EURION

European Cluster to Improve Identification of Endocrine Disruptors

EURION Progress Report

Public summary for 1/2022–6/2023
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Introduction

The *European Cluster to Improve Identification of Endocrine Disruptors*, EURION, is a cluster of eight European research projects. The projects are focusing on the development of new endocrine disruptor (ED) test methods targeting relevant endpoints that are not sufficiently covered by current regulatory tests. As the current testing tools, including regulatory *in vivo* tests and novel *in vitro* assays, do not appropriately identify ED-induced effects related to certain less studied endocrine-mediated pathways or health outcomes, the EURION projects aim to address these gaps. *EURION,* was launched by DG-RTD in Brussels, Belgium, 31 January 2019 with representatives from the eight projects as well as the International Advisory Panel (IAP) and observers from various EU agencies. The aim was to optimise synergies and avoid overlaps between the projects selected for funding from the call SC1BHC-27-2018 'New testing and screening methods to identify endocrine disrupting chemicals'.

The projects are:

- ATHENA - Assays for the identification of thyroid hormone axis-disrupting chemicals: elaborating novel assessment strategies.
- EDCMET - Metabolic effects of endocrine disrupting chemicals: novel testing methods and adverse outcome pathways
- ENDpoiNTs - Novel testing strategies for endocrine disruptors in the context of developmental neurotoxicity
- ERGO - Breaking down the wall between human health and environmental testing of endocrine disrupters: EndocRine Guideline Optimisation
- FREIA - Female reproductive toxicity of EDCs: a human evidence-based screening and identification approach
- GOLIATH - Beating Goliath: Generation of novel, integrated and internationally harmonised approaches for testing metabolism disrupting compounds
- SCREENED - A multistage model of thyroid gland function for screening endocrine-disrupting chemicals in a biologically sex-specific manner
- OBERON - An integrative strategy of testing systems for identification of EDs related to metabolic disorders

The cluster projects will:

- Deliver novel/improved ED assay candidates for regulatory use (pre-validated);
- Provide support for the OECD work on testing and assessing chemicals for ED identification, especially feeding into the OECD Endocrine Disrupter Screening and Testing Programme.
- Contribute to enhanced international cooperation.
- Contribute to the development of an international strategy and guidelines for testing EDs and assessing associated hazard and risk

As new and improved approaches are needed to increase the quality, efficiency and effectiveness of existing methods to meet demanding and evolving regulatory requirements, EURION enables effective collaboration between the projects to reach and contribute towards the main goal. Being part of the Cluster facilitates sharing of knowledge, data and expertise as well as allowing the joining of forces for common dissemination and communication activities in order to maximize impact, both at the project and cluster-level.

The EURION Cluster is coordinated by rotating teams consisting of two projects at a time to cover the 5-year life span of the projects. Each team coordinates the EURION Cluster for 15 months. The first team was GOLIATH and OBERON and the next ENDpoiNTS and ERGO followed by ATHENA and SCREENED. From October 2022 to December 2023, the EURION cluster is coordinated by EDCMET and FREIA.

This report summarises the progress and activities of the EURION Cluster and the eight projects during the 18-month reporting period, from 1st January 2022 to 30th June 2023. The first part of the report is focused on providing a progress overview of the individual projects in the form of public summaries. This is followed by an overview of the test methods to be developed, cluster highlights and working group summaries.

The EURION projects have applied for an extension to the project duration with most projects ending in June 2024.

1 Project summaries

1.1 ATHENA project summary

Assays for the identification of Thyroid Hormone axis - disrupting chemicals: Elaborating Novel Assessment strategies - ATHENA

Summary of the context and overall objectives of the project

The thyroid hormone system maintains biological functions such as growth and homeostasis. Critical during development is the delivery of thyroid hormones at the right concentration and the right time. Thyroid hormone system-disrupting chemicals (THSDCs) can interfere with these processes in many different ways. In pregnant women, THSDCs can decrease circulating thyroid hormone, which leads to lower levels reaching the foetus. The altered thyroid hormone levels can affect the foetus and be particularly damaging in the first trimester when the foetus cannot produce its own thyroid hormone and relies on the mother for this essential supply. Altered maternal thyroid hormone levels can lead to impaired psychomotor development and lowered IQ in the offspring.

The ATHENA project researches how brain development *in utero* is affected by THSDCs which disrupt the maternal thyroid hormone system.

There are several points within the thyroid hormone system where THSDCs can interact and disrupt the system. Partners within the ATHENA consortium focus on different areas within the thyroid hormone system to develop test methods to identify THSDCs, delineate their mechanism of action to construct an AOP network and to inform a comprehensive testing strategy.

The ATHENA project aims to develop new methods for incorporation into existing OECD test guidelines for the identification of THSDCs. Existing tests are insufficient to identify chemicals that pose such hazards. Further tests with new endpoints need to be established to close critical gaps in the test guidelines.

Work performed from the beginning of the action to the end of the period covered by the report and main results achieved so far

Associations of exposure to EDCs with thyroid function in pregnant women

We leverage already available human epidemiological data from the longitudinal SELMA study and the Generation R study to evaluate associations between maternal urinary and/or serum concentrations of EDCs and thyroid hormone levels. We have explored associations with phenolic compounds, phthalates, perfluorinated chemicals, PCBs and hexachlorobenzene and found that thyroid hormone levels were changed. This occurs by a variety of mechanism of action.

We further investigated families of chemicals with similar structural features. Most EDCs that were associated with lower child IQ in earlier studies from both cohorts were also identified as gestational THSDCs. Work is ongoing towards demonstrating that maternal thyroid function is associated with offspring neurocognitive outcomes. The first results of ongoing analyses indicate that altered maternal thyroid hormone, specifically T3, is associated with behavioral problems and a lower child IQ. These data could provide new insights into the role of T3, the active form of thyroid hormone, on fetal brain development.

Brain development and neural stem cell division

To understand how THSDCs interfere with brain development and neural stem cell decisions during brain development, we are developing innovative 3D *in vitro* test methods that capture key steps of vertebrate neural cell commitment. Two models are under development: human cerebral organoids and mouse neurospheres. We have progressed with characterising both models in terms of responses to activation of thyroid hormone receptors. The mouse neurosphere assay has been useful for demonstrating impairment of cell proliferation and we have analysed the effect of several chemicals. Our efforts to scale up the assays were successful, and we used machine learning technology to accelerate time-consuming measurements of neurosphere size.

High through-put screening (HTS) assays for screening interactions at non-receptor targets and development of Quantitative Structure-Activity Relationships (QSAR)

We are exploring the possibility that THSDCs can alter the concentrations of thyroid hormones in systemic circulation by acting on enzymes and transporters (non-receptor targets). At present there are no high-throughput assays to screen potential THSDCs at these non-receptor targets, a gap we will bridge by developing HTS and QSAR. To date we have screened large and comprehensive small molecule libraries of up to 70,000 compounds covering a broad spectrum of the chemical universe for potent inhibitors of DIO2, DIO3, DEHAL1 and MCT8, by using a non-radioactive semiautomatic HTS platform based on the Sandell-Kolthoff reaction.

Focus on thyroid hormone transport between mother and fetus

Based on an improved understanding of thyroid hormone transport between mother and foetus which is essential for brain development, we aim to identify new thyroid hormone transmembrane transporters (THTT) and to evaluate their suitability for HTS assays. The work focuses on three physiological barriers: the placenta for maternal to foetal TH transport and the blood-brain-barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB) for TH supply from the circulation to the developing foetal brain. We have identified further THTT capable of transporting iodothyronines and sulfated iodothyronines across the placenta, from mother to fetus, and vice versa. We have investigated the inhibition of THTT (OATP1C1 and OAT4) in cell lines stably overexpressing these transporters and screened >30 chemicals.

Capturing downstream effects on the developing brain

We are developing *in vivo* assays for downstream effects of THSDCs on brain development. We have provided a preliminary study protocol for the evaluation of neurodevelopmental endpoints of thyroid hormone action, based on results from *in vivo* studies using potent TPO inhibitors. We have followed up on this work by performing a developmental toxicity study with two chemicals that exert their thyroid hormone system-disrupting action through the induction of liver enzymes that affect thyroid hormones. Analyses of tissues from these studies are ongoing. We have also continued our search for new biomarkers by performing transcriptomic analyses of hippocampi from offspring exposed to TPO inhibitors during early development. This was identified as one major gap in our work on testing strategies. We have investigated neurodevelopmental endpoints in severely hypothyroid mice exposed to a high dose of the hormone synthesis inhibitor PTU. We conclude that periventricular heterotopia is not a useful toxicological marker of hypothyroidism in mice. This does not however diminish the potential utility of heterotopia assessment in rats.

High through-put screening by robotization of the Xenopus Eleutheroembryonic Thyroid Assay (XETA)

The XETA protocol was published as an OECD test guideline in June 2019. Work on this assay aims at further refinements and robotisations of this existing assay to reduce the number of animals used for chemical testing and to allow increasing the speed of testing. We have evaluated different technical solutions to automate the pipetting steps and the embryo sorting. To identify additional endpoints, transcriptomic experiments were performed and candidate genes to be used as biomarkers were identified. Gene inactivation experiments were performed on two of these genes. Chemical testing is currently ongoing for this assay.

Thyroid hormone system disruption in the context of human thyroid physiology, hormone cross-talk, fetal sex and iodine deficiency

Apart from its many physiological effects in forming the placenta, the "pregnancy hormone" human chorionic gonadotropin (hCG) stimulates the thyroid gland in early pregnancy to produce sufficient thyroid hormone to ensure healthy brain development. However, if certain EDCs affect the production of hCG, there might be detrimental impacts on TH production. We have completed analysis of human datasets from the SELMA study in which we investigated associations of EDCs (phenols, phthalates, PFAS and PCBs) with maternal hCG concentrations. These results show that these EDCs have the potential to decrease and disrupt hCG production and secretion by the placenta.

Validation of test methods and entry into the ECVAM modular process

The lack of validated test methods for the identification of THSDC is well recognised as a bottleneck for developing new testing strategies. We have now begun work on preparing the ground for submitting new *in vitro* test methods to the ECVAM modular process.

Developing a testing strategy for THSDCs based on Adverse Outcome Pathway (AOP) networks

We are developing a testing strategy for THSDCs based on *in vitro* testing methods for molecular initiating events (MIE) that, when activated, have a strong impact on perturbing the thyroid hormone system. In the course of this work, it has become clear that several key events (KE) on which multiple pathways converge are currently not covered by adequate test methods. We have also initiated work to fill these gaps by targeted gene expression studies.

We completed a systematic review of gestational exposures to test chemicals in which hormone profiles in both dams and offspring were recorded. The findings are relevant to the development of a testing strategy. We found that numerous chemicals lead to decreases of T4 and T3 serum levels without accompanying rises in TSH. This has important implications for the current EU approaches for identifying thyroid hormone system-disrupting chemicals which rely on altered TH serum levels as indicators of a hormonal mode of action and thyroid histopathological changes as indicators of adversity. The current regime will miss chemicals that produce TH alterations without accompanying TSH increases.

International regulation and harmonisation

The ATHENA project hopes to build strategies for thyroid disruptors for international regulation and harmonisation with non-EU trading blocs. Considerations include the differences in the regulatory management of substances deemed to be endocrine disruptors and preference for risk versus hazard assessments. We have conducted an in-depth analysis of international approaches to identifying and regulating THSDCs by way of detailed case studies of benthiavalicarb, mancozeb and triclosan. In the EU,

industrial chemicals are identified as Substances of Very High Concern (SVHC) when they show endocrine disrupting properties, and such substances are candidates for substitution. Based on risk considerations, substances are prioritised so that some can only be used in the EU market if they are authorized.

Interaction with the EURION cluster

ATHENA together with SCREENED had taken the role of coordinators of the EURION Cluster. Meetings for the Cluster project coordinators were held every 2 months and the annual Cluster meeting was prepared and held online in January 2022 with several hundred participants. There was also a meeting with stakeholders and a joint workshop with the JRC.

As a tangible result of the EURION cluster meeting, we identified specific topics of mutual interest to the ATHENA, ERGO and EnDpoiNTs project, including AOP development, thyroid hormone analytics and omics methods. A workshop addressing these topics was held in September 2022 which led to further cross-project collaborations.

Progress beyond the state of the art and expected potential impact (including the socio-economic impact and the wider societal implications of the action so far)

The ATHENA project aims to improve understanding of the mechanism of action of different THSDCs on the thyroid hormone system and insight into predicted outcomes. The test methods established in the ATHENA project will undergo the pre-validation process for incorporation into the OECD test guidelines, together with considerations for international harmonisation. We hope to cover gaps and provide a comprehensive testing strategy for THSDCs to identify chemicals that have disrupting properties, as a lack of identification means that exposure to harmful chemicals will not be eradicated.

Further information about ATHENA:
Website: https://athenaedctestmethods.net
Cordis: https://cordis.europa.eu/project/id/825161
X: @ATHENAprojectEU

1.2 EDCmet project summary

Metabolic effects of Endocrine Disrupting Chemicals: novel testing METhods and adverse outcome pathways – EDCMET

Summary of the context and overall objectives of the project

Thus far, endocrine disruptor (ED) research has mainly focused on reproductive endocrinology and related hormones, which is reflected in the regulatory test methods assessing only endocrine effects of xenobiotics. Accumulating evidence links ED exposure to increased incidence of metabolic syndrome and further, incidence of fatty liver and type 2 diabetes. EDs may exert their adverse metabolic effects via several mechanisms, such as modulation of nuclear receptors, impairment of mitochondrial respiration or excessive cellular accumulation of lipids. New and improved approaches are needed to increase the quality, efficiency, and effectiveness of existing methods to evaluate the effects of EDs and to meet the demanding and evolving regulatory requirements worldwide.

EDCMET focuses on developing novel or improved computational and *in vitro* methods, such as non-cellular assays and cell culture systems, as well as standardized *in vivo* animal models to assess the metabolic effects of EDs. Unbiased omics techniques are used to investigate tissue and plasma markers *in vivo*. Epidemiological and field monitoring data are used to gain information regarding the human exposure to EDs and related metabolic effects. EDCMET applies the adverse outcome pathway (AOP) paradigm to identify molecular initiating events (MIEs) and predict the emerging adverse biological phenotype. The interdisciplinary approach and complementary expertise of project participants aids in the identification of novel mechanisms of action and the development of novel or improved validated test methods for regulatory purposes.

Overview of progress and main results achieved so far

Interactions of EDs with their target proteins (nuclear receptors, NRs) as well as the potential mechanisms and molecular triggers behind structural changes and activation of NRs have been explored using computational approaches. An array of omics data has been produced from cellular, *in vivo*, and human cohort samples. *In vitro* optimized and pre-validated NR-coregulator interaction and NR activation assays, as well as data from over 30 known or suspected EDs, are available for several NRs. In addition, mitochondrial respiration assays and triglyceride accumulation assays in hepatic cells have been developed and used to screen EDs.

Studies on ED effects on vulnerable individuals (obesity, in utero) are ongoing. Additional compounds have been selected to be studied for further comparison of the *in vivo* and *in vitro* results, using the previously established protocols and SOPs *in vivo*, and the analyses are on-going. The previously developed predictive computational classification pipeline has been further expanded and refined to include ED exposure specific classifiers and the EDTox tool is available online. Further validation of the model by interrogation of the project omics data is underway. A systems toxicology tool to predict emergent metabolic phenotype from ED exposure, via activation of specific proteins and associated with different metabolic environments, is under development.

EDs and gene sets associated with ED molecular initiating events and metabolic endpoints, especially steatosis, have been identified. Omics data has also been used to characterize mechanisms of AOPs. The potential of the established genes, molecular initiating, and key events to be used as predictive biomarkers is under investigation. Levels of selected ED compounds have been analysed from cohort samples and

analyses on potential associations of exposure levels and systemic metabolism have been done and work towards ED risk assessment is underway.

Several scientific papers on the project findings have been published and a stakeholder workshop to discuss the project results has been planned towards the end of the project. The high-throughput, fluorescence-based triglyceride accumulation assays is under validation within the PEPPER initiative and will be available for evaluation of steatotic effects of EDs. Steps towards regulatory implementation of further assays are under consideration. Information on developed assays, ED test compounds, experimental protocols and cohorts have been shared and discussed in EURION meetings and working groups.

Progress beyond the state of the art and expected potential impact

EDCMET will provide an array of new or improved testing tools for risk assessment of metabolism disrupting EDs. These tools are expected to identify novel disrupted pathways in human liver and to increase human relevancy in testing and risk prediction and further reduce the use of laboratory animals. The developed methods have undergone preliminary experimental validation and are expected to contribute to the current OECD test systems. The methods and models will be coupled with human exposure data, linking the levels of EDs with metabolic endpoints and health outcomes. EDCMET will lay the groundwork for future testing and ED toxicity assessment and contribute to a better understanding of human exposure to chemicals and the associated burden of metabolic diseases.

Endocrine-related diseases represent a high cost for healthcare systems in the EU countries and metabolic effects of EDs are poorly understood. EDCMET will increase the knowledge on metabolic diseases by characterization of the affected critical pathways in liver using the AOP approach. EDCMET will also aid in the development of novel biomarkers for metabolic diseases and provide a relevant base for assessing adverse human health effects of environmental chemicals. The availability of fast and cheaper test systems can stimulate research around environmental chemicals and contribute to safer marketed products and thereby have a positive impact on the environment.

Further information about EDCMET:
Website: www.uef.fi/edcmet
Cordis: https://cordis.europa.eu/project/id/825762
X: @edcmet_eu

1.3 ENDpoiNTs project summary

Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity – ENDpoiNTs

Summary of the context and overall objectives of the project

ENDpoiNTs is developing a new testing strategy to meet the regulatory, scientific and societal needs for improved hazard and risk assessment of endocrine disrupting chemicals (EDCs).

A significant knowledge gap is how EDCs affect neurodevelopment, and endocrine disruption (ED)-induced developmental neurotoxicity (DNT) is hardly covered by the testing tools in regulatory use. The brain is among the most vulnerable organs with respect to toxic insults, particularly during development, and EDCs have indeed been shown to target the developing brain.

The main objective of ENDpoiNTs is to generate new scientific knowledge on how ED is linked to DNT at the molecular, cellular, tissue, and organism level. Based on this new knowledge, the project will:

- Develop predictive computational tools for chemical screening
- Develop and validate cellular testing and screening tools
- Develop novel molecular endpoints for existing animal-based test guidelines
- Ensure human relevance by linking experimental and epidemiological evidence
- Develop an integrated approach to identify EDCs inducing DNT
- Engage with key stakeholders and develop novel strategies for EDC testing and assessment into European and international chemical regulatory frameworks

Objectives of ENDpoiNTs

ENDpoiNTs is developing a new testing strategy for developmental neurotoxicity to meet the regulatory as well as scientific and societal needs for improved hazard and risk assessment of endocrine disrupting chemicals (EDCs). Globally, serious concerns have risen about the exposure to anthropogenic chemicals that can produce adverse health effects via disruption of the body's endocrine (hormone) system, known as EDCs. During a lifetime, people are exposed to numerous EDCs via food, water, air as well as various products and materials. From a scientific point of view, there is no doubt that exposure to EDCs can adversely affect the endocrine system but current chemical screening and testing tools need to be improved and harmonized to meet regulatory requirements worldwide. Certain endocrine-mediated pathways and health outcomes are less studied and hence there is a lack of basic knowledge on the events linking endocrine disruption and adverse outcomes. Yet, the new EDC criteria require information on both the adverse effects and the endocrine mode of action.

Developmental neurotoxicity (DNT) is one of the less studied outcomes in the context of endocrine disruption (ED). A significant knowledge gap is how EDCs affect neurodevelopment, and ED-induced DNT is thus hardly covered by the testing tools in regulatory use. The brain is among the most vulnerable organs with respect to toxic insults, in particular during development, and EDCs have indeed been shown to target the (developing) brain in animal models. Even in humans, several EDCs at low exposure levels have been associated with adverse effects on neurodevelopment in children. The adverse effects are manifested in changes in cognition, behaviour, and other brain functions, and even by an increased risk for neurodevelopmental disorders.

The main objective of ENDpoiNTs is to generate new scientific knowledge on correlative and causal links between ED and DNT at the molecular, cellular, tissue, and organism level. Based on this new knowledge, the project is developing an integrated battery of novel (*in silico*, *in vitro* and *in vivo*), complementary tools and methods for hazard identification, addressing endpoints relevant for human neurodevelopment (e.g. cognitive and behavioural outcomes) that are linked to a clearly defined and empirically validated endocrine mode of action. Furthermore, ENDpoiNTs is developing strategies to integrate these methods into European and international chemical regulatory frameworks, in particular of the Organisation for Economic Co-operation and Development (OECD), and support the goals defined in the EU and World Health Organisation (WHO) environment and health process. ENDpoiNTs is combining basic research needs, method development, and regulatory aspects, bringing together advanced expertise in the field of ED and DNT. The project operates at the interface between molecular and cellular toxicology, *in silico* modelling, integrative bioinformatics, and biostatistics modelling of epidemiological data to meet the demands and evolving requirements of regulators and industry.

Progress overview and main results achieved so far

A number of cellular and animal-based models exist to study DNT endpoints, addressing cellular key events and neurodevelopmental outcomes. However, the predictive value of these models to detect ED-induced DNT is largely unknown. In ENDpoiNTs, we are assessing the responsiveness of these DNT endpoints to endocrine interference. ED pathways potentially relevant for DNT cellular key events were selected using *in silico* modelling to predict targets of EDCs that are known to affect human neurodevelopment.

Using receptor agonists and antagonists, their link to cellular DNT key events was established in a number of *in vitro* models. In the previous reporting period, we had already shown that almost all key events investigated are dependent on one or several of the selected hormonal receptors. Thereby, novel roles for hitherto un- or not well studied hormonal receptors in the context of EDCs in neurodevelopmental key events were uncovered. On the other hand, the estrogen and androgen systems, that are beside thyroid hormone the most studied in the context of EDCs, were not prominently involved in DNT-related cellular key events. Some results were in accordance between human and rodent models while others diverged, underscoring species differences. Moreover, when comparing genetic sex in the cell models, some pathways were only affected in one but not the other sex. In this period, the screening efforts with the agonists and antagonists were finalised and, for the most sensitive and robust assays, applicability domains as well as positive and negative controls were defined, and standard operating procedures (SOPs) established. Additionally, brain organoids (3D induced pluripotent stem cell (iPSC)- based cortical brain cultures) have been established for ED-DNT testing by a thorough transcriptional characterisation of hormonal responses. Furthermore, derivation of multiplexed hiPSC lines from different donors was shown to be an efficient strategy to unravel the contribution of genetic variability in response to ED-induced DNT.The response of selected assays have been tested with EDCs that are associated with DNT outcomes in humans. Depending on the assays between 5 and 25 chemicals have already been tested. Results show that some of these EDCs indeed affect cellular endpoints in low doses of EDCs. One of the developed cellular assays is currently undergoing pre-validation including inter-laboratory transfer, and another one is under consideration for pre-validation as well. Additionally, a standard project submission form (SPSF) for six *in vitro* assays addressing ED-induced DNT to the OECD test guideline program is prepared. In summary the *in vitro* work and validation work conducted in WP2, WP3, and WP4 has led to successful achievement of one of the main objectives of ENDpoiNTs, to develop *in vitro* assays that address ED-induced DNT.

To link the molecular and cellular key events addressed in the *in vitro* models to adverse outcomes in whole organisms, several *in vivo* models are employed. The rat model entails a developmental exposure scheme to six model compounds (Bisphenol F (BPF), Permethrin (PMT), PFOS, Butylbenzyl phthalate (BBzP),

Triphenyl phosphate (TPP), and 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH)) and subsequent molecular as well as behavioural and cognitive measurements. All six studies were finalized, and behavioural analyses were completed. The results show sex-specific effects on learning and memory for BPF (males + females), BBzP (males), DINCH (males + females), and TPHP (females). Currently, the molecular analyses, including transcriptomics, epigenomics, metabolomics, lipidomics, and steroidomics are in progress. First results show that a number of gene networks, steroids, neurotransmitters and lipids are dysregulated by the six EDCs, and compound and sex-specific effects were observed. These molecular effects are and will be correlated to the behavioural outcomes on one hand, and transcriptional data sets from *in vitro* models, on the other hand, to provide a link to between the cellular and the organismal outcomes. In *Xenopus laevis*, a behavioral test based on remyelination has been implemented and shown to be affected by PFOS but not PFOA. Furthermore, a systematic correlation between early transcriptional dysregulation and adult adverse outcomes in this model organism is ongoing using model EDCs as well as hormone agonists and antagonists to show that DNT effects due to endocrine modes of actions. The transcriptomic signatures will also be used for comparison to the rat and the *in vitro* models. Similar analyses with more model EDCs are conducted in zebrafish embryos. This WP3 work will contribute to the objective to early molecular endpoints that can be used as early markers for ED-induced DNT in animal-based tests.

For the management and handling of the experimental data, a standardised database platform had been developed in the last reporting period, which has been complemented with a statistical pipeline to analyse all the *in vitro* data. It includes data capture, curation procedures and data treatment and statistical analysis approaches relevant for ENDpoiNTs. All current ENDpoiNTs data is contained in the database (currently 2.316.378 data records) and will be complemented with the still to be generated data. When the project is finalised, this data will be made available as an invaluable resource for the community. Furthermore, 12 first tier screening high confidence Quantitative Structure Activity Relationships (QSARs) were developed and published for predicting the agonistic and antagonistic modes of action of the selected receptors. This is the basis for *in silico* models identifying chemicals that can induce DNT via these interactions. To extrapolate *in vitro* to *in vivo* concentrations of EDCs, a PBTK approach has been developed, predicting fetal brain concentrations. Furthermore, Data produced in ENDpoiNTs is integrated into existing Adverse Outcome Pathways (AOPs) and AOP networks are built. Currently, an AOP network connecting interference with retinoic acid receptors with impaired learning and memory is finalised using project results and data from the public domain, and submission to AOP wiki is planned. Other AOP networks for glucocorticoid receptor and LXR-interference are underway. To establish human relevance of the test methods, doses producing an adverse effect in test systems are currently being compared with human exposure data from the ENDpoiNTs cohorts and other international data sets for both single reference EDCs and for their mixture. For comparison, the metrics have been evaluated using established regulatory values translated to biomonitoring equivalent concentrations. Furthermore, a mixture study of EDCs is being finalised, with the aim to compare effects of a real-life mixture, established based on human data, to single compounds in the developed *in vitro* models. To further align experimental with human evidence, molecular data on the level of metabolomics and epigenomics will be compared. To this end, analyses in the epidemiological data from the LINC and the SELMA studies have been conducted to provide links between molecular patterns and both prenatal exposures and DNT outcomes (cognition and other behaviour). In summary, these efforts in WP5 and WP6 have been and will be instrumental to integrate the experimental data and prove human relevance for the established ED-induced DNT endpoints.

To ensure the uptake of the developed assays and strategies into the regulatory context, ENDpoiNTs is actively engaging with key stakeholders. Within the EURION cluster, another stakeholder workshop was organised, discussing the views, needs and expectations related to EDC test method development and international strategies and guidelines. Furthermore, communication channels with relevant working groups of the OECD have been strengthened to enable continuous discussion of the readiness of the

developed assays and endpoints for validation and regulatory implementation. This is of particular relevance in the context of the planned SPSF. Finally, to disseminate the project's results to the scientific community, key stakeholders, and the public at large, ENDpoiNTs has produced project flyers and two animated short films about the aims and first results of the project shown at YouTube (ENDpoiNTs project - YouTube). It is actively maintaining its webpage (https://endpoints.eu/) and X account (@ENDpoiNTs_EU), as well as continuously publishing its results in open access scientific journals. Together, this work, conducted in WP7 and WP8, will contribute that the novel methods developed in ENDpoiNTs will be acknowledged and taken up by the relevant communities.

Progress beyond the state of the art and expected potential impact

ENDpoiNTs will advance the state of the art in several ways. **Scientifically**, it has already contributed with significant new knowledge on hormonal involvement in neurodevelopmental key events, which is the basis for understanding ED-induced DNT and targeted methods for this kind of toxicity. Scientific dissemination at conferences with academic, industry and regulatory audiences have contributed to convey these important novel insights that shift the focus of the EDC field from the estrogen-, androgen, and thyroid hormone signalling pathways to other, less studied ones. In the coming months, these insights will be enriched with molecular understanding of the established links by extensive OMICs analysis and integration, both on the cellular and whole organism level. ENDpoiNTs is also contributing with tools **advancing chemical testing beyond the state of the art**. As of now, 12 QSARS have been established to interrogate modes of actions identified as relevant for ED-induced DNT. Furthermore, 6 well-characterised novel assays for ED-induced DNT have been established, and several more are in the pipeline. The OMICs analyses will reveal novel early molecular endpoints that can be used in whole organism models to predict later adversity, as well as human biomarkers of exposure and adverse outcomes. Other tools are PBTK models to predict fetal brain concentrations from maternal serum concentrations and for *in vitro-in vivo* extrapolations, and the ENDpoiNTs database including statistical analyses tools that will be an invaluable tool to make all the produced data FAIR. In summary, ENDpoiNTs will develop endpoints, tools, and methods to test for ED-induced DNT that are based on mechanistic understanding rather than descriptive observations, and to marshal evidence across different levels of complexity to develop testing strategies that derive their solidity and public health relevance through the integration of those levels. Notably, the impact of ENDpoiNTs goes beyond the EDC field, since some of the *in silico*, *in vitro* and *in vivo* assays, methods and procedures developed in the project can be applied to any chemical or chemical mixture. Ultimately, this will have an **impact on policy making and regulations** and enable better protection of vulnerable populations and particularly, the developing brain.

Further information about ENDpoiNTs:
Website: https://endpoints.eu/
Cordis: https://cordis.europa.eu/project/id/825759
X: @ENDpoiNTs_EU
YouTube: https://www.youtube.com/channel/UC-7hPA8eVthZ4ZgICeDj0nw

1.4 ERGO project summary

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

Summary of the context and overall objectives of the project

ERGO project will break down the wall between mammalian and non-mammalian vertebrate regulatory testing of endocrine disrupting chemicals (EDCs) by identifying, developing and aligning thyroid-related biomarkers and endpoints (B/E) for linkage of effects between different vertebrate classes. To achieve this, an adverse outcome pathway (AOP) network covering various modes of thyroid hormone system disruption (THSD) in multiple vertebrate classes will be developed. An AOP starts from a molecular initiating event (MIE) and outlines the sequence of key events (KE) leading to a relevant adverse outcome at the organism or population level. The AOP network will provide the scientifically plausible and evidence-based foundation for the selection of B/E and assays in lower vertebrates predictive of human health outcomes. These assays will be prioritized for validation in ERGO.

ERGO will re-think ED testing strategies from *in silico* methods to *in vivo* testing and develop, optimize and validate existing *in vivo* OECD guidelines and (if required) new *in vitro* protocols with novel TD endpoints and consequently reduce requirements for vertebrate animal testing by preventing duplication of testing in mammals and non-mammalian vertebrates. The ERGO Integrated Approaches to Testing and Assessment (IATA) strategy will increase the screening capacity to enable more chemicals to be tested for ED properties.

To achieve its goals of providing stakeholders and businesses with better tools and strategies needed to ensure improved management of EDCs, ERGO has four overarching objectives:

- Investigate, develop and validate thyroid hormon system disruption (THSD) sensitive B/E predicting effects across vertebrate classes for inclusion in new *in vitro* and existing *in vivo* OECD test guidelines (TGs) for improved identification and safer assessment of thyroid disrupting chemicals.
- Develop an AOP network across vertebrate classes for identification of THSD B/E applicable for assessment of cross-class thyroid disrupting KE.
- Transform new data, tools and understanding into a harmonized IATA testing strategy for regulation of EDCs by inclusion of stakeholders at the global level in the incorporation of the cross-vertebrate class testing approach.
- Publish a guidance document on extrapolation of TD effects across mammalian, fish and amphibian OECD TGs.

Overview of progress and main results achieved so far

In collaboration with two other projects in the EURION cluster; the ATHENA and the ENDpoiNTs projects, ERGO has held a joint workshop on 15-16 September 2022 in Madrid to increase synergies between the projects and consolidate common interests. The focus was on presentation of topical results in an interactive manner through poster sessions and discussions. Small groups discussed a number of topics and brought thoughts and conclusions to a main session. Topics discussed were (among others), the use of OMICs data, AOPs and PBTK modelling, assay validation criteria as well as specific endpoints in common like hormone measurement methodologies and sensitivity. The workshop was a great place to exchange experiences and get inspired by new ideas and approaches and several follow up activities are ongoing (e.g., a thyroid hormone analysis validation).

ERGO members are actively involved in I) development of a guidance for implementing the newly introduced ED hazard classes under the CLP regulation and the REACH review addressing ED specific standard data requirements and how to implement them in a tiered and efficient way. II) the OECD Thyroid Disruption Methods Expert Group and III) the PEPPER project led by France to enhance selection and pre-validation of ED relevant test systems. These crosslinks ensure that the ERGO results are directly fed into regulatory relevant processes and at the same time the needs and discussions from the ongoing activities are mirrored back to the ERGO consortium.

ERGO partners have submitted a report on the entry of an AOP network to the AOP-Wiki for OECD review. It presents the status of AOPs describing THSD with specific focus on the progress relative to development, availability in the AOP-Wiki and endorsement by the Working Party on Hazard Assessment - Working Group of the National Coordinators for the Test Guidelines Programme (WPHA-WNT)

A number of AOP related activities and goals has been achieved by ERGO partners:

- Swim bladder AOP network has been endorsed and published by the OECD.
- AOP linking TPO inhibition to impaired visual function in zebrafish has been published as AOP report in "Environmental Toxicology & Chemistry" in September 2022 and is currently under review at OECD ESCA.
- The impact of THSD on the posterior swim bladder chamber of zebrafish occurs during the development of the swim bladder and not during the inflation process. Hence, the effect on posterior swim bladder inflation occurs before the onset of the endogenous TH synthesis and is therefore less likely caused by mechanisms targeting TH synthesis.
- The taxonomic domain of applicability (tDOA) assessment in the THSD AOP network showed a high potential for applicability of (parts of) AOPs across vertebrate taxa. This publication serves as a catalogue that summarizes plausible and empirical evidence for future cross-species AOP development and usage.
- OECD projects 1.35 on the OECD AOP development work plan (led by ERGO-partners from BE, DE and DK in collaboration with the US-EPA) and 2.64 on the OECD test guidelines work plan (led by ERGO partners from DK, BE, DE and NL) were aligned and integrated to support addition of AOP-based endpoints to fish test guidelines.

During the last year, a large number of *in vitro* assays, embryo assays (zebrafish/Japanese medaka) and *in vivo* tests with fish (zebrafish/Japanese medaka) and amphibians (Xenopus laevis) has been performed to identify/pre-validate new assays and biomarkers for THSD and to investigate the applicability across species of the effect markers. Samples from many of the experiments are still under evaluation for effects of model THSD chemicals:

- Several *in vitro* assays are now characterized for their pre-validation potential, SOPs are being standardized and optimized. The assay with the best performance, reproducibility, and complementarity with international validation activities (OECD/JRC) soon goes into the second pre-validation phase.
- Possible fish THSD sensitive endpoints for validation were submitted to OECD for the second commenting by the OECD EDTA AG in May 2022. Based on research and pre-validation in ERGO, a detailed plan is being prepared and developed with OECD VMG-Eco on an upcoming validation of 4 selected THSD endpoints in OECD TG 236 and OECD TG 210 (as a part of OECD TG work program project 2.64 under VMG-Eco and project 1.35 on AOP development under ESCA). The endpoints selected for the validation are swim bladder inflation (posterior and anterior), eye development, thyroid hormone measurements and thyroid morphology/histopathology. An official validation invitation was prepared by ERGO partners and distributed by OECD during summer 2023.

Standardization of SOPs for fish tests is continuing in ERGO and will be finalized mid-September 2023 to be ready for use in the OECD validation starting late 2023. Several experienced laboratories (outside ERGO) have signed up for validation participation.

During this reporting period, there were a total of 27 ERGO scientific publications, bringing the total number of publications from the project to 42. Several partners have attended high profile conferences, workshops and events representing the ERGO project and promoting its results, in this period a total of 128 dissemination and communication activities have been recorded by ERGO partners, with an estimated reach of 44,124 stakeholders.

Progress beyond the state of the art and expected potential impact

ERGO is a coordinated attempt to contribute to filling the gaps in the field of TD. It will allow to identify both disturbance of the thyroid hormone axis and its potential adverse effects in different vertebrate classes. ERGO has already improved several methodologies for using cell *in vitro* tests and fish and amphibian assays for early screening of substances and is far in the process of developing new *in silico* models for predicting internal dose of TDCs to design physiologically based toxicokinetic modelling (PBTK) models and to link MIEs with AOs within an AOP network.

ERGO experimental and analytical work increases basic knowledge on the detailed role of THSD disturbances. The ERGO approach would be of significant interest for the safety assessment of existing chemicals lacking endocrine and developmental toxicity data and new chemicals at an early stage of their industrial development. This approach allows:

- To simultaneously screen chemicals for their potential human THSD effects, as well as their environmental impact with similar negative effects on fish and amphibians.
- Significantly reduce the requirement of vertebrate animal testing, with respect to animal welfare.
- A relevant assessment at the *in vitro* scale opens the door to automation and higher throughput screening of chemicals, which would further reduce the cost of their assessment.

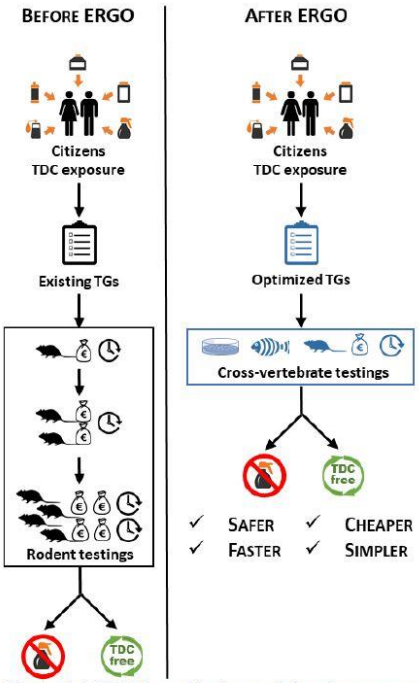

Figure 1.1 ERGO investigates and develops a cross-vertebrate class AOP/testing approach that will deliver optimized and novel ED TGs

Further information about ERGO:
Website: ergo-project.eu
Cordis: www.cordis.europa.eu/project/id/825753
X: @ERGO_EU

1.5 FREIA project summary

FREIA: Female Reproductive toxicity of Endocrine disrupting chemicals (EDCs): a human evidence-based screening and Identification Approach

Summary of the context and overall objectives of the project

The FREIA project aims to provide better test methods to identify human-made chemicals that disturb hormones and their actions on development and function of the reproductive system in women. Currently available test methods are not fit for purpose, which is partly the reason why the effects of such endocrine disrupting chemicals (EDCs) on female reproductive health are often overlooked in regulatory chemical safety assessments. This means that women's reproductive health is at risk globally. Our EU-funded project FREIA will increase our understanding of how EDCs can harm female reproductive health. We will use this information to provide better test methods that enable fit-for-purpose chemical regulation. We will also share our knowledge widely to improve the reproductive health of women globally.

Main results of the work performed between January 2019 and June 2023

In the FREIA project, we first looked for biological characteristics (biomarkers) for female reproductive toxicity using two well-understood EDCs, diethylstilbestrol (DES, a potent estrogen receptor activator) and ketoconazole (KTZ, a blocker of steroid hormone production). Studies with cell cultures of fetal and adult human ovaries, bovine oocytes and immortalized ovarian cells show changes in several genes and proteins that may be good indicators of exposure to these EDCs. Some promising candidates are involved in cholesterol biosynthesis, which is needed to produce steroid hormones. We are currently testing with another potential EDC, propylparaben, in our cell models and animal models to verify the predictive value of our candidate biomarkers. We previously showed that endpoints that are currently being assessed in rat studies in regulatory testing are not sufficiently sensitive to detect an endocrine disrupting effect. Our studies did reveal some findings that may improve existing test guidelines:

- Our data show that mammary glands shortly after birth (postnatal day 6) and in young adult (postnatal day 22) rats exposed in the womb to DES and KTZ were different compared to mammary glands of unexposed pups. Together with the platform for international standard-setting Organisation for Economic Co-operation and Development (OECD), we are assessing if inclusion of mammary gland analysis in existing test guidelines is valid and feasible.
- In test guidelines, effects on rat ovaries are evaluated by traditional histological assessment. We explored the possibility to use a relatively quick screening process called surface photo counting (SPC). Our data show that the SPC method has good predictive value in the assessment of ovulations and is simpler, faster, and more cost-effective than traditional histological assessment. SPC might open new possibilities for a fast and operator-friendly assessment of effects on ovaries that can help to prioritize exposure groups for more thorough histological evaluation (Li et al., 2023).
- We evaluated whether circulating steroid hormones may be an indicator of EDC effects. Clear age dependent changes in hormone levels were observed in plasma of rats. However, exposure to KTZ and DES in the womb did not result in changes in circulating steroid hormone levels in female rats after birth. These data do not support inclusion of circulating sex steroid hormones in test guidelines. This does not mean that effects of chemicals on steroid hormone formation are irrelevant. In contrast, many of the effects of EDCs observed in our cell models revealed changes in steroid hormone formation, indicating an effect on reproductive cells directly. Our data do not indicate that an ovarian specific cell model would improve EDC identification, but the existing steroidogenesis assay H295R may be improved by measuring more steroid hormones. Under supervision of the OECD, we are performing a study with other labs to investigate this. ii Ultimately,

we aim to integrate our newly identified biomarkers and sensitive endpoints with existing test systems from OECD to improve on future test methods and a strategy to determine the effect of an EDC on female reproductive development and health. To provide further evidence on the effects of EDCs on female fertility, we explored this association in women attending fertility clinics in Sweden and Estonia. Levels of 59 known and suspected EDCs were analysed in follicular fluid, the biological fluid surrounding oocytes, of 185 Swedish women and 148 Estonian women undergoing fertility treatment. Multiple chemicals were detected in all follicular fluids. In >90% of the follicular fluids, 3 metabolites of the phthalate DEHP, methylparaben, and 6 PFAS (PFOS, PFOA, PFHxS, PFUnDA, PFNA and PFDA) were detected and used to link with female fertility parameters. The ovaries of women with higher levels of DEHP, methylparaben and possibly PFUnDA and PFOA responded less to fertility treatment, established by calculating the ovarian sensitivity index (OSI). There were indications that some PFAS lowered the success of fertility treatment, determined by chance of establishing a pregnancy or live birth. Overall, this study provides additional evidence that DEHP can negatively influence female fertility. In addition, several other chemicals, i.e. methylparaben and some PFAS, were identified that may harm ovarian function and contribute to female infertility (Bellavia et al, 2022). We also studied how these women were exposed to these chemicals. The Swedish women from this study answered a questionnaire that contained information on home-environment, occupation, lifestyle and diet. We found that frequent use of perfumes was associated with higher phthalate (MEP) levels. Henn's egg consumption led to higher PFAS exposure. PFAS levels were also associated with certain fish consumption. We did not observe any correlation between the semipersistent chemicals and use of plastics in microwave heating of food or flooring material (Hallberg et al., 2023). We are currently collecting available scientific data on how humans, and women in particular, can be exposed to EDCs and what actions effectively can be taken to avoid exposure. On the FREIA website (www.freiaproject.eu), general background information on EDCs and female reproductive health can be found as well as project specific information, including webinar recordings, peer-reviewed scientific publications and databases, the FREIA factsheet and infographics. FREIA is one of the eight projects on test method development for EDC identification within the EURION cluster (www.eurion-cluster.eu).

Progress beyond the state of the art and potential impacts

FREIA uniquely provides the opportunity to investigate hormonal processes in human ovaries from fetal to adult age in order to improve scientific knowledge on the causes of female reproductive toxicity. Our committed collaborators for policy, advocacy and communicating actions to promote women's health and a healthy society allow FREIA to have a huge societal impact. The FREIA approach will strongly support the work of European regulatory agencies, or even globally through the EURION cluster activities. The tools we are developing perfectly fit the needs of modern-day toxicity testing with a clear regulatory application in mind. Together, the FREIA outcomes will support testing, identification and assessment of EDCs that are toxic for female reproduction.

Further information about FREIA:
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1.6 GOLIATH project summary

Beating Goliath: Generation Of NoveL, Integrated and Internationally Harmonised Approaches for Testing Metabolism Disrupting Compounds

Summary of the context and overall objectives of the project

The incidence of metabolic disorders such as obesity, diabetes and metabolic associated fatty liver disease has reached 'Goliathan' proportions. The worldwide increase in metabolic disorders cannot be explained by lifestyle and genetic factors alone; the role of environmental factors in these disorders has been increasingly acknowledged. Exposure to endocrine disrupting chemicals (EDCs) that disrupt metabolism – chemicals collectively referred to as 'metabolism disrupting chemicals' (MDCs) – is an environmental risk factor that urgently requires more attention. MDCs are natural and anthropogenic chemicals that have the ability to promote metabolic changes that can ultimately contribute to the development of obesity, diabetes and/or fatty liver in humans. Considering the important role these metabolic alterations can play in the global epidemics of metabolic disorders, it is essential that international chemicals regulations require the identification of MDCs and the assessment of the risk associated with exposure.

Within European chemicals regulations, criteria to identify EDCs have been proposed that require information on a chemical's endocrine mode of action (MoA) and related adverse effects relevant for human health. However, currently no regulatory *in vivo* or *in vitro* tests exist to identify the potential metabolism disrupting effects of chemicals. The need for these tests has been internationally recognized, as without them, comprehensive hazard and risk assessment of chemicals for potential metabolism disrupting activity is virtually impossible.

The overall aim of the GOLIATH project (www.beatinggoliath.eu) is to improve hazard and risk assessment of EDCs by generating novel, optimised, integrated and internationally harmonised approaches for testing metabolic disruption. The GOLIATH project spans the entire spectrum of testing, from *in silico* predictive modelling and high-throughput screening, to the development of robust ready-to-use *in vitro* assays and optimisation of current *in vivo* testing guidelines. By incorporating novel omics technologies to translate *in vitro* and *in vivo* assay outcomes into human health effects, GOLIATH will generate new testing strategies for MDCs, and provide novel insights in the mechanisms by which MDCs disrupt metabolic pathways and induce adverse effects on human health. With a consortium comprised of world-leading experts in endocrinology, physiology, molecular biology, endocrine disruption, toxicology, epidemiology, bioinformatics, test method development, validation and chemical regulation, GOLIATH will be pivotal in the development of an internationally harmonised strategy for testing MDCs.

The overall objectives of the project are:

- To improve the understanding of the endocrine modes of action of MDCs.
- To develop assay candidates for metabolic disrupting chemicals based on confirmed MoA and key biological effects in target tissues.
- To select and develop assay candidates into (pre-)validated test methods, in collaboration with OECD, ensuring test method definition, transferability, inter-laboratory testing and assessment of predictivity, which are prerequisites for their regulatory use.
- To develop an internationally harmonised, integrated approach to testing and assessment (IATA) of MDCs, using an Adverse Outcome Pathway (AOP) conceptual framework.

Work performed from the beginning of the project to the end of the period covered by the report and main results achieved so far

Overview of the main tasks and results of the first 56 months:

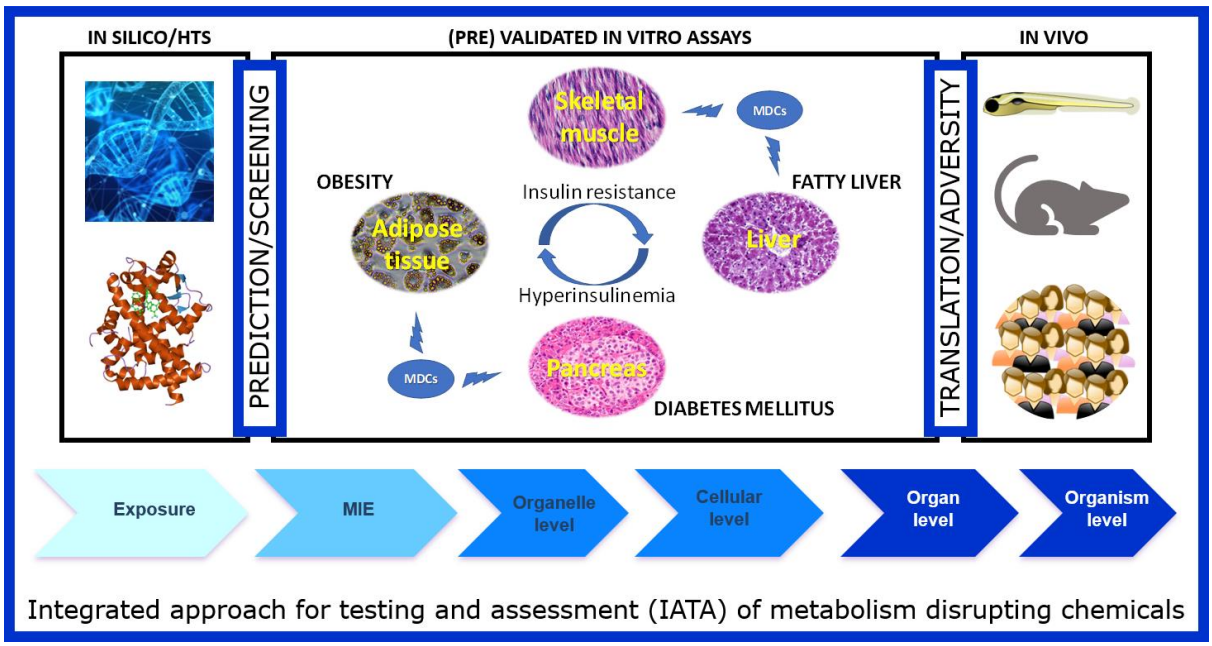
1. Review paper on MDCs: A review on current state of science and overview of human exposure to MDCs was published (doi: 10.3390/ijms21103480), to be further elaborated as a detailed review paper with OECD.
2. Chemical selection: Model test chemicals for assays were chosen, as well as chemicals for pre-validation studies in GOLIATH (doi:10.14573/altex.2013.3.331).
3. Advanced in silico screening: EDMON platform is enhanced (http://atome.cbs.cnrs.fr/ATOME_V3/SERVER/EDMon_v3.html), in silico models developed for MDC interaction prediction (doi:10.1021/acs.chemrestox.2c00267).
4. PPAR gamma activity characterization: Importance of species-specific models for endocrine disruption study shown (doi: 10.1021/acs.est.1c04318).
5. Aryl Hydrocarbon characterization: AhR ligand binding domain elucidated (doi: 10.1038/s41467-022-34773-w).
6. Advanced in vitro assay development: Progress on SOPs, test definitions of hepatocyte assays (steatosis, insulin resistance, CYP induction), pancreatic assays (10.3390/ijms24010231, 10.3390/ijms23095040), adipocyte differentiation.
7. (Pre)validation of in vitro assays: PPAR gamma and alpha, CYP induction, white adipogenesis assays (pre)validated, manuscripts in preparation.
8. Metabolic profiling: Developing DEXOM algorithm for cell-specific metabolic network characterization (10.1021/acs.est.1c04318), FORUM for chemical-health data mining (10.1093/bioinformatics/btab627).
9. Proteomics progress: Implementing proteome-based thermal shift assay for MDCs in zebrafish (doi: 10.1016/j.jprot.2021.104382), hepatocytes, adipocytes.
10. In vivo relevance: Optimizing transgenic zebrafish model for metabolism disruption post early life MDC exposure.
11. Human relevance: Epidemiological analysis of anthropogenic measures and markers in cohorts relative to prenatal MDC exposure found five overlapping genes (10.3390/ijms24087607).
12. Multi-omics progress: Standardized protocols used for gene expression, metabolomics and lipidomics in various samples.
13. IATA development: Mechanistic networks developed for GOLIATH endpoints, enhancing IATAs for MDCs.
14. Weight of evidence assessment for MDCs: Expert elicitation finalized for defining chemicals as endocrine disruptors.
15. Augmentation of test guidelines: Progress made to include metabolic disruption measures in animal study guidelines.
16. Collaborating with EURION partners (https://eurion-cluster.eu/) within working groups.
17. International outreach within EURION through stakeholder meetings, Advisory Board, GOLIATH presentations at events.

Progress beyond the state of the art, expected results until the end of the project and potential impacts (including the socio-economic impact and the wider societal implications of the project so far)

In order to progress beyond the state of the art, the expected results of the GOLIATH project are to:

- Generate a set of robust, well-characterised and ready-to-use *in vitro* test methods for the effects of MDCs on key target cells in metabolic disorders that will be (pre-)validated and further implemented beyond the duration of the project

- Realize an integrated approach for testing and assessment of MDCs, that will exceed the duration of GOLIATH, by bringing together new and existing test methods, in a framework that will be internationally harmonised and relevant for regulatory purposes
- Describe systematically in an AOP framework the mechanisms by which MDCs disrupt metabolism and contribute to metabolic disorders in humans, thereby providing information on the endocrine MoA which is essential for defining endocrine disruption criteria
- Generate a significant knowledge base on the exposure to and effects of MDCs relevant to the European population, providing a substantial contribution to the weight of evidence for the role of chemicals in metabolic disorders



Integrated approach for testing and assessment (IATA) of metabolism disrupting chemicals

Further information about GOLIATH:
Website: https://beatinggoliath.eu
Cordis: https://cordis.europa.eu/project/id/825489
X: @beating_goliath

1.7 OBERON project summary

An integrative strategy of testing systems for identification of EDs related to metabolic Disorders - OBERON

Summary of the context and overall objectives of the project

The main objective of the OBERON project is to develop a new battery of tests to detect endocrine disruptors having an impact on metabolic disorders, without the use of animal experimentation. Based on the concept of integrated approach for testing and assessment (IATA), OBERON will combine 1) experimental methods, (2) high throughput omics technologies, 3) epidemiology and human biomonitoring and 4) advanced computational models on functional endpoints related to metabolism.

- WP1 aims at integrating epidemiology and human biomonitoring (HBM) studies with the ED test systems for metabolic disorders, in order to increase the relevance of the ED test system. Various time windows will be considered, including developmental periods of high susceptibility (pregnancy-childhood-adolescence) and adulthood. Sex specificity will be studied. This WP draws on large-scale, population-based study populations including ongoing human biomonitoring (HBM) studies and some of Europe's most informative prospective birth cohorts.
- WP2's goal is to develop whole organism test systems to identify EDCs implicated in metabolic disorders. The main objectives are: 1) to perform the pre-validation of the zebrafish obesogenic test (ZOT) to screen EDCs and mixtures acting as obesogens, 2) to set up and pre-validate zebrafish-based bioassays to screen EDCs involved in NAFLD progression. The endpoints used for these whole-organism alternative testing assays are adiposity, liver steatosis and markers of steatohepatitis.
- The global aim of WP3 is to improve/develop innovative in vitro cellular models which best represents the three main organs and tissues (liver, adipose tissue and pancreas) involved in the etiology of metabolic disorders. These models will serve as a tool for the identification of endocrine disruptors which may increase the risk of developing metabolic diseases like obesity, diabetes or hepatic steatosis. Identification will be based on toxicity mechanisms, omics data and metabolic networks.
- The main objective of WP4 is to develop or adapt existing in silico methods for endocrine disruptor compounds. WP4 will develop QSAR and PK/PBPK models in support of the evaluation and integration of the data streams of the project.
- WP5 aims at integrating information from WP2 (*in vivo* models), WP3 (in vitro models) and WP4 (in silico models) in order to interpret the WP1 data. Overall objectives of WP5 include the (a) data infrastructure development, (b) development and application of a bioinformatics workflow of cross-omics and the respective biomarkers towards ED exposure/disease pathways, (c) development of new AOPs relevant EDs outcomes, and (e) development of an integrative testing strategy.
- The WP6 will ensure the scientific project management, and that the project reaches the defined objectives. Specifically, WP6 sets up an effective management framework for the consortium, acts as the interface between the consortium and the European Commission, performs strategic decision making.
- WP7 organizes a well targeted dissemination effort to ensure a maximum impact of the project and release the full exploitation potential of the results.

Overview of progress and main results achieved so far

During the first period, we started establishing and analysing several experimental tests across the different EU laboratories. During this period, expected deliverables including ethics, and publications were achieved. In the second period, biomonitoring studies and birth cohorts of exposure levels of EDS in Europe were finished and respective deliverable were submitted. Articles were published. Model were studied and used to measure the effects of OBERON ED compounds. The VEGA platform set up by partners at IRFMN was updated: https://www.vegahub.eu/. The OBERON Integrated Data Management (IDM) system has been developed as well and is currently being implemented in support of collection and provision of access to all datasets collected/developed in OBERON. Metabolomics and transcriptomics have been launched. The COVID-19 crisis slowed down the whole project, however some experimental results have been produced. An AOP working group has been created in order to conjugate the WP efforts and start designing AOPs in accordance with the early results.

In the third period:

- WP1: Metabolic health effects of ED exposure in humans: Follow-up and analysis of cohorts were completed. Multi-omics network analysis has been useful to identify several biologically relevant molecular signatures related to non-persistent ED exposure in childhood. Studies in adults have also observed positive associations between PFAS and hepatic enzymes.
- WP2: Disease models using zebrafish: Cell lines stably transfected with zebrafish PPAR evaluated agonist or antagonist activity toward zfPPARγ. A detailed optimized zebrafish obesogenic test (ZOT) protocol was delivered. It has been used to perform the screening of OBERON selected substances in order to determine their obesogenic potential. The optimization of a bioassay to evaluate the potential of selected EDCs to induce liver steatosis (StAZ) has been performed. The screening of selected EDCs for their potential to induce steatosis has been performed. The chemical analyses of selected compounds during time-course studies of ZOT and StAZ were delivered. In addition, metabolomics data were obtained from samples derived from ZOT as well as transcriptomic analyses of eleutheroembryos exposed to positive StAZ controls in order to increase toxicological knowledge.
- WP3: Along the project up to now we have successfully improved and established optimal culture conditions for different liver, adipose and pancreas cellular models. Based on the results of this extensive functional assay, relevant metabolic endpoints were identified and the best cellular model for each organ was selected to be further analysed using high throughput omics technologies. Omics analysis is in progress. In addition, we continue working on the development of validated in vitro test systems to be used for regulatory aspects regarding ED assessment in relation to metabolic disorders.
- WP4: In silico models will feed databases with new and important parameters and make these tools more precise and relevant. QSAR models for endocrine disruption (ED) activity and metabolism disruption were reviewed, optimized and will be freely available to the public in the next VEGA release. New models for thyroid peroxidase, steroidogenesis and glucocorticoid receptor perturbation were developed. A PBPK model for bisphenols in the Zebrafish eleuthero embryo was finalized and showed good predictive capabilities. The human PBPK model has been parameterized for all the compounds and was applied to translate exposure doses into doses in tissues. In addition, biokinetic interactions of co-exposure to 4 phthalates has been elaborated and many metabolic networks have been added to the PBPK model.
- WP5: Bioinformatics infrastructure was established and analysis of the omics data were delivered. AOP-helpFinder, was updated and added to the AOP-Wiki database. Decision trees based on AI methods were developed for IATA methodology development. ReadEDTest was developed to assess the readiness of in vitro test methods: https://readedtest.u-paris-sciences.fr/.

Progress beyond the state of the art and expected potential impact

Research conducted in OBERON uses longitudinal epidemiological studies with data on body composition, blood pressure, lipid profiles etc. in addition to applying novel statistical methods. All this to reflect a real-life scenario of effects of EDCs. New and convenient screening test methods for EDCs will be developed for regulatory use, *in vivo* and in vitro. In silico models feed into the previously mentioned experiments and help guide them contributing to the 3R. A comprehensive and usable IATA will be developed resulting in more efficient public health protection and reducing test chemicals cost as well as socioeconomic impact of ED exposure.

Further information about OBERON:
Website: https://oberon-4eu.com/
Cordis: https://cordis.europa.eu/project/id/825712/fr
X: @OBERON_4EU

1.8 SCREENED project summary

A multistage model of thyroid gland function for screening endocrine-disrupting chemicals in a biologically sex-specific manner - SCREENED

Summary of the context and overall objectives of the project

There is growing evidence that endocrine disrupting chemicals (EDCs) interfere with thyroid functioning and cause changes in thyroid hormone concentrations, their peripheral metabolism and the signalling of their receptors. Yet, the mechanism by which EDCs act on the thyroid axis is still far from being elucidated, in part due to the limitations of existing tests.

SCREENED aims to develop 3D in vitro tests, supported by modelling, to characterise the effects of EDCs on thyroid function. SCREENED will deliver innovative "organ-on-a-chip" models, where thyrocyte cells organised in a 3D structure will be hosted in a microfluidic cell culture device (a microfluidic bioreactor). This device will mimic the microenvironment of the thyroid gland, by simulating tissue- and organ-level physiology. The first 3D models consist of thyroid organoids able to recapitulate the thyroid hormone production functionality of the native thyroid (mouse and human models). We work also on ECM-scaffold-based and bioprinted 3D models, which represent an even more complex version of the 3D thyroid models, where the organ-on-a-chip will also be supported by a vascularized network.

Our solutions have the potential to overcome the limitations of existing in vitro tests by being more sensitive at low doses of exposure and supporting the prediction of toxicity on human health in a sex-specific manner.

Work performed from the beginning of the project to the end of the period covered by the report and main results achieved so far

Work on the three 3D in vitro cellular models, able to mimic the microenvironment of a thyroid gland at different levels of anatomical complexity, continued:

- Beyond the purified thyroid cells/progenitors from murine origin coaxed into thyroid follicles, we have established the derivation of thyroid follicles from both human embryonic (hESC) and induced pluripotent stem cells (iPSC). Excitingly, our results demonstrated that we can generate structures capable to organise three-dimensionally in follicles, produce thyroid hormone in vitro, and rescue the levels of TH when transplanted in thyroid-ablated mice.
- Recellularization of decellularized scaffolds with these 3D organoids has been accomplished and allows to reproduce the biological composition of a native thyroid. We have also developed two simpler models based on the repopulation of a collagen scaffold and of a cell-laden hydrogel "cookie" configuration. Both models allowed to recreate functional rat thyroid function.
- Bioprinted constructs comprising the 3D organoids have shown successful maintenance of murine and human thyroid follicles derived from pluripotent stem cells. In addition, vascularization strategies to obtain a vascularized bioprinted thyroid model have been finalised showing the successful formation of a primitive vascular network, able to mimic the spatial and geometrical architecture of a native thyroid.

In parallel, we continued to develop the modular microbioreactor, compatible with organoid culture, sensing technology and plug-and-play microfluidics, able to host the 3D in vitro assays. In period 2, the microfluidic bioreactor platform developed could host up to eight 3D thyroid tissue constructs in parallel, under flow

conditions and meeting the requirements for high throughput screening. In the current period, the platform has been successfully used with mESC- and hESC-derived thyroid follicles, and has been upscaled to host up to 54 culture chambers in parallel. This supports the validation of the microfluidic platform, designed to be compatible with industry demands and translatable for commercialization.

Using this platform, we completed screening of 16 different EDCs for 24h and the top three compounds for 10 days. We were able to elucidate key thyroid responses to EDC exposure. Representatives per class of screened chemicals were selected, and comparative studies between the 3D in vitro models are now being performed on the representatives of the class. Sex-specific experiments have also been performed by simulating a sex-specific environment in the 3D in vitro models, resulting in significant differences at the single cell level. These results are now being further analysed.

To help elucidate the capacity of EDCs to interfere with thyroid development and function, two mechanisms of action (MoA) have been identified:

- activation of the aromatic hydrocarbon receptor by PAHs (a class of chemicals that occur naturally in, and from the burning of, coal, crude oil and petroleum products) and planar PCBs (industrially-generated fat-soluble substances that persist in the environment and living organisms), and
- inhibition of succinate dehydrogenase by phthalates and phthalate esters (a group of chemicals used to make plastics more durable or to help dissolve other materials).

Three adverse outcome pathways (AOPs) initiated by these MoAs have been created, and are under development in the AOP-Wiki. Additionally, mathematical models have been developed linking changes in thyroid function to observed changes in circulating thyroid hormones in vivo.

We are now working on the development of a biomarker able to reflect the interference of a chemical product on the thyroid function. Unbiased liquid chromatography with tandem mass spectrometry (LC-MS/MS) proteomics is used to identify a protein candidate biomarker signature of EDC action on the thyroid proteome. We used the literature and the Human Protein Atlas to identify thyroid-specific proteins and targeted protein multiple reaction monitoring (MRM) assays developed to measure the proteins in mouse and human thyroid cells. Analytical validation of the MRM assays is in progress.

Regular discussions with regulatory and industry representatives through the SCREENED Stakeholder Group and the EURION cluster have given us a better understanding of the requirements for the use of our assays for regulatory purposes. To support this aim, efforts have been devoted to ensure that methods are well documented and standardised. As an in vitro assay is rarely used as stand-alone test method but rather in a battery of assays, the possible combination with other assays is further investigated in the frame of EURION.

Progress beyond the state of the art, expected results until the end of the project and potential impacts

The establishment of human thyroid in vitro models constitutes a major breakthrough in thyroid research and will be very useful to study the thyroid gland. These models also bring a new fast, cheaper and animal-free alternative to screen EDCs for toxic effects, using a system that resembles human physiology.

SCREENED is advancing the field of “organ-on-a-chip” devices, fostering its adoption by industries and regulators. The development of reversibly sealed bioreactor chips is compatible with high-throughput screening platforms, a major requirement for industrial applicability. Our system enables integration of sensing technology for continuous monitoring of physical and biochemical parameters during culture. The progress made on the 3D in vitro cellular assays has led to the successful generation and characterisation of functional human thyroid follicles, derived from mESC, hESC and iPSC. This gives us the opportunity to analyse

the effects of EDCs on these in vitro models of the human thyroid at a gene (transcript) and protein level, and by elucidating the MoA and AOPs caused by the interference of an EDC with thyroid development and function. Besides, we started to develop targeted protein assays to transcripts that have been shown to change and attempt to circumvent the limitations of protein-based discovery experiments.

Further information about SCREENED:
Website: https://www.screened-project.eu/
X: https://X.com/ScreenedH
LinkedIn: https://www.linkedin.com/in/screened-project-8625611b4/
Cordis: https://cordis.europa.eu/project/id/825745

2 Overview of test methods to be developed

The EURION projects are developing and (pre-)validating methods and novel tools for better hazard identification of chemicals, focusing on thyroid system, developmental neurotoxicity, metabolic effects and female reproduction.

Table 1: Overview of test methods to be developed

	ATHENA	EDCmet	ENDpoiNTS	ERGO	FREIA	GOLIATH	OBERON	SCREENED
Definitive tests								
Addition of:								
Molecular readouts to existing test guidelines			X	X		X		
Endpoints to existing test guidelines	X			X	X	X		
More sensitive endpoints to existing test guidelines	X		X	X	X	X		
Biomarkers to existing test guidelines	X		X	X	X	X		
Screening tests								
Cell-based assays	X	X	X	X	X	X	X	X
Cell membrane transporters assays	X							
3D cell models							X	X
Stem cell assays	X		X			X		X
Reporter gene assays	X	X	X	X	X	X		
Non-cellular assays		X						
In vitro biotransformation			X					
Whole organism embryo		X	X	X	X	X	X	
Computational tools for screening (QSAR or other)	X	X	X	X	X	X	X	
Blood cerebrospinal fluid barrier (BCSFB) assay	X							
Blood brain barrier (BBB) assay	X							
High throughput assays	X	X	X	X	X	X	X	X
Molecular mechanism based assays (omics readouts)		X	X	X		X	X	X

3 Cluster highlights

EURION Cluster Annual Meetings

The EURION Cluster annual meetings are held to present and discuss the latest advances in research by the Cluster towards improving the identification of endocrine disruptors, to facilitate cross-project collaboration on topics of common interest and to maximize synergies between projects. Additionally, the meetings inform and engage stakeholders and the EURION International Advisory Panel for Regulatory Affairs (IAP). ATHENA and SCREENED arranged the 3rd Cluster Annual Meeting online on 20–21 January 2022. Several sessions on 20 January were open to everyone interested. EDCMET and FREIA arranged the 4th Cluster Annual Meeting on 30–31 January 2023 at the Vrije Universiteit, Amsterdam. The meeting minutes are found in EURION website. Two sessions on 30 January 2023 were open online to the interested stakeholders. A summary of the meeting as well as research highlights from the projects are available on the EURION website.

EURION Stakeholder workshops

The EURION stakeholder workshops gather representatives from research, regulatory authorities, industry, civil society, contract research organisations, policymakers as well as other experts working in the field of EDCs. The purpose of the workshops is to present and discuss stakeholder views, needs and expectations related to endocrine disrupting chemical (EDC) test method development and international strategies and guidelines. A virtual EURION stakeholder workshop was held on 2 December 2022 to present and discuss stakeholder views, needs and expectations related to endocrine disrupting chemical (EDC) test method development and international strategies and guidelines. Attendees included representatives from research, regulatory authorities, industry, civil society, contract research organisations, policymakers as well as other experts working in the field of EDCs. After short highlight presentations from the eight cluster projects, there were talks by representatives from EFSA, CEFIC, CHEM Trust and ECHA.

EURION Policy brief 2024

The first Cluster policy brief was released in January 2022. In the policy brief one can read the Clusters' recommendations to policymakers to advance the field of ED research, in the context of the EU Chemicals Strategy for Sustainability and the revision of the REACH Annexes. A second policy brief is released in 2024.

IATA workshop

On 8 March 2023, Sharon Munn (JRC) led an online IATA-workshop with 34 participants from the EURION cluster. It included break-out sessions on overarching IATA-topics. The afternoon started of with some excellent presentations. It was suggested to focus on data-rich EDCs when building IATAs, looking into connections in the developed AOPs, and common biological endpoints, biomarkers and compounds. For this, several inventories are needed: biomarkers and assays, as well as ED compounds and AOPs. The need of all projects to participate in creating these inventories of assays (test methods), biomarkers, ED compounds tested within and across the projects was discussed. The list of reference EDCs used by the projects indicated some overlap but also showed that there is no single chemical that is used by all EURION projects. It was suggested that an inventory of existing AOPs, under development by the new PARC project, could possibly support the EURION inventory development, if available in time. The collected information will be shared later on.

Presentations at the Annual Forum on Endocrine Disrupters

The DG Environment organises an Annual Forum on Endocrine Disrupters. The Forum brings together scientists, policy makers, public and private stakeholders with expertise on endocrine disruptors to exchange information and best practices, identify challenges and build synergies, in order to inform the Commission's reflections. The EURION cluster overview, update and emerging results were presented at the 4th Annual Forum on Endocrine Disruptors on 21–22 September 2022.

PEPPER

PEPPER is a public-private platform made to accelerate production of validated tests in Europe. The EURION Cluster maintains active collaboration with the PEPPER platform. This helps to progress assays towards a successful regulatory process.

4 Working groups

There have been altogether 14 working groups within the EURION cluster. Each WG has had a special thematic focus in accordance with the Cluster mandate to optimise synergies and avoid overlaps between the projects. The WGs have leaders from the eight projects as well as from the Joint Research Centre, JRC.

The EURION Cluster WGs:

- ADVERSE OUTCOME PATHWAYS (AOPS) WG
- CHEMICALS WG
- ANIMAL STUDIES: RODENTS WG
- ANIMAL STUDIES: AQUATIC ORGANISMS WG
- IN VITRO MODEL STUDIES WG
- REGULATORY AFFAIRS AND POLICY (RAP) WG
- INTEGRATED APPROACHES TO TESTING AND ASSESSMENT (IATA) WG
- EVIDENCE-BASED TOOLS WG
- IN SILICO WG
- EPIDEMIOLOGY WG
- DATA MANAGEMENT WG
- DISSEMINATION AND COMMUNICATION WG
- OMICS WG
- VALIDATION WG
- THYROID HORMONE ANALYTICS WG *

*) Set up in the EURION Annual meeting 2022.

4.1 ADVERSE OUTCOME PATHWAYS (AOPs) WG

AOPs (WG chair: Dries Knapen, ERGO)

Purpose and objectives of the WG:

- To support and facilitate AOP-related activities in the EURION projects by
    - Providing general AOP training
    - Organising discussion groups and meetings focused on specialised topics
- To bring together AOP-structured information and data across projects by
    - Providing generic templates for data entry and sharing
- Organising AOP development workshops

Progress to date:

- An online AOP WG meeting was organized on January 20 2022, aligned with the 2022 EURION annual meeting. All projects provided a status update on their ongoing AOP development activities, and AOP publication strategies were discussed.
- An online AOP WG meeting was organized on April 26 2022. All projects provided a status update on their ongoing AOP development activities. Challenges encountered during AOP development activities in the different projects were discussed. A number of follow-up action items were identified, including building an inventory of AOPs under development or completed across the different projects, and promoting the publication of EURION AOPs in the scientific literature.
- At the joint ERGO-ENDpoiNTs-ATHENA workshop on 15-16 September 2022 in Madrid, an AOP WG breakout session was organized. The main topic of the discussions was how to deal with inconsistencies in experimental data when compared to the mechanisms outlined in AOPs and AOP networks.
- In Spring 2023 a JRC/EURION thematic workshop on IATA development took place. The EURION AOP WG actively participated in this workshop, and was involved in the workshop preparations. Partners presented case studies for IATA development and suggestions were made on how to progress IATA development by leveraging the EURION AOP development activities. The main discussions during the breakout sessions related to how to best integrate new approach methodologies (NAMs), and how to facilitate uptake and application of the project outputs related to AOP and IATA development (e.g., for regulatory purposes, or by subsequent projects) after the EURION cluster has ended.

Activities in near future:

- As a follow-up of the IATA thematic workshop, the organization of a joint EURION AOP WG - IATA WG meeting is being considered. Given the link between AOP and IATA development, this will foster the uptake by future projects and other initiatives of all efforts on AOP, assay and IATA development resulting from the EURION cluster.
- Given the delays in the various EURION projects due to the Covid pandemic, it was decided to give the projects some time to further progress with their AOP development activities before planning another AOP WG meeting or workshop.

4.2 CHEMICALS WG

Chemicals (WG chair: Pim Leonards, ENDpoiNTs)

Purpose and the objectives of the WG:

- to provide an actual overview of EDCs studied by the projects,
- to provide an overview of EDCs used by multiple projects, and
- to exchange information on the analytical quality and protocols of the studied EDCs.

Activities in the near future:

- update list of EDCs studied in all EURION projects

4.3 ANIMAL STUDIES: RODENTS WG

Animal studies: rodents (WG chair: Sofie Christiansen [Julie Boberg until mid-2021] and Majorie van Duursen, FREIA)

Purpose and objectives of the WG:

To gain knowledge amongst the EURION-cluster members on planned in vivo studies.
To give input to the study design of planned studies.
Especially to

- share experience and expertise on in vivo rodent studies,
- share tissues among the EURION projects and beyond, whenever possible, and to
- give an overview of chemical compounds used in the different EURION projects.

Progress to date:

Several online meetings were held throughout the period of 2019-2022 with presentations from FREIA, ERGO and ATHENA. During the EURION annual meeting in Amsterdam, January 2023 an In vivo breakout group was established (Sofie Christiansen was Chairing). The group discussed the following questions:
1. Which new endpoints have you progressed within your project?
2. What are the future plans regarding in vivo studies?
3. Please mention some lessons learned regarding in vivo studies.
4. Which method/biomarker/endpoint have shown most regulatory readiness?

Participants were representing the following projects: FREIA, EDCMET, ATHENA, ENDPOINTS and ERGO.

Activities in the near future:

No planned meetings as the projects are finalizing, so all in vivo studies have been finalized. Answers and discussion points from the breakout group from January 2023 will be input for the final reports. This is because test strategy and regulatory readiness is key to the EURION-project outcome.

4.4 ANIMAL STUDIES: AQUATIC ORGANISMS WG

Animal studies: aquatic organisms (WG chairs: Jean-Baptiste Fini, ATHENA / Lisa Baumann, ERGO)

Purpose and objectives of the WG:

- Insight in models and outcomes used across projects
- Share and compare protocols and chemicals
- Explore cross-project collaboration in ring studies
- Discuss new scientific results

Progress to date:
The WG meets regularly to exchange information about different topics related to experiments with aquatic species, as well as to discuss new scientific results. The meetings were opened to external experts and audience who work in the field of endocrine disruption with aquatic species to increase attendece and broaden topics. In the period from January 2022 to June 2023, multiple online meetings with presentations of different scientists took place:

- 23.02.22: no speaker, general discussion
- 29.03.22: George Ruck (Viewpoint), zebrafish behavior assays, Toxmate
- 26.04.22: John Green (ERGO), the use of solvent controils in aquatic toxicity testing
- 31.05.22: Tamara Tal (UFZ), "Exploring mechanisms by which alkyl sulfonic acid PFAS cause hyperactivity in larval zebrafish"
- 28.11.22: Sarah Johann (Univ. Frankfurt), the marine medaka model
- 26.04.23: Chedi Erradhouani (OBERON) „Investigating the effects of metabolic endocrine disruptors on CYP expression in the intestine of zebrafish"
- 28.06.23: Jean-Baptiste Fini (ATHENA), "Chemical mixture matters_ lessons from amphibian models"

Changes in the WG practicalities:
Jessica Legradi (ENDpoiNTS) resigned as co-chair in autumn 2022 and was replaced by Jean-Baptiste Fini (ATHENA).

Activities in near future:
The online meetings will continue in autumn/winter 2023/2024.

4.5 IN VITRO MODEL STUDIES WG

In vitro models (WG chair: Lorenzo Moroni, SCREENED)

Purpose and objective of the WG:
The working group has gathered online and in person in the annual meeting of Paris 2020 and Amsterdam 2023. Activities aim at generating a database of cells and protocols used in the different projects of EURION to individuate possible synergies and additional collaborations in the context of *in vitro* models for ED screening.

Progress to date:
The WG had a very proficient meeting during the EURION 2023 annual meeting in Amsterdam. The number of cross-project collaborations increased. Discussions and interactions aimed at better define the use of negative compounds or "non-EDCs", which is still a pressing item in setting solid in vitro assays. Maximal tested concentrations, batteries of reference compounds, local versus systemic effects, and limitation of experimental set-up under which conditions chemicals are tested, have been identified as important variables to consider in uniforming *in vitro* assays.

Direct correlation between human *in vitro* assays and human population data is highly desired, but use of improper cell lines impinges on proper assessment of such direct correlation. This is a point of high attention for future studies improvement.

Activities in the near future:
Gather more information on collaborations across EURION cluster projects. Continue harmonization of use of negative chemicals/non-EDCs, as well as of human advanced *in vitro* models that may have a better potential to correlate directly with human population datasets.

4.6 REGULATORY AFFAIRS AND POLICY (RAP) WG

Regulatory Affairs and Policy (WG chair: Andreas Kortenkamp, ATHENA)

No updates.

4.7 INTEGRATED APPROACHES TO TESTING AND ASSESSMENT (IATA) WG

IATA (WG chair: Miriam Jacobs, GOLIATH)

Purpose and objectives of the WG:
To build understanding of IATA development, and develop collaborative approaches amongst the EURION cluster members, IATA's are a main deliverable for all EURION projects.

- To review and understand existing IATA guidance
- To review and learn from i) case studies of relatively simple IATAs: e.g eye and skin irritation ii) case studies of more complex IATAs: Non-Genotoxic carcinogenicity
- To agree common understanding of terminology and applications
- To build harmonised bridges across the EURION projects

Progress to date:
After the initial meetings held in 2019/2020, in March 2023 together with JRC, GOLIATH partner DH (Dr. Miriam Jacobs), organized an IATA workshop. The workshop consisted of earlier case studies and presentations on EURION activities on IATAs by ERGO, FREIA, ENDpoiNTs and Oberon, with breakout sessions to look for synergies between projects and how to bring methods forward within IATAs.

Activities in the near future:
Planned a combined AOP/IATA workshop.

4.8 EVIDENCE-BASED TOOLS WG

Evidence-based WG (previous WG chair: Martin Olwenn, Elias Zgheib, ATHENA)

Purpose and objectives of the WG:
The evidence-based tool working group was created following discussions during the EURION Annual Cluster meeting in 2021. Its main objectives were:

- To share knowledge and review the use and utility of evidence-based tools and methods, particularly for the development of Endocrine AOPs
- To disseminate knowledge about such tools and methods

The WG chair(s) no longer work in their earlier positions. The working group has not been active.

4.9 IN SILICO WG

In Silico (WG chair: Maria Sapounidou, GOLIATH; ENDpoiNTs)

Purpose and objectives of the WG:

- To share analysis approaches, intelligence around test chemicals etc. and to provide a platform for scientific discussion and inter-laboratory validation
- To generate joint publications exploring the utility of *in silico* methodologies for risk assessment within the ED space

Progress to date:
A number of meetings have been organised, where the potential of collaborative work has been discussed. Our latest meeting took place in Amsterdam, during the EURION annual meeting. Two initiatives were discussed. The first one was a co-authored overview of the *in silico* tools and approaches developed within EURION. It was agreed that all interested parties will share 1-2 paragraphs with to present their work, which will then combined into a manuscript. Submission is expected before spring 2024. The second initiative was proposed by ERGO partner from Aarhus University on *in silico* screening of non-target screening read-outs using approaches from three EURION partners. Two follow-up meetings were organised.

Activities in the near future:
A final chemical list is processed between EURION partners on which the *in silico* screening will be performed.

4.10 EPIDEMIOLOGY WG

Epidemiology (WG chair: Carl-Gustaf Bornehag, ENDpoiNTs)

The purpose and objectives of the Epidemiology WG, EWG: The overall aim is to compile information on epidemiological studies in the cluster, and more specifically:

- to coordinate the involved epidemiological studies within the cluster
- to establish a data management plan for how to share information/results from different epidemiological studies.

Progress to date:
Growing evidence from multiple pregnancy cohort studies suggests exposure to mixtures of environmental chemicals are associated with important adverse health and developmental effects. In addition, experimental studies of mixtures show causal relationships between exposures and outcomes. Combining the human relevant evidence with experimental studies therefore provides important confirmation of the potential adverse effects of environmental exposures.

Caporale et al (Science, 2022) developed a mixture-centered risk assessment strategy that integrates epidemiological and experimental evidence using a Similar Mixture Approach (SMACH) in the EDC-MixRisk consortium, a Horizon 2020 funded grant. The strategy included epidemiological data from the SELMA pregnancy cohort with prenatal endocrine-disrupting chemical (EDC) exposures measured with childhood neurodevelopmental outcomes. Weighted quantile sum (WQS) regression was used to characterize combinations of EDCs associated with an adverse outcome. A human-relevant typical mixture (relative proportions and total concentrations) was identified and synthesized for experimental testing of a reference mixture. The mixture included 8 EDCs: MEP, MBP, MBzP, BPA, PFHxS, PFNA, and PFOS. Experimental evidence identified molecular pathways and dose-responses with points of departure in OECD validated *in vivo* models. For subjects with exposures determined to be “sufficiently similar” to the reference mixture, exposures were

compared to the mixture point of departure using a similar mixture risk index (SMRI) where SMRI>1 indicates exposure ranges of concern.

This strategy provides an opportunity to evaluate how relevant the reference mixture is to the exposures in other populations. In the Epi Working group several cohorts are collaborating.

The SMACH strategy was presented in a draft form at the EURION Cluster meeting in Amsterdam in January 2023, using data from US NHANES of women of reproductive age. The results showed that 66% of the women in reproductive age in the US (corresponding to roughly 38 million women) had exposures sufficiently similar to the reference mixture, and, 1.1 million of these women concentration levels of concern (i.e., SMRI>1). These results are published by Sapounidou et al., (2023). Their R code for conducting the SMACH approach for this mixture code is publicly available for other investigators to use at https://www.mdpi.com/article/10.3390/toxics11040331/s1. Other investigators in the EURION Cluster indicated interest in collaborating within the working group to conduct a similar analysis of their own data. A limitation is that some of these cohort studies do not have biomonitoring data from all 8 chemicals in the EDC-MixRisk reference mixture.

Activities in the near future:

- An invitation to a meeting to discuss how to conduct a similar strategy will be sent out
- The discussion will include how to solve the problem when levels for the full set of chemicals are not available
- The ambition is that working group members will publish their results. These publications will provide evidence of the generalizability of the approach

5.11 DATA MANAGEMENT WG

Data Management (WG chair: Anna-Liisa Levonen, EDCMET)

Purpose and objectives of the WG:

- To discuss and evaluate data management solutions and approaches, including data documentation and databases
- To provide templates and/or instructions how to make data findable and interoperable
- To provide support in data management for projects and aid in the development of project specific data management plans
- Integration and harmonization of data infrastructure of EURION – EURION data management roadmap for environmental exposure /ED health effects

Progress to date:

Status of data management and DMPs of projects, sharing of data and use of common databases as well as the functions of the working group have been discussed in the WG meetings during previous reporting periods. As there are many differing needs for data management and re-use, these topics have been discussed also in meetings of other WGs as well as at the EURION Annual Cluster meeting in Jan 2023. Individual project DMPs have been collected in EURION Teams.

Activities in the near future:

Further discussions on themes related to data management will be had with the Cluster project coordinators and with the Commission representatives as well as within and between relevant WGs (especially *in silico*, omics and epidemiology) and e.g., PARC.

5.12 DISSEMINATION AND COMMUNICATION WG

Dissemination and communication (WG chair: Avril Hanbidge, ERGO, with the EURION Cluster chairmanship)

Purpose and objectives of the WG:

- Consists of communication experts from each of the eight EURION projects to ensure a focused and dynamic approach.
- The main objective of this WG is to harmonise communication and dissemination activities amongst all sister projects, to maximise their combined impact.

Progress to date:

- EDCMET currently maintain the EURION X account and AquaTT support by providing content or retweeting through the ERGO account. As of June 2023, the EURION X account has 291 followers (up 33 since December 2021) and 174 tweets.
- AquaTT staff attended the EURION Annual Cluster Meeting on 20-21 January 2022 and provided progress updates and upcoming activities from the WG for the coordinator to present at the meeting and prepared content to include on the EURION website.
- AquaTT reviewed, proofread and designed the first EURION Policy Brief: 'Towards safer chemicals – Reliable test methods to identify endocrine disruptors' of which overall development was led by ERGO and ENDPoiNTS. The brief was published in January 2022, AquaTT supported its dissemination and promotion and coordinated a social media pack for EURION partners.
- AquaTT is contributing to the organisation of the ERGO interview for the EURION Coordinator Lunchtime Webinar series, a citizen engagement activity to target the public. The series is planned to continue in the final year of the cluster and AquaTT will support its promotion. OBERON have completed their webinar which is available on YouTube.
- The most recent Dissemination and Communication WG Meeting was held in M49 – 26 January 2023. Discussion was had about recent and upcoming communication and dissemination activities within the eight projects that we will support and collaborate on together as a cluster. It was agreed to have email check ins from AquaTT to see how the WG is getting on with activities, any updates, if support is needed and if it is necessary to hold a meeting to discuss activities etc. Coordinating the email check ins with the coordinator meetings so that feedback to and from the coordinators and the WG can be easily organised.

Activities in the near future:

- AquaTT send WG email check ins in alignment with EURION Coordinator Meetings in September and December 2023.
- The EURION Coordinator Lunchtime Webinar series. All projects have been encouraged to keep the WG informed of progress so that we can support each interview's promotion as a cluster.
- Finalise an update of the EURION Communication & Dissemination Plan, currently with the chairmanship for review.
- The second and final EURION E-Newsletter will be produced by ATHENA and SCREENED, it was originally scheduled for 2022, we expect it to be published in 2023.
- Joint policy brief: the next brief will be produced by FREIA and EDCMET and was provisionally scheduled for 2023, it should be close to the end of the projects so early 2024 as the project extension has been granted.
- Potential significance of the activities and achievements are a larger awareness and impact of the ERGO project results, as well as of the combined cluster projects, making them available to a wider public.

5.13 OMICS

OMICS (WG chair: Karine Audouze, OBERON)

Purpose and objectives:
Purpose and objectives: The main objective is to harmonize the data in order to be able to use them across the different projects, and to establish an 'EURIONOMICS protocol' to have comparable data.
The OMICS working group led by K. Audouze (OBERON), includes 32 participants from six of the seven other ED projects (the project ATHENA does not plan any omics activity), and Jukka Sund, JRC will be part of the working group as EU representative.

Progress to date:
Since the start of activities in this WG, an EURION omics leaflet was created, a couple of meetings have been held and 4 webinars have been organized. The webinar series is now finished.

During the EURION Annual Meeting in 2023, a WG Omics meeting was organised where it was concluded that a comparative study of all project should be done.
Since then, a meeting was set up in March 2023 and several tasks were agreed:

- All projects will ask their respective PI for the use of TOXsIgN (https://toxsign.genouest.org/)
- Interesting data will be uploaded
- People from TOXsIgN (FREIA) will run the comparisons as private data cannot be run online

Activities in near future:
The future of this WG is linked to the availability of its participants. Project are coming to an end and this WG has a low activity rate.

5.14 VALIDATION WG

Validation (WG chair: Sharon Munn, JRC)

Purpose and objectives of the WG:

- To provide a platform for cross-cluster discussion on issues relevant to validation through identification of issues and sharing of best practices
- To develop and provide relevant training

Progress to date:
The Validation WG has been active throughout the project through the provision of relevant training by the JRC, kicking off with a workshop held prior to the EURION annual meeting in Paris in 2020. The core of the training was on establishing an assay in-house, particularly focusing on in vitro assays following the OECD guidance document on 'Good in vitro methods Practice' (GIVIMP) with emphasis on good documentation and inclusions of all relevant parameters in the Standard Operating Procedures (SOPs) to facilitate transfer of the method to another laboratory. The process of validation leading to regulatory acceptance at OECD was also described. This was followed-up with the distribution of a template describing seven criteria, with a number of sub-criteria, relevant to the evaluation of an in vitro method's readiness for validation adapted from a publication by Bal-Price et al, 2018, with a scoring scheme, allowing project partners to evaluate their own methods against the criteria. The collective experiences of evaluating methods against these 'technical readiness' criteria was the basis for a further validation workshop held online in June 2022. Comments received on the criteria were used to update the template and an automated Test Readiness Template developed by OBERON to facilitate a rapid self-evaluation was shared with the cluster. A dedicated session on validation at the 2023 EURION annual meeting in Amsterdam gave the opportunity to share experiences

and concerns related to the deliverables of a research project and how they could be best designed to facilitate the necessary further work on method standardisation/validation and regulatory uptake.

Activities in the near future:
There are no further activities planned beyond completing the Test Readiness Criteria template and providing SOPs and method descriptions for the most promising methods developed by the EURION projects.

5.15. THYROID HORMONE ANALYTICS WG

Thyroid Hormone Analytics (WG chair: previous Rikke Poulsen, new chair Martin Hansen, ERGO)

Purpose and objectives of the WG:

- Encourage knowledge sharing about thyroid hormone analytics within EURION, potentially with exchange visits between laboratories.
- Explore collaborations and knowledge sharing with non-EURION members including the OECD expert group on TH methods.
- The main output of the WG is to organize an interlaboratory study for TH quantifications.

Progress to date:
The WG had its first meeting in August 2022. The activities have been focussed on sharing knowledge of the currently applied methods in the laboratories of WG members and identify knowledge gaps. It was decided that an interlaboratory study, which can compare the sensitivity and specificity of currently applied methods, is highly needed and the subsequent activities have been focussed on establishing this study. This includes the definition of samples to be measured, recruitment of participants external to EURION, pre-evaluation of samples and planning of sample distribution.

Activities in the near future:
The samples for the interlaboratory study are planned to be distributed to the 25 recruited partners during autumn 2023 and results returned in February 2024 for data evaluation and publication.

Changes in the WG practicalities:
Martin Hansen (ERGO, Aarhus University) will take over as chair of this working group.