

EFSA (European Food Safety Authority), Craig, P., Dujardin, B., Hart, A., Hernández-Jerez, A. F., Hougaard Bennekou, S., Kneuer, C., Ossendorp, B., Pedersen, R., Wolterink, G., & Mohimont, L. (2020a). Cumulative dietary risk characterisation of pesticides that have acute effects on the nervous system. *EFSA Journal*, *18*(4), 6087. https://doi.org/10.2903/j.efsa.2020.6087

EFSA (European Food Safety Authority), Craig, P., Dujardin, B., Hart, A., Hernández-Jerez, A. F., Hougaard Bennekou, S., Kneuer, C., Ossendorp, B., Pedersen, R., Wolterink, G., & Mohimont, L. (2020b). Cumulative dietary risk characterisation of pesticides that have chronic effects on the thyroid. *EFSA Journal*, *18*(4), 6088. https://doi.org/10.2903/j.efsa.2020.6088

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. (2021). Scientific report on the cumulative dietary risk assessment of chronic acetylcholinesterase inhibition by residues of pesticides. *EFSA Journal*, *19*(2), 6392. https://doi.org/10.2903/j.efsa.2021.6392

EFSA (European Food Safety Authority), Anagnostopoulos, C., Anastassiadou, M., Castoldi, A. F., Cavelier, A., Coja, T., Crivellente, F., Dujardin, B., Hart, A., Hooghe, W., Jarrah, S., Machera, K., Menegola, E., Metruccio, F., Sieke, C., & Mohimont, L. (2022). Scientific report on retrospective cumulative dietary risk assessment of craniofacial alterations by residues of pesticides. *EFSA Journal*, *20*(10), 7550. https://doi.org/10.2903/j.efsa.2022.7550

EFSA (European Food Safety Authority). (2023). *Scientific Report on the prioritisation method of substances and organs or systems to be considered in cumulative risk assessment (for publication at the end of 2023)*.

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues). (2008). Scientific opinion to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risks from pesticides to human health with a view to set MRLs for those pesticides in the frame of regulation (EC) 396/2005. *EFSA Journal*, *6*(4), 705. https://doi.org/10.2903/j.efsa.2008.705

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues). (2012). Guidance on the use of probabilistic methodology for modelling dietary exposure to pesticide residues. *EFSA Journal*, *10*(10), 2839. https://doi.org/10.2903/j.efsa.2012.2839

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues). (2013). Scientific opinion on relevance of dissimilar mode of action and its appropriate application for cumulative risk assessment of pesticides residues in food. *EFSA Journal*, *11*(12), 3472. https://doi.org/10.2903/j.efsa.2013.3472

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues). (2014). Scientific opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile (2014 update). *EFSA Journal*, *11*(7), 3293. https://doi.org/10.2903/j.efsa.2013.3293

EFSA Scientific Committee. (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

EFSA Scientific Committee. (2022). Guidance on the use of the benchmark dose approach in risk assessment. *EFSA Journal*, *20*(10), 7584. https://doi.org/10.2903/j.efsa.2022.7584

EFSA-SANTE. (2021). *EFSA-SANTE action plan on cumulative risk assessment for pesticide residues*. https://food.ec.europa.eu/system/files/2021-03/pesticides_mrl_cum-risk-ass_action-plan.pdf

Endocrinology Website. (online). *Thyroid disorders*. http://www.drharper.ca/new_page_12.htm

European Commission, Joint Research Centre, Bernasconi, C., Langezaal, I., Bartnicka, J., Asturiol, D., Bowe, G., Coecke, S., Kienzler, A., Liska, R., Milcamps, A., Munoz Pineiro, A., Pistollato, F., & Whelan, M. (2023). *Validation of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system*. Publications Office of the European Union. https://doi.org/10.2760/862948

Fliers, E., Bianco, A. C., Langouche, L., & Boelen, A. (2015). Thyroid function in critically ill patients. *The Lancet Diabetes & Endocrinology*, *3*(10), 816–825. https://doi.org/10.1016/S2213-8587(15)00225-9

Georg, B., Fahrenkrug, J., Jørgensen, H. L., & Hannibal, J. (2021). The circadian clock is sustained in the thyroid gland of VIP receptor 2 deficient mice. *Frontiers in Endocrinology*, *12*, 737581. https://doi.org/10.3389/fendo.2021.737581

Groeneweg, S., Visser, W. E., & Visser, T. J. (2017). Disorder of thyroid hormone transport into the tissues. *Best Practice & Research Clinical Endocrinology & Metabolism*, *31*(2), 241–253. https://doi.org/10.1016/j.beem.2017.05.001

Haigis, A.-C., Vergauwen, L., LaLone, C. A., Villeneuve, D. L., O'Brien, J. M., & Knapen, D. (2023). Cross-species applicability of an adverse outcome pathway network for thyroid hormone system disruption. *Toxicological Sciences*, *195*(1), 1–27. https://doi.org/10.1093/toxsci/kfad063

Hernández, A. F., Bennekou, S. H., Hart, A., Mohimont, L., & Wolterink, G. (2020). Mechanisms underlying disruptive effects of pesticides on the thyroid function. *Current Opinion in Toxicology*, *19*, 34–41. https://doi.org/10.1016/j.cotox.2019.10.003

Hernández, A. F., Gil, F., & Lacasaña, M. (2017). Toxicological interactions of pesticide mixtures: An update. *Archives of Toxicology*, *91*(10), 3211–3223. https://doi.org/10.1007/s00204-017-2043-5

Hu, X., Wang, X., Liang, Y., Chen, X., Zhou, S., Fei, W., Yang, Y., & Que, H. (2022). Cancer risk in Hashimoto's thyroiditis: A systematic review and meta-analysis. *Frontiers in Endocrinology*, *13*, 1664–2392. https://doi.org/10.3389/fendo.2022.937871

Huisinga, M., Bertrand, L., Chamanza, R., Damiani, I., Engelhardt, J., Francke, S., Freyberger, A., Harada, T., Harleman, J., Kaufmann, W., Keane, K., Köhrle, J., Lenz, B., Marty, M. S., Melching-Kollmuss, S., Palazzi, X., Pohlmeyer-Esch, G., Popp, A., Rosol, T. J., … Yoshida, M. (2020). Adversity considerations for thyroid follicular cell hypertrophy and hyperplasia in nonclinical toxicity studies: Results from the 6th ESTP international expert workshop. *Toxicologic Pathology*, *48*(8), 920–938. https://doi.org/10.1177/0192623320972009

Jing, L., & Zhang, Q. (2022). Intrathyroidal feedforward and feedback network regulating thyroid hormone synthesis and secretion. *Frontiers in Endocrinology*, *13*, 992883. https://doi.org/10.3389/fendo.2022.992883

Knapen, D., Angrish, M. M., Fortin, M. C., Katsiadaki, I., Leonard, M., Margiotta-Casaluci, L., Munn, S., O'Brien, J. M., Pollesch, N., Smith, L. C., Zhang, X., & Villeneuve, D. L. (2018). Adverse outcome pathway networks I: Development and applications. *Environmental Toxicology and Chemistry*, *37*(6), 1723–1733. https://doi.org/10.1002/etc.4125

Lerro, C. C., Beane Freeman, L. E., DellaValle, C. T., Kibriya, M. G., Aschebrook-Kilfoy, B., Jasmine, F., Koutros, S., Parks, C. G., Sandler, D. P., Alavanja, M. C. R., Hofmann, J. N., & Ward, M. H. (2018). Occupational pesticide exposure and subclinical hypothyroidism among male pesticide applicators. *Occupational and Environmental Medicine*, *75*(2), 79–89. https://doi.org/10.1136/oemed-2017-104431

Meek, M. E., Boobis, A., Cote, I., Dellarco, V., Fotakis, G., Munn, S., Seed, J., & Vickers, C. (2014). New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis. *Journal of Applied Toxicology*, *34*(1), 1–18. https://doi.org/10.1002/jat.2949

Miller, M. D., Crofton, K. M., Rice, D. C., & Zoeller, R. T. (2009). Thyroid-disrupting chemicals: Interpreting upstream biomarkers of adverse outcomes. *Environmental Health Perspectives*, *117*(7), 1033–1041. https://doi.org/10.1289/ehp.0800247

Mughal, B. B., Fini, J. B., & Demeneix, B. A. (2018). Thyroid-disrupting chemicals and brain development: An update. *Endocrine Connections*, *7*(4), R160–R186. https://doi.org/10.1530/EC-18-0029

Nielsen, E., Nørhede, P., Boberg, J., Isling, L. K., Kroghsbo, S., Hadrup, N., Bredsdorff, L., Mortensen, A., & Larsen, J. C. (2012). Identification of cumulative assessment groups of pesticides. *EFSA Supporting Publications*, *9*(4), 303. https://doi.org/10.2903/sp.efsa.2012.EN-269

Noyes, P. D., Friedman, K. P., Browne, P., Haselman, J. T., Gilbert, M. E., Hornung, M. W., Barone, S., Jr., Crofton, K. M., Laws, S. C., Stoker, T. E., Simmons, S. O., Tietge, J. E., & Degitz, S. J. (2019). Evaluating chemicals for thyroid disruption: Opportunities and challenges with in vitro testing and adverse outcome pathway approaches. *Environmental Health Perspectives*, *127*(9), 95001. https://doi.org/10.1289/EHP5297

Paczkowska, K., Otlewska, A., Loska, O., Kolačkov, K., Bolanowski, M., & Daroszewski, J. (2020). Laboratory interference in the thyroid function test. *Endokrynologia Pololska*, *71*(6), 551–560. https://doi.org/10.5603/EP.a2020.0079

Peeters, R. P., & Visser, T. J. (2017). Metabolism of thyroid hormone. In K. R. Feingold, B. Anawalt, M. R. Blackman, A. Boyce, G. Chrousos, E. Corpas, W. W. de Herder, K. Dhatariya, K. Dungan, J. Hofland, S. Kalra, G. Kaltsas, N. Kapoor, C. Koch, P. Kopp, M. Korbonits, C. S. Kovacs, W. Kuohung, B. Laferrère, … D. P. Wilson (Eds.), *Endotext*. MDText.com, Inc. https://www.ncbi.nlm.nih.gov/books/NBK285545/

Ramhøj, L., Axelstad, M., Baert, Y., Cañas-Portilla, A. I., Chalmel, F., Dahmen, L., De La Vieja, A., Evrard, B., Haigis, A.-C., Hamers, T., Heikamp, K., Holbech, H., Iglesias-Hernandez, P., Knapen, D., Marchandise, L., Morthorst, J. E., Nikolov, N. G., Nissen, A. C. V. E., Oelgeschlaeger, M., … Svingen, T. (2023). New approach methods to improve human health risk assessment of thyroid hormone system disruption – A PARC project. *Frontiers in Toxicology*, *5*, 1189303. https://doi.org/10.3389/ftox.2023.1189303

Rouquie, D., Tinwell, H., Blanck, O., Schorsch, F., Geter, D., Wason, S., & Barset, R. (2014). Thyroid tumor formation in the male mouse induced by fluopyram is mediated by activation of hepatic CAR/PXR nuclear receptor. *Regulatory Toxicology and Pharmacology*, *70*(3), 673–680. https://doi.org/10.1016/j.yrtph.2014.10.003

Soldin, O. P., & Soldin, S. J. (2011). Thyroid hormone testing by tandem mass spectrometry. *Clinical Biochemistry*, *44*(1), 89–94. https://doi.org/10.1016/j.clinbiochem.2010.07.020

US EPA (US Environmental Protection Agency). (2003). *Framework for cumulative risk assessment*. U.S. Environmental Protection Agency, Office of Research and Development, Center for Public Health and Environmental Assessment (CPHEA), formerly known as the National Center for Environmental Assessment (NCEA), Washington Office. https://www.epa.gov/sites/production/files/2014-11/documents/frmwrk_cum_risk_assmnt.pdf

US EPA (US Environmental Protection Agency). (2005). *Guidelines for carcinogen risk assessment EPA/630/P-03/001F*. https://www.epa.gov/sites/default/files/2013-09/documents/cancer_guidelines_final_3-25-05.pdf

US EPA (US Environmental Protection Agency). (2022). *Computational toxicology (CompTox) chemicals dashboard*. https://www.epa.gov/system/files/documents/2022-06/chemicals_dashboard_march2022.pdf

Van Klaveren, J. D., Kruisselbrink, J. W., de Boer, W. J., van Donkersgoed, G., te Biesebeek, J. D., Sam, M., & van der Voet, H. (2019). Cumulative dietary exposure assessment of pesticides that have chronic effects on the thyroid using MCRA software. *EFSA Supporting Publications*, *16*(9), 85. https://doi.org/10.2903/sp.efsa.2019.EN-1708

Villeneuve, D. L., Angrish, M. M., Fortin, M. C., Katsiadaki, I., Leonard, M., Margiotta-Casaluci, L., Munn, S., O'Brien, J. M., Pollesch, N. L., Smith, L. C., Zhang, X., & Knapen, D. (2018). Adverse outcome pathway networks II: Network analytics. *Environmental Toxicology and Chemistry*, *37*(6), 1734–1748. https://doi.org/10.1002/etc.4124

SUPPORTING INFORMATION

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

How to cite this article: EFSA (European Food Safety Authority), Crivellente, F., Hernández-Jerez, A. F., Lanzoni, A., Metruccio, F., Mohimont, L., Nikolopoulou, D., & Castoldi, A. F. (2024). Specific effects on the thyroid relevant for performing a dietary cumulative risk assessment of pesticide residues: 2024 update. *EFSA Journal*, *22*(3), e8672. https://doi.org/10.2903/j.efsa.2024.8672

APPENDIX A

Mechanistic tests for chemicals interfering with the hypothalamic–pituitary–thyroid axis

The European Commission's European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) has launched a study to validate a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the TH system (i.e. chemicals interfering with the hypothalamic–pituitary–thyroid, HPT, axis), which could be considered as building blocks for the development of OECD test guidelines (European Commission, 2023). EURL ECVAM selected the methods and laboratories responsible for conducting the validation, verified the methods in terms of human relevance, specificity for the measured endpoint, commercial availability and possible intellectual property issues and coordinated the whole study. Several in vitro/ex vivo methods covering the most relevant mechanisms for TH system disruption by chemicals are now available or under development (Table A1).

TABLE A1 List of in vitro/ex vivo methods covering the mechanisms for TH system disruption by chemicals as selected by EURL ECVAM for entering the validation process (Bernasconi et al., 2023).

MIE or KE investigated[a]	TSAR method #; title	Link to the method in TSAR[b]	Validation status
Thyrotropin-Releasing Hormone (TRH) receptor activation	1a; PATHHUNTER® BETA-ARRESTIN thyrotropin-releasing hormone receptor activation (beta-galactosidase) measuring agonist and antagonist activities	https://tsar.jrc.ec.europa.eu/test-method/tm2019-02	Stopped
TSH receptor activation	1b; Thyrotropin-stimulating hormone receptor activation based on cyclic adenosine monophosphate (cAMP) measurement	https://tsar.jrc.ec.europa.eu/index.php/test-method/tm2019-03	Ongoing
TPO inhibition	2a; Thyroid peroxidase (TPO) inhibition based on oxidation of Amplex UltraRed®	https://tsar.jrc.ec.europa.eu/test-method/tm2019-04	Ongoing
TPO inhibition	2b; Thyroid peroxidase (TPO) inhibition based on oxidation of Luminol	https://tsar.jrc.ec.europa.eu/index.php/test-method/tm2019-05	Stopped
Inhibition of tyrosine iodination	2c; Tyrosine iodination using liquid chromatography	https://tsar.jrc.ec.europa.eu/index.php/test-method/tm2019-06	Ongoing
Inhibition of NIS activity	2d; Activation of the sodium iodide symporter (NIS) based on Sandell-Kolthoff reaction	https://tsar.jrc.ec.europa.eu/test-method/tm2019-07	Ongoing
Binding to serum proteins TTR and TBG	3a; Thyroxine-binding prealbumin (TTR)/ thyroxine-binding prealbumin (TBG) binding using fluorescence displacement (ANSA)	https://tsar.jrc.ec.europa.eu/test-method/tm2019-08	Ongoing
Binding to serum protein TTR	3b; Thyroxine-binding prealbumin (TTR) binding using fluorescence displacement (FITC-T4)	https://tsar.jrc.ec.europa.eu/test-method/tm2019-09	Ongoing
Inhibition of Deiodinase 1 activity	4a; Deiodinase 1 activity based on Sandell-Kolthoff reaction	https://tsar.jrc.ec.europa.eu/test-method/tm2019-10	Ongoing
Inhibition of THs glucuronidation	4b; Inhibition of THs glucuronidation using liquid chromatography/mass spectrometry (LC/MS)	https://tsar.jrc.ec.europa.eu/test-method/tm2019-11	Ongoing
Inhibition of THs sulfation	4c; Inhibition of THs sulfation using liquid chromatography	https://tsar.jrc.ec.europa.eu/test-method/tm2019-12	Stopped
Inhibition of TH transporter MCT8	5a; Inhibition of monocarboxylate transporter 8 (MCT8) based on Sandell-Kolthoff reaction	https://tsar.jrc.ec.europa.eu/test-method/tm2019-13	Ongoing
TRα and TRβ receptor activation (agonist activity)	6a; Human TH receptor alpha (TRα) and Human TH receptor beta (TRβ) reporter gene transactivation measuring agonist activities	https://tsar.jrc.ec.europa.eu/test-method/tm2019-14	Ongoing
Human TRβ receptor (in)-activation (agonist and antagonist activity)	6b; TRCALUX human TH receptor beta (TRβ) reporter gene transactivation measuring agonist and antagonist activities	https://tsar.jrc.ec.europa.eu/test-method/tm2019-15	Ongoing

(Continues)

TABLE A1 (Continued)

MIE or KE investigated[a]	TSAR method #; title	Link to the method in TSAR[b]	Validation status
Alteration of intrafollicular T4-content in Zebrafish eleutheroembryos	7a; Measurement of intrafollicular T4 using Zebrafish eleutheroembryos	https://tsar.jrc.ec.europa.eu/test-method/tm2019-16	Ongoing
Alteration of cell proliferation of TH-responsive cells	8a; T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin	https://tsar.jrc.ec.europa.eu/index.php/test-method/tm2019-17	Ongoing

Note: Although detailed information on the aforementioned mechanistic methods available to assess chemicals with the potential to disrupt the HPT axis may be retrieved from the TSAR website, validation through the EURL ECVAM study is expected to enhance regulatory acceptance.

Abbreviations: cAMP, cyclic AMP or 3′,5′-cyclic adenosine monophosphate; hiPSC, human induced pluripotent stem cells; KE, key event; MIE, molecular initiating event; NIS, sodium iodide symporter; TBG, thyroxine-binding globulin; TPO, thyroid peroxidase; TRH, thyrotropin-releasing hormone; TSAR, Tracking System for Alternative methods towards Regulatory Acceptance; TSH, thyrotropin-stimulating hormone; TTR, thyroxine-binding prealbumin.

[a]Molecular Initiating Event or Key Event, as defined by Noyes et al. (2019).

[b]Accessed: 30-07-2023.

Reference

European Commission, Joint Research Centre, Bernasconi, C., Bartnicka, J., & Asturiol, D. (2023). Validation of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system. Publications Office of the European Union. https://doi.org/10.2760/862948

ANNEX A

Data collection template

Annex A can be found in the online version of this output (in the "Supporting information" section): https://doi.org/10.2903/j.efsa.2024.8672.