ERGO

Breaking down the wall between human health and environmental testing of endocrine disrupters: EndocRine Guideline Optimisation

Deliverable Report

D3.4 Proposal for AOP network-based assay battery for THSD testing

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1. INTRODUCTION

The increasing number of potential thyroid hormone system disrupting compounds poses a threat for both human and environmental health. Since the Hypothalamus-Pituitary-Thyroid (HPT) axis is well conserved across vertebrate taxa (Holzer et al., 2016, 2017; Lalone et al., 2018; Mengeling et al., 2017), there is great potential to share information between the areas of human and environmental toxicology. To identify compounds that interfere with the normal function of the thyroid hormone (TH) system, efficient and reliable tools for screening are required. Establishing whether a compound is causing THSD requires evidence of the compound's endocrine mode of action (MoA), its capability to cause an adverse outcome (AO) as well as evidence demonstrating that the observed adverse outcome is a result of the endocrine mode of action as implemented in the Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009" by the European Chemicals Agency (ECHA) and European Food Safety Authority (EFSA) (ECHA-EFSA, 2018). The adverse outcome pathway (AOP) framework is especially suited to establish this kind of relationships as an AOP links a molecular initiating event (MIE, i.e. interaction between molecular target and chemical) to an AO at the organism or population level through a series of causally linked key events (KE, i.e. measurable biological state) (Ankley et al., 2010; OECD, 2018). AOPs can thus help support endpoints for THSD assessment.

Here, we put forward an AOP network-based selection of endpoints for thyroid hormone system disruption (THSD). Throughout this document, an endpoint is defined as an individually measurable (quantifiable), biological response such as altered thyroid hormone levels. An endpoint can be measured in one or several assays and Test Guidelines (TGs). For example, the endpoint 'posterior swim bladder inflation' can be observed both in the Fish Embryo Acute Toxicity test (FET, OECD TG 236; OECD, 2013a) and in the Fish Early-Life Stage test (FELS, OECD TG 210; OECD, 2013b). We propose a set of endpoints complementing currently available endpoints in TGs to aid in the assessment of THSD for both human and environmental health. For the selected endpoints, a summary of existing TGs that already include these endpoints is provided, also taking optional endpoints into account. For the endpoints that have been developed in ERGO, we discuss to which existing OECD TGs the endpoints can be added or are currently being added. Further, this deliverable does not aim to propose a testing strategy for THSD. Instead, it intends to provide an overview of endpoints and assays that could be used in THSD assessment. Work package 7 of the ERGO project goes deeper into how these endpoints and assays could be used. The results presented in this deliverable are also taken up in the context of other international efforts to develop testing strategies, such as the work on Integrated Approaches to Testing and Assessment (IATA) development in the Partnership for the Assessment of Risks from chemicals (PARC, funded by Horizon Europe) and the OECD Thyroid Disruption Methods Expert Group.

1.1. The THSD AOP network

The THSD AOP network in the AOP-Wiki (March 16, 2022) is built of 50 user-defined AOPs originating from 32 AOP pages. This network includes 13 MIEs leading to THSD and the majority of AOPs in the network are initiated by MIEs that lead to hypothyroidism through decreased TH synthesis (thyroperoxidase (TPO) inhibition, sodium/iodide symporter (NIS) inhibition, iodotyrosine deiodinase (IYD) inhibition, pendrin inhibition, and dual oxidase (DUOX) inhibition (Annex 1). Other AOPs in the network lead to hypothyroidism through increased TH clearance (displacement of TH from TH binding plasma proteins, increasing free TH available for clearance, or via hepatic nuclear receptor (HNR) activation) or through deiodinase (DIO) 1 or 2 inhibition. Additionally, there is one AOP initiated by DIO 3 inhibition and two are initiated by thyroid receptor antagonism (Annex 1; Haigis et al., 2023).

Further, the THSD AOP network contains nine AOs. One set of AOPs leads to neurodevelopmental outcomes such as decreased cognitive function or impaired learning and memory, while a second set leads to impaired neurosensory development related to cochlear function and altered visual function.

Lastly there is a third set of AOPs linking to developmental transitions in fish and amphibians, specifically reduced swimming performance resulting from impaired swim bladder inflation and altered amphibian metamorphosis (Annex 1; Haigis et al., 2023).

1.2. Considerations for AOP network-based selection of endpoints

Considerations related to the selection of endpoints and hence assays for inclusion in the assay battery may relate to opportunities for using assays to support the assessment of endocrine activity or adversity, and to opportunities for sharing information across different species and thus between human and environmental health.

1.2.1. Opportunities for evaluating endocrine activity and adversity

The topology of the THSD AOP network can aid in the selection of endpoints assessing endocrine activity or adversity in such a way that it is possible to, on the one hand, distinguish among different mechanisms and, on the other hand, provide good coverage of relevant mechanisms.

In vitro assays may be used to address the MIEs in the network (e.g., a TPO inhibition assay). Such in vitro assays have the advantage of distinguishing among different mechanisms without relying on animal testing. They can be utilised to support a conclusion on endocrine activity and can aid in a MoA analysis which is mandatory in certain cases as described in the joint EFSA-ECHA guidance document (ECHA-EFSA, 2018). However, multiple in vitro assays need to be combined in an in vitro test battery in order to provide sufficient coverage of THSD mechanisms and this may pose a challenge. Therefore, selecting endpoints for which the underlying mechanisms are well understood can be a main factor to consider.

In the AOP network, different THSD mechanisms converge in a small number of hub KEs representing alterations in TH levels. These central endpoints may be especially valuable since they integrate several MIEs into only a few downstream hub events (i.e., decreased/increased T4/T3 levels) and therefore provide considerable coverage of THSD mechanisms, including those for which a suitable in vitro assay is missing (not developed, not sensitive enough, not specific enough to clearly distinguish endpoints etc.). Intermediate KEs such as these integrative hub KEs are important in supporting the biologically plausible link in endocrine disruptor (ED) identification.

On the right-hand side of the AOP network, pathways diverge again, and different AOs result from these central alterations in TH levels. In a regulatory context those AOs can be used to inform on adversity. It should be noted that there is an important distinction between adversity assessment for human and environmental health. While for human health, adversity is established on the level of the individual (e.g., altered TH levels and thyroid morphology), adversity in an environmental context should have population relevance.

Besides the rather traditional use of the THSD AOP network, where MIEs are used to predict an AO in a left-right approach, AOP networks with a point of convergence (e.g., altered TH levels) can also be used in a right-left approach (Knapen et al., 2015, 2018). Using this approach, one is able to narrow down a set of potentially induced MIEs based on the observation of a certain AO or intermediate KE. This may be considered an advantage of AOP network-based testing approaches if running a whole battery of in vitro assays to conclude on an MIE is not possible.

1.2.2. Opportunities for cross-species extrapolation

Historically, AOPs on THSD in the AOP-Wiki have been developed in a specific context (either human/mammalian or environmental health) and have been based on a particular taxon (mammals vs. fish or amphibians). Those AOPs do, however, share multiple KEs and thus form a cross-species THSD AOP network that, for example, connects AOPs developed in fish to AOPs developed in

mammals. This network could provide a scientifically plausible and evidence-based foundation for (1) identifying new endpoints for THSD for in vitro or in vivo testing and (2) measuring endpoints of THSD in fish and amphibian assays to raise concern for mammalian, including human, health effects or vice versa. To increase the network's usefulness for cross-species extrapolation, we assessed the taxonomic domain of applicability (tDOA) of MIEs and AOs in the network in the context of altered TH levels, which form hub KEs in the network (Haigis et al., 2023). This assessment showed that MIEs, including those that were initially described in the AOP-Wiki as being putatively applicable to amphibians, were potentially applicable to mammals and, with limitations, to fish and amphibians (Figure 1). Similarly, the tDOA assessment highlighted the potential applicability of AOs related to neurodevelopmental or neurosensory outcomes across vertebrate species.

The cross-species THSD AOP network provides a graphical representation of potentially measurable endpoints (in vitro or in vivo) on different levels of biological organisation (molecular mechanisms to organism or population response) which can support ED identification and at the same time may present an opportunity for cross-class extrapolation. In this regard, the measurement of THs across vertebrate taxa as integral part of the network should be considered a pivotal part of any battery aiming to assess THSD. As such, we use the AOP network to highlight the coverage of THSD mechanisms and effects by the in vitro and in vivo endpoints across vertebrate taxa and utilise this coverage to prioritise endpoints in the network.

In the context of selecting endpoints for an assay battery and in the light of cross-species comparisons, we chose to visualize AOs in the network on the individual level for both human and environmental health. At this point, the larval amphibian growth and development assay (LAGDA, TG 241) is the only non-mammalian assay available in the Conceptual Framework (OECD, 2018) that measures adverse effects (ECHA-EFSA, 2018). Measuring impaired swim bladder inflation and impaired eye development in fish assays would be a significant step forward as they may be considered AOs on the individual level with population relevance. For this reason, these endpoints are currently under OECD validation for inclusion in a thyroid FET (tFET) or FELS (tFELS), i.e. FET and FELS tests with addition of THSD-sensitive endpoints (project 2.64 of the OECD Work Plan for the Test Guidelines Programme). Additionally, the THSD AOP network can be used to highlight endpoints with a high potential for cross-species extrapolation, for which assays are yet missing, thereby aiding in identifying opportunities for the development of new, potentially relevant assays.

2. SELECTION OF ENDPOINTS

Based on the above considerations, the aim here is to use the THSD AOP network described in section 1.1 to select endpoints for inclusion in an assay battery to assess THSD. THSD endpoints and assays that are not covered by the ERGO AOP network are not part of the scope of this document.

2.1. In vitro assays measuring MIEs

From a scientific perspective, having in vitro assays for all MIEs is important to obtain a full mechanistic understanding of THSD. From an implementation perspective this may, however, not be feasible which makes it necessary to focus on those MIEs that are considered most relevant in a given context. In general, the MIEs in the THSD AOP network can be grouped into five mechanistic categories: Inhibition of thyroid hormone synthesis (Inhibition of TPO, NIS symporter, dual oxidase, pendrin, iodotyrosine deiodinase), inhibition of TH transport in serum (Binding to thyroid binding globulin or transthyretin), disruption of metabolism or biotransformation (Activation hepatic nuclear receptor(s) or pregnane-x-receptor(s), Inhibition of deiodinases 1-3) and disruption of TH signalling (Antagonism of thyroid receptor). Including endpoints informative of MIEs in the above mentioned categories can aid to cover a broad range of THSD mechanisms while at the same time providing distinctive mechanistic information to support MoA analyses. Across the mechanistic categories, MIEs such as TPO, NIS and

DIO1-3 inhibition as well as thyroid receptor antagonism and TTR binding have a potentially broad tDOA (Figure 2). Measuring these MIEs as part of an assay battery could therefore be informative for both human and environmental health. It should be recognized, however, that the extrapolation potential of a given assay (e.g., based on a human versus fish cell line) depends on the level of phylogenetic conservation of the molecular target across taxonomic groups. Tools such as SeqAPASS can be used to determine the acceptable limits of extrapolating from one taxonomic group to another based on accepted cut-off values (Haigis et al., 2023). The in vitro assay development and assay characterization work in ERGO has been mostly focused on these MIEs (shown in dark colour in Figure 2). Some work on AhR agonism was also included in ERGO. For a detailed discussion of the cross-species applicability of the MIE 'activation of hepatic nuclear receptors', the reader is referred to Haigis et al. (2023).

In ERGO, in vitro work on assay development or further characterization has been done for TPO, NIS, DIO1, DIO2, DIO3, TTR and TR. This includes the development of a number of new in vitro models overexpressing the individual target genes, characterizing gene and protein expression as well as activity, selecting the best models and developing/optimizing assays for detection of the inhibition. Assays measuring inhibition of DIO1 and DIO3 have been prioritized for more detailed prevalidation (indicated with solid arrows in Figure 2) because a DIO3 assay is not included in the ongoing validation of 18 in vitro assays, measuring a set of THSD MIEs (Joint research centre (JRC)-European Union Network of Laboratories for the Validation of Alternative Methods (NETVAL)) and because the included DIO1 assay based on human liver microsomes from donors has been recommended to be preferentially replaced by an assay based on an in vitro enzyme source. The work in ERGO on TPO, NIS, DIO2, TR and TTR has been supportive of the NETVAL validation effort (indicated by open arrows in Figure 2) through the further characterization of the in vitro models and assays and the investigation of the compatibility with an external biotransformation system (Liu et al., 2024).

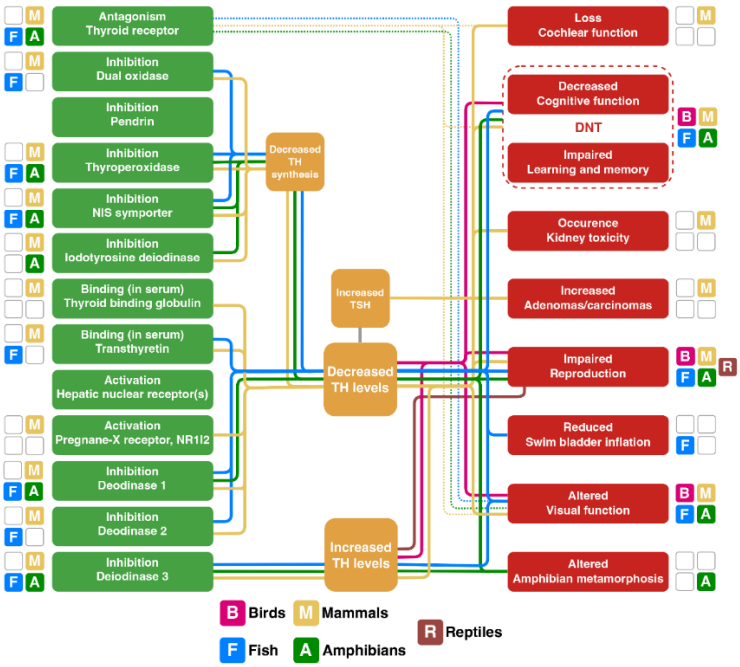

Figure 1: Conceptualized THSD AOP network after evaluation of the taxonomic domain of applicability. The colored squares and connections indicate the applicability to birds (B, pink), mammals (M, yellow), fish (F, blue), amphibians (A, green), and reptiles (brown). Only those colored squares and connections reflecting a moderate or high level of confidence in the taxonomic applicability of the MIE-to-THSD and THSD-to-AO relationships are shown. The dotted lines distinguish THSD-to-AO relationships initiated by thyroid receptor antagonism. DNT= Developmental neurotoxicity. From Haigis et al., 2023. For more information on Activation of hepatic nuclear receptors, the reader is referred to Haigis et al. (2023).

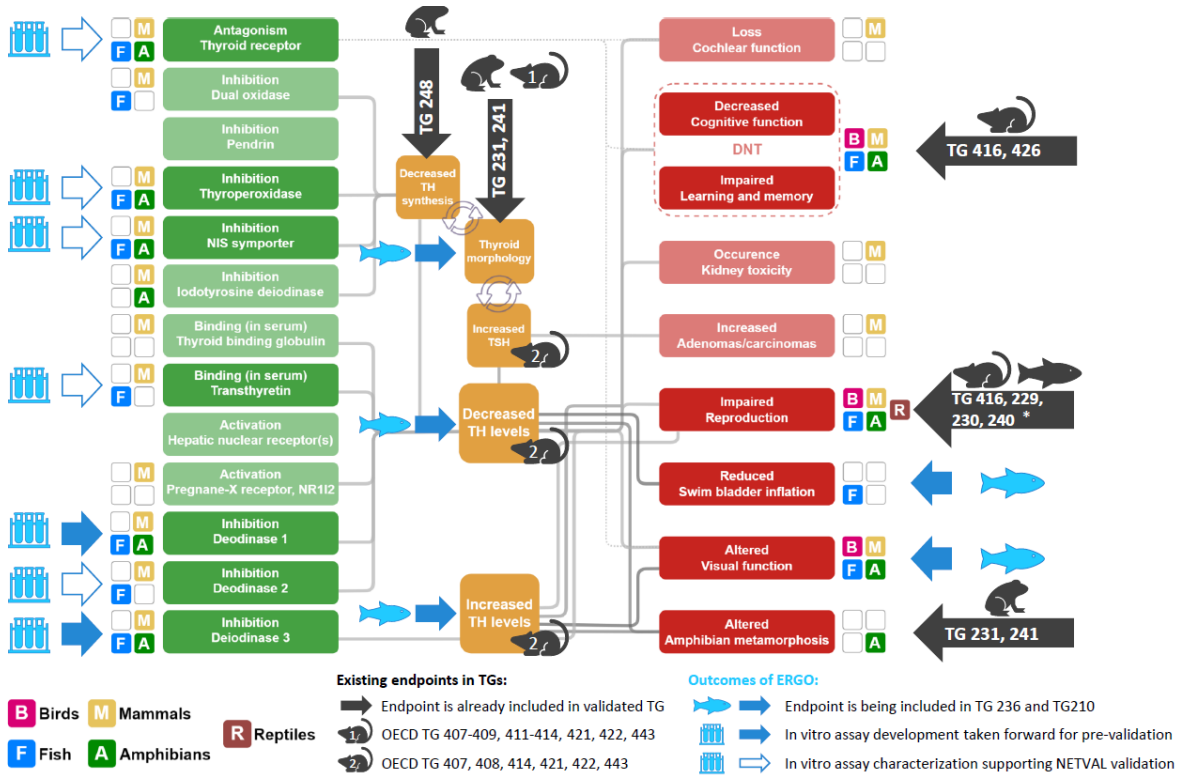

Figure 2: Conceptualised cross-species THSD AOP network highlighting selected endpoints based on the AOP network. Endpoints that are selected are indicated by dark coloured MIE, KE or AO boxes. Black symbols and arrows indicate the availability of existing TGs already including a respective endpoint with the animal icon symbolising the taxonomic group. In vitro assay development in ERGO that is being taken forward for pre-validation is indicated by glass vials and solid arrows. In vitro assay characterization and further development in support of the ongoing NETVAL validation is indicated by glass vials and open arrows. For the in vitro assays, new TGs will need to be developed. In vitro assays included in the NETVAL effort but not advanced in ERGO are not shown. New endpoints developed in ERGO for addition to existing fish TGs are indicated as blue fish icons. For the fish endpoints, existing TGs to which the endpoints are currently being added are indicated. The endpoint thyroid morphology (measured as thyroid histopathology) has been added to this figure in-between "Decreased Th synthesis" and "Increased TSH" as it is connected to both KEs via feedback mechanisms (indicated by circulating arrows). For better visualisation, paths are shown in grey and are shown only once instead of separately for every class (see Figure 1 for reference). DNT= Developmental neurotoxicity.= This is not an exhaustive list of TGs addressing impaired reproduction. Figure adapted from Haigis et al., 2023.*

2.2. In vivo endpoints

2.2.1. In vivo endpoints measuring intermediate KEs

Thyroid hormone synthesis

Impairments related to thyroid hormone synthesis are, based on the THSD AOP network, relevant across different taxonomic groups (Figure 1). In the AOP network, decreased thyroid hormone synthesis represents an intermediate KE integrating different MIEs and linking them to the main hub KEs (altered TH levels). This makes the endpoint valuable for MoA analyses related to functional alterations in the thyroid. Amphibian and fish assays that investigate thyroid hormone synthesis are available or undergoing validation, respectively (XETA, OECD TG 248; ZETA, JRC under validation, Figure 2). The Xenopus eleutheroembryo thyroid assay (XETA) uses genetically modified (TH/bZip-eGFP) tadpoles and measures the expression of the genetic construct, which is directly regulated by THs at the time of metamorphosis, thus deducing information on TH production (Fini et al., 2007; OECD, 2019). In the zebrafish eleutheroembryo thyroid assay (ZETA), zebrafish eleutheroembryos are used in a whole-mount immunohistochemistry approach which stains intrafollicular T4, thereby informing on the follicular T4 synthesis (Raldua & Babin, 2009; Thienpont et al., 2011).

Thyroid hormone measurements

In the THSD AOP network altered TH levels are cross-vertebrate class hub KEs, that in an assay battery function as point of convergence for several mechanisms/MIEs. With respect to non-mammalian vertebrates, assessment of the T-modality is currently only done using level 3/4 amphibian assays in

which measurement of TH levels is not included (ECHA-EFSA, 2018). However, recent technological advances as well as the recently started OECD validation to include this endpoint in the tFET and tFELS may trigger a re-evaluation of the inclusion of TH measurements in the relevant TGs. In light of 3Rs and sustainability, including TH level measurements in these TGs, especially in the tFET, could support reducing the need for multiple tests and thus reduce the usage of resources and animals. Measuring TH levels as part of existing fish and amphibian TGs (e.g., FELS, LAGDA, Amphibian metamorphosis assay, AMA), which are frequently requested under different regulatory frameworks, could aid the mechanistic understanding for MoA analyses as TH level alterations lead to apical endpoints such as impaired swim bladder inflation and metamorphosis, and potentially also to neurodevelopmental or visual impairments in the THSD AOP network (Figure 1 & 2). Additionally, and based on the conservation of the HPT-axis, measuring TH levels, which are hub KEs in the THSD AOP network, in fish may provide a unique opportunity to assemble data supporting cross-species extrapolation efforts (see 1.1).

Thyroid follicle morphology

Morphological changes of the thyroid gland or follicles are routinely assessed in the context of existing level 3/4 TGs for THSD in amphibians and mammals (e.g., TG 231, 241 and 407-409, 414, 421), and are now included in the OECD validation of THSD endpoints for addition to existing fish TGs (tFET and tFELS) (Figure 2). In addition to altered TH levels, thyroid histopathology is a key endpoint in determining T-mediated adversity in mammals (EFSA-ECHA, 2018). Thyroid follicles of juvenile and adult fish, and especially zebrafish, have been shown to respond with a similar sensitivity than the anuran thyroid to compounds disrupting the thyroid hormone system (Gardell et al., 2017; Mukhi & Patiño, 2007; Petersen et al., 2015; Schmidt et al., 2012, 2017), thus demonstrating that thyroid follicle morphology can also be a sensitive endpoint to identify THSD in fish. Histopathological investigations of other fish tissues are already part of existing fish OECD TGs (229, 234). In the OECD validation effort, thyroid histopathology is being added to the often required FELS test and evaluation of thyroid follicle development using transgenic zebrafish eleutheroembryos (*tg(tg:mCherry*) is being added to the FET test for a fast and efficient screen.

In an AOP network context, thyroid morphological alterations typically reflect the consequences of feedback mechanisms (Figure 2) rather than being a direct and essential event in a particular toxicological pathway. This is of particular relevance as thyroid stimulating hormone (TSH), included in several mammalian assays, is not regularly measured in fish or amphibians and hence feedback mechanisms might not be represented adequately in tests using these taxa. Prioritising thyroid morphology as endpoint in a THSD assay battery could be considered an approximation of feedback mechanisms making it a valuable mechanistic component across taxa.

2.2.2. In vivo endpoints measuring Adverse Outcomes

Swim bladder inflation

The responsiveness of swim bladder inflation to altered TH levels as a result of TPO and/or DIO inhibition has been described in the AOP-Wiki (AOPs 155-159) (Vergauwen et al., 2022e, 2022c, 2022d, 2022b, 2022a). In several fish species, THSD has been linked to impaired posterior and/or anterior swim bladder inflation, thereby affecting the fishes buoyancy and motility, making it an endpoint of population relevance able to indicate adversity (Cavallin et al., 2017; Czesny et al., 2005; Horie et al., 2023; Stinckens et al., 2016, 2018; Woolley and Qin, 2010). In the THSD AOP network, swim bladder inflation represents an AO on the organism level which can be easily assessed using a stereomicroscope. For certain species, e.g., the zebrafish, assessment of posterior chamber inflation can be done in the eleuthero-embryonic life stage, in the context of the FET test (extended until 5 days post fertilization), when zebrafish are still non-protected animals (Strähle et al., 2012; EU Directive 2010/63/EU). Anterior chamber inflation can be observed at around 21 dpf and may be assessed in

the context of e.g., the FELS test. Swim bladder inflation was shown to respond to different THSD MIEs in a life-stage dependent manner (Stinckens et al., 2016, 2018; Vergauwen et al., 2022e, 2022c, 2022d, 2022b, 2022a) but may also be affected by various, endocrine or non-endocrine mechanisms. For inclusion as an endpoint in the assay battery it is therefore recommended to combine swim bladder inflation with a thyroid-specific endpoint such as TH measurements which places the endpoint in a THSD context (Knapen et al., 2020).

As an example of how the mechanistic understanding available in the AOP network can be used to integrate information on the MIEs, hub KEs and AOs to support a MoA analysis and establish a plausible link, a distinction can be made between the consequences of TPO and DIO inhibition in fish. DIO inhibition is known to impair swim bladder inflation both in the embryonic stage (inflation of the posterior swim bladder chamber in the FET time frame) and in the larval stage (inflation of the anterior swim bladder chamber in the FELS time frame), while posterior chamber inflation is expected to be less responsive to TPO inhibition because this process mainly relies on maternally transferred THs (Knapen et al., 2020). Using the THSD AOP network in a right-to-left approach, in vitro assays could be used to confirm the mechanism underlying the observed AO. Note that often to observe the absence of a swim bladder effect due to TPO rather than DIO inhibition, a follow-up until two days beyond the time frame of the FET may be required to account for a delay in inflation rather than actual impaired inflation (Stinckens et al., 2016, 2018).

Eye development

Thyroid hormones are involved in vertebrate eye development with the structure and organisation of the retina being susceptible to perturbation by TH system disrupting chemicals (Baumann et al., 2016; Gölz et al., 2022; Houbrechts et al., 2016; Marsh-Armstrong et al., 1999; Ng et al., 2010; Trimarchi et al., 2008; Vancamp et al., 2019). Therefore, eye development is an endpoint with a high potential for cross-species extrapolation. In embryonic and juvenile life stages of fish, histopathological examinations of the retinal layers proved to be a reliable and responsive endpoint for THSD when combined with THSD diagnostic endpoints such as TH measurements (see Deliverable 5.4). In mammals, OECD TG 405 is available to assess the irritative/corrosive potential of substances in the eye. This TG does, however, not include endpoints relevant for eye development or structure and may thus be of limited relevance for eye development. Specific assays investigating an impact on eye development in mammals are currently not available. In the THSD AOP network, impaired eye development, resulting in altered visual function, is considered an AO on the individual level with implications for the population level (see AOP 363; Gölz et al., 2022; Haigis et al., 2023). Eye development as an endpoint in a fish TG (e.g., tFET or tFELS) may thus be utilised as an AO on the individual level providing mechanistic information on a plausible linkage with THSD across vertebrate species. Currently, eye development is under OECD validation for inclusion in the tFET and the tFELS tests.

Neurodevelopment

In the THSD AOP network, AOPs developed for mammals are heavily focusing on neurodevelopmental AOs as a result of e.g., NIS or TPO inhibition and subsequently altered TH levels (Crofton et al., 2019; Rolaki et al., 2019). Assays to evaluate neurodevelopmental mechanisms/ MoAs in mammals are readily available (e.g., OECD TG 416, 426; Figure 2). Evaluating potential linkages between altered TH levels as hub KEs and neurodevelopmental impairments (AOs) has indicated that these AOs are plausibly applicable to multiple vertebrate taxa, including fish and amphibians (Figure 1; Haigis et al., 2023). However, assays specifically measuring neurodevelopmental perturbations like impaired learning and memory in amphibians or fish are currently lacking. Therefore, in many cases the general integrity and functionality of the nervous system is evaluated by means of behaviour assays (which can have more generic causes, Haigis et al., 2023). It is assumed that a severe impact on the neurodevelopment of these species would adversely reflect on their ability to receive external stimuli

and respond to their environment and hence develop adaptive behaviours, which may be considered equal/comparable to an impairment of learning and memory in mammals. Keeping the high potential for the cross-taxa applicability of THSD-induced neurodevelopmental outcomes in mind, testing for neurodevelopmental AOs in the context of e.g., an AMA or FELS test could be a step towards bridging the gap between human and environmental health assessment (Figure 1). Currently, efforts are ongoing to standardise the light-dark transition test with zebrafish eleutheroembryos to explore how it can be used to investigate neurological development. This effort is led by UFZ Leipzig, Germany with participation of several ERGO partners.

Reproduction

The cross-species applicability analysis of the AOP network suggested that THSD-induced impaired reproduction is potentially relevant across taxonomic groups (Haigis et al., 2023). The impact on the population level is the major driver to conclude adversity in an environmental health context. Assays measuring this endpoint are already included in fish (TG 229, 240) and mammalian (TG 416) TGs (Figure 2). Hence, guidance on how to assess the reproductive outcome is already available for some taxonomic groups and could plausibly be included in tests for endocrine disruption as is aimed for example by the integrated fish endocrine disruptor test (iFEDT). This test combines endpoints for THSD, reproduction, early development, and sexual differentiation to identify multiple endocrine-disruptive effects based on the integration of TGs 229 and 234 (Fagundes et al., 2024; Pannetier et al., 2023). It should be noted that reproduction is also strongly driven by other hormonal systems and the extent to which THSD affects reproductive endpoints is not fully understood yet.

3. Discussion and conclusion

Based on the initial considerations and the endpoints introduced above, an AOP network-based THSD assay battery is proposed (Figure 2). Several assays that are included in this assay battery are already available, focusing on the assessment of TH levels, thyroid morphology, and AOs such as impaired neuronal development, impaired reproduction or impaired metamorphosis. Test guidelines investigating thyroid adenomas/carcinomas (TGs 421, 422) do exist but are not discussed in the context of this document due to the limited taxonomic applicability of this endpoint (Figure 2, Haigis et al., 2023). Adding to this set of existing assays, (1) ERGO proposes the inclusion of a set of in vitro assays, targeting different MIEs in the network for which we have a good mechanistic understanding and that, at the same time, have a potentially broad tDOA, (2) ERGO partners advanced assays to measure TH levels and thyroid morphological alterations in fish, whereas impaired swim bladder inflation and altered visual function are selected as AOs on the individual level with consequences at the population level. The OECD validation to include those THSD responsive endpoints into TGs 236 and 210 for fish has been initiated in March 2024 with 15 laboratories participating in the context of project 2.64 of the OECD Work Plan for the Test Guidelines Programme and is being coordinated by Denmark (Henrik Holbech, ERGO coordinator), and co-led by Belgium (Dries Knapen, ERGO partner), the Netherlands (Lisa Baumann, ERGO partner), and Germany (Thomas Braunbeck, ERGO partner). Adding those endpoints to more fish TGs such as the fish sexual development test (TG 229), the Medaka Extended One-Generation Reproduction Test (MEOGRT, TG 240) and others (TGs 230, 234, 250, and the newly proposed iFEDT; Pannetier et al., 2023) may also be explored in the future. Further, the AOP network-based assay battery prioritises endpoints, like impaired reproduction or impaired neuronal development, for which standardised assays across taxonomic groups are not yet available, but for which a high cross-species applicability is assumed (Figure 1, Haigis et al., 2023).

The endpoints highlighted above address different levels of biological organisation that, from an AOP network-based perspective can be utilised in a modular approach. It is envisioned that by combining assays/endpoints along the knowledge captured in the THSD AOP network and according to the respective need(s) of a user, an efficient testing strategy for potential thyroid hormone disrupting compounds can be achieved.

An integrative part of this modular design is the fact that several assays within or across modules are combined to form an assay battery. Within one level this becomes necessary for example to ensure sufficient coverage of THSD mechanisms, for example when focusing on the MIEs. Across levels, combining assays into a battery is needed because many of the endpoints under consideration are responsive to, but not diagnostic of, THSD. For instance, this is the case for swim bladder inflation or eye development which need to be combined with a thyroid specific endpoint such as TH measurements to place them in a THSD context. By combining e.g., TH measurements and swim bladder inflation one is able to obtain mechanistic information as well as information on adversity.

There is a strong interest to move to non-animal based testing, while at the same time in vivo data will continue to be a regulatory requirement in the foreseeable future. Modular testing approaches that are developed based on this assay battery will allow combining non-animal endpoints with endpoints measured in animals thereby acknowledging the regulatory requirements for in vivo testing as well as supporting a way forward towards non-animal testing. In this regard, in vitro and/or fish embryo data from the assay battery are intended to support activity assessments and MoA identification. At the same time, the assay battery acknowledges the regulatory framework using mammalian tests for informing on human health on the one hand and mammalian as well as non-mammalian non-target organisms for informing on environmental health on the other hand. Based on the knowledge that is now available on the cross-vertebrate applicability of THSD mechanisms and effects, information can be shared between human and environmental health assessments. Ultimately this will support a gradual phase out of in vivo mammalian testing as increasing experience with e.g., in vitro or fish embryo data will increase the confidence in those endpoints.

Finally, in general, any remaining uncertainties/inconsistencies related to the endpoints should be taken into account. Underlying AOPs highlighting such uncertainties/inconsistencies may be used as a resource for this. Inconsistencies may for example relate to responses across life stages and species or non-monotonic dose-response relations.

The results of the ERGO project, and specifically the progress in the development of in vitro and fish assays, are taken up in the context of different follow-up projects. The work on exploring the potential for cross-species extrapolation is being continued in PARC. The AOP network is used as a basis for further AOP development in PARC Task 5.3.2 on AOP development. Specialty group 2, led by Dries Knapen (ERGO partner) and Henrik Holbech (ERGO coordinator), is focusing on AOP development for endocrine and metabolic disruption with important attention for THSD. Further, the ERGO assays will be used in PARC Task 6.1.1 on the development of IATAs. The project on the development of IATAs for endocrine disruption, led by Dries Knapen and Miriam Jacobs (GOLIATH), is focusing on THSD and is taking up ERGO outcomes. As part of the IATA development, a case study making use of both the in ERGO vitro assays and the THSD endpoints in fish TGs (in conjunction with the OECD validation of these endpoints) will be performed using REACH-relevant chemicals. These efforts are specifically aimed towards generating confidence in those new endpoints. The outcomes of ERGO will also be taken up in the “Network for cross-disciplinary ED assessment: training the next generation of toxicologists” (NeXED, MSCA Doctoral network, funded by Horizon Europe).

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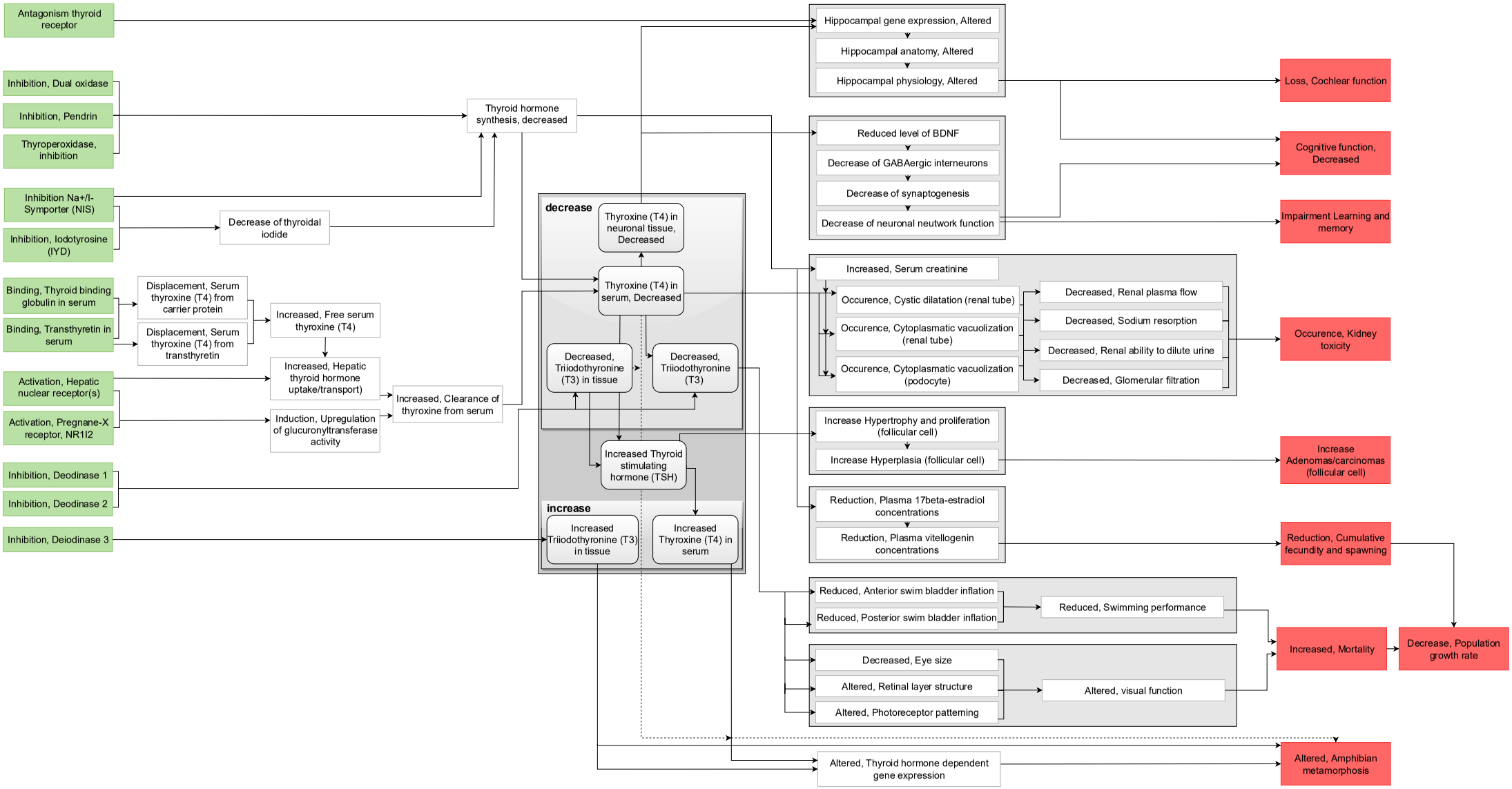
Annex 1

Visualization of the complete THSD AOP network as available in the AOP-Wiki on March 16, 2022



Visualization of the complete THSD AOP network as it was present in the AOP-Wiki on March 16, 2022 with different MIEs converging towards altered TH levels (hub KEs, dark grey box) and diverging towards multiple AOs. MIEs are shown as green nodes and AOs are shown as red nodes. Full and dashed lines show adjacent and non-adjacent KERs respectively. For a detailed description see Haigis et al., 2023 (https://doi.org/10.1093/toxsci/kfad063).