SECOND YEAR ANNUAL REPORT FOR ASPIS

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EXECUTIVE SUMMARY

ASPIS is a confluence of three Horizon 2020-funded projects: PrecisionTox, ONTOX and RISK-HUNT*3R*. Its goal is to unite three distinct ideas to better understand chemical toxicity and together provide innovative methods of assessing and regulating hazardous chemicals without traditional toxicity testing using animals. During its second year, ASPIS continued to collaborate, learn and advance regulatory science through its eight working groups. Each working group (WG) is defined by its specialised area of research or activity applied towards the development of new approach methodologies (NAMs). These have now grown to nine WG with the addition of the ASPIS Academy, whose activities focus on the career development of Early-Stage Researchers (ESRs). This new activity reflects a need for ASPIS to ensure that the results that are produced from this work extend beyond the funding period to ultimately improve the practice of safeguarding human health and the environment in both private and public sectors. Aligned with the ASPIS Academy is the initiation of an ASPIS sustainability plan. The overall aim is to ensure that investments in the research on NAMs produce lasting information resources.

The work of ASPIS during its second year is centred on the development of the ASPIS Next Generation Risk Assessment framework (ASPA) and related concepts, via the pursuit of case studies. These include the study of toxic substances producing steatosis and developmental neurotoxicity. The substances are chosen to exploit the diversity of ideas and approaches of all three projects, by sharing data produced from a shared set of study chemicals, which are also prioritised by our stakeholders.

The outcome of Year Two includes:

- An ASPIS consensus working understanding of NAMs
- A strategy towards artificial intelligence-based probabilistic risk assessment
- The introduction of ASPA to key stakeholders for its co-development with the Commission (e.g., JRC), the OECD Working Party on Hazard Assessment (WPHA), members of the PARERE network, the European Medicines Agency 3RsWP and the Partnership for Alternative Approaches to Animal Testing (EPAA)
- Publishable criteria and protocols for chemical selection for research towards NAMs
- Awareness building via international scientific events and public outreach
- Development of knowledge resources including a database infrastructure
- Development of analysis strategies for heterogeneous omics data for understanding biological variation in toxicological responses (both within and among species)
- Development of models that quantify Molecular Initiating Events (MIEs) or Key Event Relationships (KERs) within Adverse Outcome Pathways (AOPs)
- Orthogonal computational tools that improve the success of the modelling activities
- Development of a tiered approach to exposure and kinetics assessment to improve quantitative *in vitro-in vivo* extrapolation.

The outlook for 2024 includes the first peer-reviewed publications from investigations of the ASPIS WGs, a growing list of opportunities for its ESRs, implementation of its sustainability plans and a continuing focus on the co-development of ASPA and related concepts with our key stakeholders. Efforts are still underway to further connect ASPIS with other major European initiatives including the Partnership for the Assessment of the Risks of Chemicals (PARC).

BACKGROUND

ASPIS is a collaboration of the Horizon 2020 funded projects ONTOX, PrecisionTox and RISK-HUNT*3R*. It represents Europe's €60M effort towards the sustainable, animal-free and reliable chemical risk assessment of tomorrow. ASPIS includes more than 70 institutions across 16 countries of the European Union plus the United Kingdom and the United States. The mission of ASPIS is **to establish a next generation risk assessment framework based on new approach methodologies, encompassing *in vivo* to *in silico* technologies**.

ASPIS was launched in July 2021; under the leadership of Bob van de Water, Leiden University, RISK-HUNT*3R* coordinator. This year the ASPIS leadership role was inherited by John Colbourne, University of Birmingham, PrecisionTox coordinator.

ASPIS maintains its commitment to improving the accuracy, speed and affordability of chemical safety testing without the use of laboratory animals. Building on advances within the three consortia and close interactions with its stakeholders, it provides NAMs to accelerate and improve chemical risk assessment. Through these activities, informed decisions that safeguard human health can be made to facilitate the development of safe and sustainable products. The goals of ASPIS are being met through international cooperation, methods development and guidance documents for chemical safety testing and risk assessment.

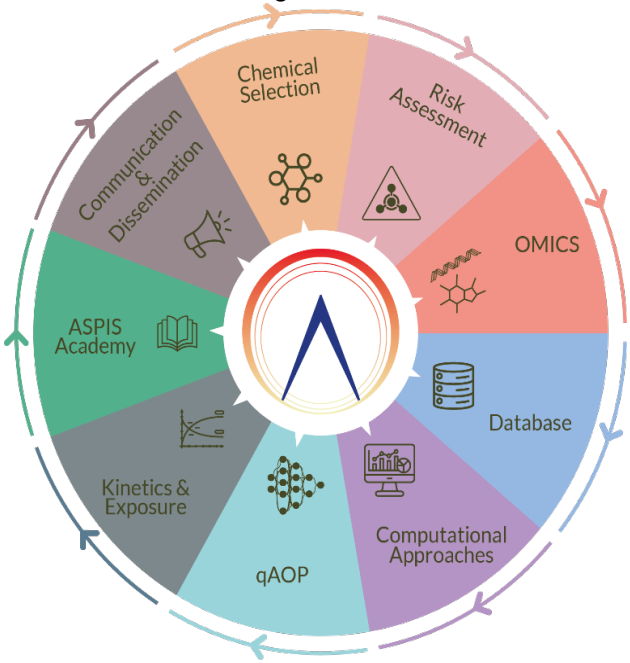


Figure 1. ASPIS Working Groups

ASPIS collaborations function through eight Working Groups (WGs) composed of investigators from all three consortia that specialise in activities that are relevant to the cluster's mission: Chemical Selection, Risk Assessment, quantitative Adverse Outcome Pathway, Omics, Kinetics & Exposure, Computational Approaches, Database and Communication & Dissemination. A ninth group was established this year, the ASPIS Academy, whose activities focus on career development of Early Stage Researchers (**Fig. 1**).

The WGs are co-chaired by a member from each consortium and includes members from each of the three projects, the ASPIS Academy and the Joint Research Centre (JRC). WGs have regularly scheduled monthly or bimestrial virtual meetings, as well as *ad hoc* meetings when needed. Meeting recordings and other ASPIS documentation (manuscripts, posters, PowerPoint presentations and meeting slide decks) are maintained on the ASPIS MS Teams/SharePoint. Additionally, there are dedicated Slack, GitHub and web sites. The ASPIS Working Group Coordinator (WGC) continues to play a critical role in the development and reporting on ASPIS inter-working group projects. as well as a link among the WGs, ASPIS leadership, Scientific/Regulatory Advisory Boards and the Regulatory Forum. Furthermore, the WGC communicates WG activities to the international community through their attendance/presentations at international meetings, EU workshops and publications.

ASPIS-WIDE ACTIVITIES

Successes and Opportunities

During the first year of ASPIS, formal organisational and WG structures were established. Its mission and goals were developed and areas for cross-consortium collaboration were identified. In its second year, ASPIS built on this foundation with each WG pursuing its individual activities, as well as several ASPIS-wide projects.

Organisational meetings continue to be held on a regular base including:

- Coordinators meeting (monthly) is attended by the ONTOX, PrecisionTox and RISK-HUNT*3R* coordinators, project managers and the WGC. Additionally, representatives from the European Commission (EC) are invited to attend.
- WG chairs meeting (monthly) includes consortia coordinators, project managers, WGC, chairs of each WG and the ASPIS Academy Core team.
- Regulatory Forum (RF) (*ad hoc)* is composed of policy and regulatory experts from across the European Commission and ASPIS. The JRC is responsible for the organisation of the RF. The RF discusses regulatory issues related to revisions of REACH towards adopting NAMs. ASPIS supervisors, SRAB chair and the ASPIS Coordination Team are invited to the RF. The RF shares documents and communicates *via* a dedicated TEAMS channel hosted by the JRC. During this reporting period, the RF did not formally meet. However, the JRC invited ASPIS to participate in the joint PARERE-ASPIS workshop in Ispra in March 2023. Additionally, Julia Malinowska, Officer at the JRC (ECVAM), gave a presentation on standardisation issues at the Third Annual ASPIS Open Symposium in September 2023 in Ljubljana, Slovenia.

ASPIS continued to expand its efforts in the steatosis case study. Initiated by the Chemical Selection Working Group, each WG is now actively engaged in this case study. Steatosis was originally selected as the first case study for several reasons: its inclusion as a biological endpoint in several of the consortia, the large amounts of available chemical and toxicological data, the multiple AOPs affected by steatotic chemicals and to demonstrate that the three consortia can work together to address a common problem. Through this study, WGs worked together to develop information pipelines for data generation and information sharing among ASPIS WGs, stakeholders and the public. Details of each WGs contribution to the steatosis case study can be found below.

The second major ASPIS-wide project is the development of the ASPIS Next Generation Risk Assessment (NGRA) framework. This framework, called the ASPIS Safety Profiling Algorithm (ASPA), is a tiered approach to identify tools and methods, when to obtain and evaluate data and how to put data into a context of a hazard or risk assessment scenario. ASPA defines a decision logic with multiple entry and exit points, activating/deactivating specific modules and prioritising and filtering of information. (**Fig. 2**) This framework is the basis of the ASPIS steatosis case study that is guided by such exchanges and will be delivered as a whitepaper with scientific findings before the end of the project.

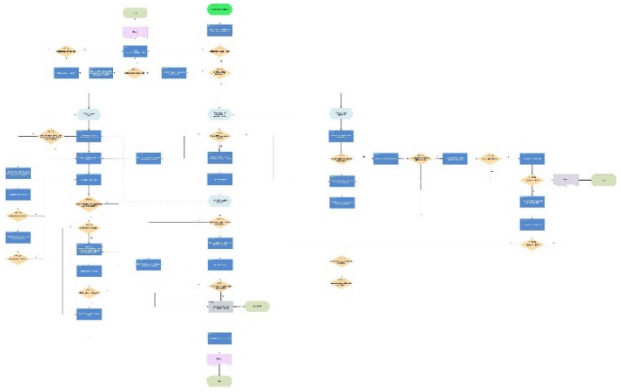


Figure 2. ASPA Workflow Version 1.9

The presentation of ASPA was at the centre of the 2nd Annual ASPIS Open Symposium (Sitges, Spain, November 2022). Additionally, ASPA has been presented at several international meeting including the 62nd Annual Meeting of the Society of Toxicology (Nashville, Tennessee, USA, March 2023), PARERE - ASPIS Workshop (Ispra, Italy, March 2023), 12th World Congress on Alternatives and Animal Use in the Life Sciences (Niagara Falls, Canada, August 2023) and the 3rd Annual ASPIS Open Symposium (Ljubljana, Slovenia, September 2023). ASPA will become publicly available at least by the end of the project; meanwhile intermediate versions will be communicated. A dashboard version (i.e. electronic) to be accessible via a web page to facilitate application is being developed and currently applied. It will provide a formal NAM-based process for the safety assessment of chronic adverse health effects associated with chemical exposure.

Through the activities among the ASPIS WGs on steatotic chemicals, the Partnership for the Assessment of Risks from Chemicals (PARC) and ASPA, a smaller case study was initiated focused on a collection of conazoles; antifungal compounds that have been associated with developmental toxicity. A list of conazoles, metabolites and control compounds and their cognate physical-chemical and toxicological data has been collected by the CSWG. This information has been distributed to the other WGs who are beginning to incorporate this information into their group's. activities

ASPIS-wide Challenges

The first challenge faced by ASPIS and its three consortia is sustainability. A major concern is the loss of accumulated knowledge, data, SOPs, etc. when the cluster ends. This concern is shared by members of our advisory board and stakeholders. A major effort of the ASPIS Leadership and stakeholders is to ensure that this cluster does not follow the same path as other previous consortia.

Although this past year as seen a resurgence in NAMs and NGRA discussion in the toxicological science and regulatory/policy arenas, they are not universally accepted in risk assessment and regulatory communities and changes are very slow in coming. NAMs and NGRA are dynamic fields, with constantly and slowly changing needs and goals from stakeholders and the regulatory community. This leads to conflicts that can blur the goals of ASPIS. Another challenge is that stakeholders (e.g., academia, industry, regulatory bodies) have yet to define a single position regarding NAMs.

Aims for 2024

A request was made by our stakeholders that ASPIS should develop its definition of a NAM. A small work group prepared a draft document and presented it as a poster at the 3rd Annual ASPIS Open Symposium for external input. In the future, the document will be distributed to the ASPIS membership for their input and a final version prepared. Ultimately, the ASPIS NAMs definition will be posted on the ASPIS webpage and submitted to a peer-reviewed journal for publication.

ASPIS is currently developing a third case study focused on developmental neurotoxicants (DNT). The CSWG has collected lists of DNTs from the three consortia and stakeholders including the JRC, National Toxicology Program (NTP), PARC, US Environmental Protection Agency (EPA) and the European Chemicals Agency (ECHA). Information on physical-chemical properties, *in vivo* and *in vitro* toxicity and modes of action are being collected and will be distributed to the other WGs.

This year we will begin to prepare and submit ASPIS-wide publications. Considerable progress in the areas of chemical selection, Omics, qAOP development and computational methods has been made centred on steatosis and conazoles. Several WG are currently preparing manuscripts that will include members from the three consortia and multiple WGs. These manuscripts will be submitted for publication during the coming year. In addition to publications, ASPIS will utilise available opportunities to present its work at workshops, symposia and regional, national and international meetings.

ASPIS will continue to expand its presence and championing of NAMs acceptance by presenting at local, national and international meetings; participating in workshops focused on NGRA and NAMs (e.g., US NIH Complement-Animal Research In Experimentation (ARIE) Challenge program) and organising workshops and meeting sessions. Additional activities are presented below by the Communication & Dissemination WG.

Finally, ASPIS will begin discussions on information sustainability. Many of the members of ASPIS have participated in other consortia-based programs. One of the unfortunate realities is that once a program ends, data and accumulated knowledge disappears or remains in non-curated websites and databases. During the coming year, ASPIS will collect a small group to develop and initiate an ASPIS sustainability plan.

Dissemination Activities

- Oral Presentations; 3Rs Working Party (3RsWP) plenary meeting - Public session on the 2023 work plan, Virtual
- Oral Presentation; *21st Century Toxicology: Sneak Preview of Ongoing Relevant Activities* at the 62nd Annual Meeting and ToxExpo of the Society of Toxicology Nashville, Tennessee, USA.
- Exhibitor's booth; 62nd Annual Meeting and ToxExpo of the Society of Toxicology, Nashville, Tennessee, USA
- Oral Presentation; PARERE-ASPIS Workshop, JRC, Ispra, Italy
- Platform Presentation; *From Molecular Toxicology to Regulatory Acceptance, the Next Step for Animal-free Testing* at the12th World Congress on Alternatives and Animal Use in the Life Sciences, Niagara Falls, Canada
- Exhibitor's booth; 12th World Congress on Alternatives and Animal Use in the Life Sciences, Niagara Falls, Canada
- Oral and Poster Presentations; 57th Congress of the European Societies of Toxicology, Ljubljana, Slovenia
- Exhibitor's booth; 57th Congress of the European Societies of Toxicology, Ljubljana, Slovenia.
- Oral Presentation; OpenTox Virtual conference
- Oral Presentation; Workshop on the Commission Roadmap Towards Phasing Out Animal Testing for Chemical Safety Assessments, Brussels Belgium.

INDIVIDUAL WORKING GROUP ACTIVITIES

Chemical Selection Working Group (CSWG)

Co-chairs: Sylvia Escher (RISK-HUNT*3R*), Jonathan Freedman (PrecisionTox) and Mathieu Vinken (ONTOX)

Background

The goal of the Chemical Selection Working Group (CSWG) is to coordinate chemical selection among the ASPIS partners. Members of this WG are responsible for collecting and distributing information on chemicals being used by the three consortia. Through its coordinated effort, the CSWG minimises duplication of efforts and identifies inter-consortia activities. The CSWG is involved in the development of ASPIS-wide case studies and assisting other working groups by providing physical-chemical and toxicological information on individual or groups of chemicals. Additionally, the CSWG serves as a link to other 3R's and toxicological projects to identify inter-consortia activities.

Successes and Opportunities

The CSWG has collected and combined chemical lists from the three consortia. Additionally, lists have been obtained from the JRC, US NTP, US EPA, ECHA, the EU co-funded project PARC and several stakeholders. These chemicals and cognate information have been provided to all WGs and will be included in the ASPIS database. The CSWG led the development of the steatosis case study and is supporting the conazoles case study. It is supporting the development of the ASPA by providing physical-chemical, toxicological and exposure information to the Risk Assessment WG. To expedite data mining, the CSWG created several KNIME workflows to collect information from public and private databases. (**Fig. 3**)

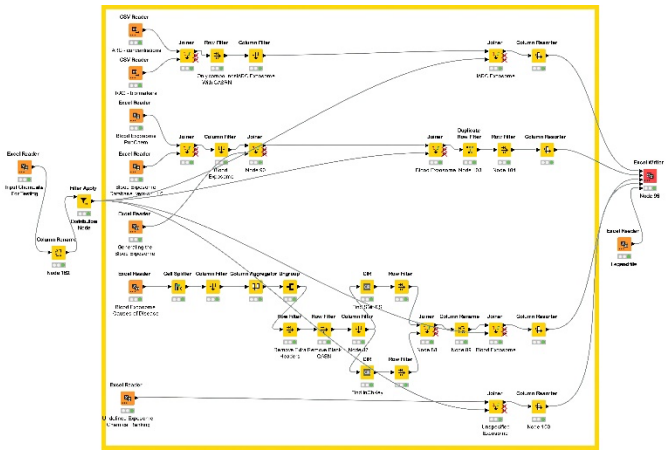


Figure 3. KNIME Exposome Workflow

Aims for 2024

The CSWG aims to complete several projects this coming year. First it will complete compiling and distributing a finalized list of DNTs, plus associated physical-chemical, toxicological, transcriptomic and exposure information; to the other ASPIS WGs. The CSWG will begin this task in January 2024.

Second, this WG is to begin working with the JRC to create an ASPIS chemical library. These chemicals will be made public and provided to interested investigators with the goal of

expanding toxicological information on the ASPIS compounds. Following the protocols currently being finalized through an analogous project by PrecisionTox, the ASPIS CSWG will compile a list of chemicals being used thought out the cluster.

Finally, the CSWG will begin preparing manuscripts on chemical selection procedures used by the three consortia. We anticipate that the release of the ASPIS Chemical library will coincide with the publication of this manuscript.

The CSWG will continue to help building the ASPIS database with the Database WG. It will also support the Risk Assessment WG in the development of ASPA. Additionally, it will assist other WGs regarding issues associated with chemical selection and characterization.

Dissemination Activities

- Poster at the 3rd ASPIS Open Symposium Ljubljana, Slovenia.
- The majority of the CSWG Dissemination Activities are incorporated into the activities of the other WGs.

Communication and Dissemination Working Group (C&DWG)

Co-chairs: Francois Busquet (ONTOX/PrecisionTox) and Giorgia Pallocca (RISK-HUNT*3R*)

Background

The Communication and Dissemination Working Group (C&DWG) aims to harmonise dissemination activities and maximise the impact of ASPIS. Communication and dissemination activities are coordinated by the three consortia with the shared mission to unbiasedly inform on how NAMs-based strategies can rapidly accelerate and improve chemical risk assessment without the use of animals.

The communication teams of the three consortia work together to synergise dissemination efforts and positively impact ASPIS visibility and the outreach of its key messages. In particular, the goal of the C&DWG is to build, beyond projects' specificities, a single ASPIS to have a stronger voice to vehicle its outcome to regulatory stakeholders, policy makers, non-governmental organisations and the lay public.

ASPIS speaks jointly online *via* a coherent social media presence to facilitate message amplification. The communication teams of each consortium echo news and outcomes of each other and disseminate those *via* their channels. ASPIS also jointly communicates *via* official press declarations and policy briefs, as support statements of policy legislative initiatives or involvement in public consultations. Common publications, op-eds and press coverage in European journals are also used to inform stakeholders about ASPIS.

The WG organizes also face-to-face activities, such as participation in joint conference sessions at international conferences (e.g., SOT annual conferences, EuroTox) and the organisation of the ASPIS annual open symposium. The symposium series involves consortia members, stakeholders and the ASPIS regulatory advisory board to discuss crucial achievements and challenges in the implementation of NAMs into chemical risk assessment in Europe and beyond. This allows ASPIS to support EU Green Deal objectives, such as a toxic-free environment.

Successes and Opportunities

Building on the sustainable foundation established in 2022-2023, the C&DWG generated multiple operational activities:

The C&DWG supported the creation of ASPIS Academy (AA) core team that will further lead action. It included contacting and defining ESR status, organising teleconferences and coordinating future AA work programs. In terms of support, the C&DWG contacted external initiatives for collaborations and funding (e.g. EPAA and young TPI). A follow-up is foreseen.

Figure 4. ASPIS members at the WC12 conference
Oral presentations (*top right*), dedicated exhibited booth space (*middle left*), recording video interviews (*middle right*) and ASPIS open symposium participants (*bottom*)

The C&DWG coordinated the presence of the cluster at the main scientific events such as the Society of Toxicology annual conference, the 12th World Congress on Alternatives and Animal Use in the Life Sciences and the EuroTox congress. (**Fig. 4**) On all those occasions, ASPIS was represented at the respective exhibition. At the booth, we distributed projects and cluster material and generated discussion with the participants. Taking advantage of the presence of multiple partners, it was the opportunity to generate half a dozen short videos that were disseminated on social networks.

The C&DWG organised the ASPIS Open Symposium in Ljubljana, Slovenia, which follow the EuroTox conference. For this occasion, the C&DWG prepared the communication plan accordingly and developed visual materials and "goodies" for the participants. Up to 100 participants joined in person. In parallel, the WG organised a training on grant writing workshop for ESRs and poster exhibitions for the three consortia including a poster prize.

The C&DWG voiced its concerns or support on *ad-hoc* occasions to public initiatives or statements to be vocal about the robustness and relevance of NAMs' whenever appropriate by publishing declarations on the cluster's website.

Last, the C&DWG took action in involving several ASPIS partners with EU institutions events such as the EMA 3RsWP Stakeholder meeting (Online 28 February 2023), JRC-PARERE workshop (Ispra 30-31 March 2023), ECHA workshop on NAMs (Helsinki 31 May- 1 June) and the EC roadmap workshop to "phase out animal testing." (Brussel 11-12 December 2023).

Aims for 2024

In 2024 ASPIS will continue to maintain its presence in multiple scientific events, such as the SOT, ESTIV and EuroTox annual meetings by having exhibition booths, coordinating the participation of the cluster partners, disseminating the cluster scientific input to diverse programs and strengthen its collaboration with EU institutions. The C&DWG is also supporting the organization of the next ASPIS OS that will take place at EuroTox in Copenhagen, Denmark.

The C&DWG will support the training events for the AA including "How to bridge the gap: from cell-based test methods development to regulatory applications" at the RISK-HUNT*3R* general assembly in February 2024. The possibility of organizing events in collaboration with external initiatives, such as the Young TPI, is under discussion as the first summer school in June 2024.

Dissemination Activities

- ASPIS Exhibitor booths at Exhibitors booth at the 62nd Annual Meeting and ToxExpo of the Society of Toxicology, Nashville, Tennessee, USA; 57th Congress of the European Societies of Toxicology, Ljubljana, Slovenia; 12th World Congress on Alternatives and Animal Use in the Life Sciences, Niagara Falls, Canada.
- ASPIS Platform Session (seven speakers) at the 12th World Congress on Alternatives and Animal Use in the Life Sciences, Niagara Falls, Canada.
- Third ASPIS Open Symposium, Ljubljana, Slovenia

ASPIS Academy

Co-chairs: Eliska Kuchovska (ONTOX), Luiz Ladeira (ONTOX), Barira Islam (RISK-HUNT*3R*), Kirsten Veltman (RISK-HUNT*3R*), Ruben Martinez (PrecisionTox) and Gaelle Hayot (PrecisionTox)

Background

The purpose of the ASPIS Academy (AA) is to build a viable research network focused on the use of NAMs for chemical risk assessment. The AA promotes the careers of ESRs by providing specialized training, promoting equal opportunity for all the members and creating a platform devoted to the voices and aspirations of a new generation of scientists who will become natural experts and future leaders of NAMs and their applications.

The AA Core Group was established during the 2nd half of 2023, which includes two ESR representatives from each consortium, completed by two advisors and three project managers forming the ASPIS Academy Board, the governing body of the AA (**Fig. 5**). The duties of AA Board and reporting requirements to the ASPIS C&DWG, respective projects and the ASPIS coordinator are described in the drafted AA Governance document. Currently, AA

membership is restricted to the ASPIS cluster, however there are plans to include non-ASPIS researchers in the future.

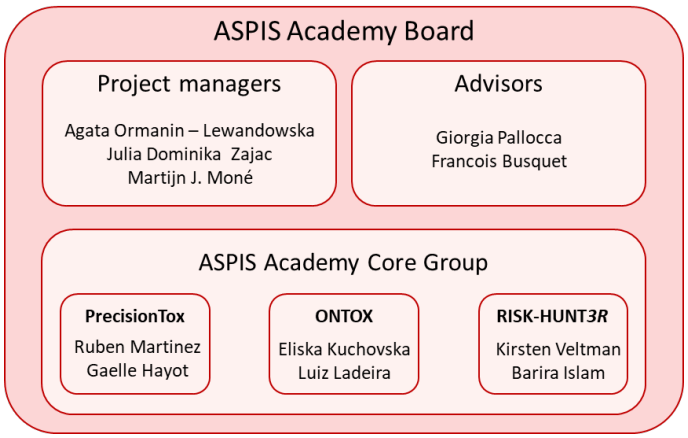


Figure 5. ASPIS Academy Leadership

Successes and Opportunities

The AA held one on-site training for the ESRs entitled Grant Writing Masterclass at the ASPIS Open Symposium in September 2023 in Ljubljana, Slovenia. On the same occasion, a networking sightseeing event was organized by members of the Academy. The AA inaugural meeting was planned for the middle of December 2023, when the AA Core Group introduced the AA aims, structure, programs and plans to ASPIS ESRs. Furthermore, AA Core Group created an AA LinkedIn group as a online ASPIS networking platform.

Aims for 2024

Planned activities for AA members include a mentoring program (one-on-one mentoring from senior mentors to ESRs but also from senior ESRs to junior ESRs), a twinning program (ESR visits at hosting institutions, open days at companies), a mirroring program (matching ESRs with other ASPIS WGs) and training program (webinars and on-site trainings). All new activities will start at the beginning of 2024. Moreover, a communication plan was drafted describing the strategy, target audiences, channels, tools and C&D activities. This communication plan was created based on the ONTOX communication plan.

The ASPIS Academy Core Group plans to organize several online trainings beginning with a career development session in January 2024. They have invited four guests from diverse backgrounds (academia, industry, intergovernmental organization and NGO) to enable a discussion with ESRs. The organization of the first AA summer school is under discussion and will be coupled with another ESR network. Moreover, the AA Core Group will organize a poster session for the ESRs as well as on-site training at the ASPIS Open Symposium in September 2024. The AA Core Group also plans to initiate an ESR C&D activity consisting of short videos/articles/memes to reach target audiences as described in the AA Communication plan, being able to actively involve other members in AA coordinated actions. Finally, though the AA has been conceived with ESRs in mind, ASPIS is considering to further broaden the scope of the AA network to support ongoing NAM-focused research and researchers.

Database Working Group (DbWG)

Co-chairs: Barry Hardy (RISK-HUNT*3R*), Tomasz Ignasiak (PrecisionTox) and Thomas Luechtefeld (ONTOX)

Background

The ASPIS DbWG was formed in early 2023 to support collaboration with regards to data-driven resources supporting the ASPIS goals. The group had several initial meetings during Spring 2023 establishing the group and presenting and discussing the different activities of group members.

Success and Opportunities

A focus goal that the group has established is to work together to develop knowledge resources supporting ASPIS case studies starting with the steatosis case study. Following up on the work of the ASPIS CSWG, the DbWG assembled a number of inputs on compound lists, including selection work on ONTOX, network modelling on RISK-HUNT*3R,* inputs from the EC JRC and public resources. We are building a knowledge graph to represent current knowledge of the steatosis network and the relationships between key events and the adverse outcome (**Fig. 6**). This has in turn raised the issue of needing a common ontology for the terms describing nodes and relationships in the steatosis network model, which is currently a work in progress.

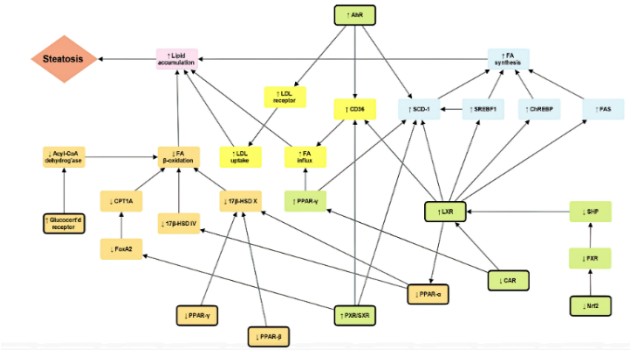


Figure 6. Steatosis Network Model

Once the harmonised knowledge graph for steatosis is in place, our plan for follow-on work is to connect compounds to the graph. We will then work on the task of connecting data to the knowledge graph. A related scientific goal we are pursuing is establishing a data-driven methodology for determining true MIEs. We are also interacting with other working groups (e.g., qAOP, omics, risk assessment) to align our knowledge resource activity with support for their goals and efforts.

Aims for 2024

A new activity starting in December 2023 and progressing into 2024 is to repeat our process for the planned ASPIS developmental neurotoxicity case study.

An important goal and activity planned for 2024 is to release knowledge resources related to the DbWG first for ASPIS access and then broader public access. This will include the compound database, test methods database and knowledge graph resources including linked biological data. We plan to release, present and discuss these resources at conference events during the year.

Dissemination Activities

- Participation in ASPIS stand at the 62nd Annual Meeting and ToxExpo of the Society of Toxicology, Nashville, Tennessee, USA

- SaferWorldbyDesign stand at 57th Congress of the European Societies of Toxicology, Ljubljana, Slovenia, combined with adjacent Altertox stand to cover ASPIS
- Participation in ASPIS OS after EuroTox, including poster

Omics Working Group (OWG)

Co-chairs: Florian Caiment (ONTOX), Giulia Callegaro (RISK-HUNT*3R*) and John Colbourne (PrecisionTox)

Background

The ASPIS Omics work group (OWG) is dedicated to promoting the transition of omics into NGRA. For this, all data produced by ASPIS will be made compliant with the OECD Transcriptomics and Metabolomics Report Framework (TRF and MRF, respectively). These frameworks have been designed to allow regulatory agencies to assess the quality of omics datasets. The R-ODAF (Omics Data Analysis Framework for regulatory application) established, as a reference pipeline for comparing the output of two different transcriptomics datasets, will also be presented as a possible reference analysis pipeline to be used in ASPIS. Regrouping all the omics experts of the three consortia, the OWG is organising a monthly meeting to engage in discussion on data analysis strategies, software presentation and statistical best practices. Every member will be welcome to present their specific omics-related problem and benefit from the expertise of the group to obtain ideas and possibly a consensus. Lastly, the OWG will contribute to the general ASPIS case study, by assuring that any omics dataset selected by other WGs are processed and analysed according to the state of the art.

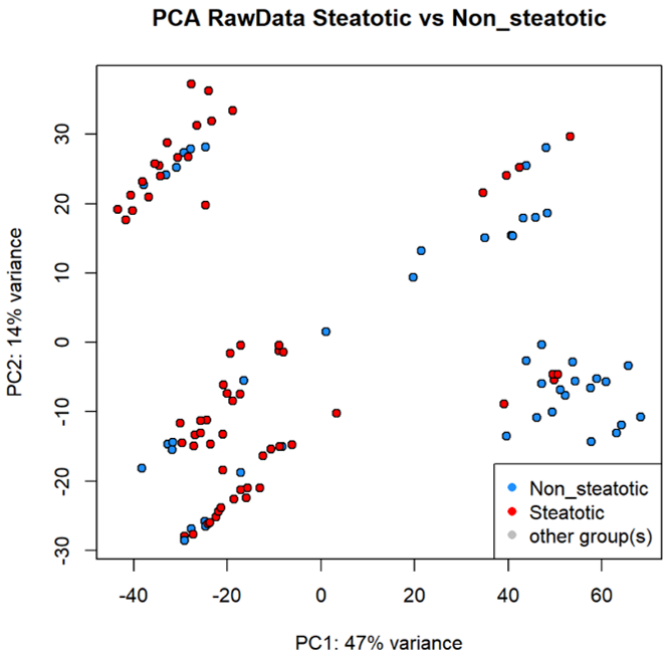


Figure 7. PCA Plot to exclude the post-processing outlier replicates

Successes and Opportunities

For the steatosis case study, we compiled a core dataset consisting of over 1200 microarrays. These were assembled, normalized and analysed before being distributed among all members of the OWG. The selection of these microarrays was based on the steatotic chemical list curated by the CSWG. To address the overarching question; 'Can heterogeneous transcriptomics datasets effectively identify the steatosis state of a compound?, we formed sub-teams tasked with pursuing distinct analysis strategies. (**Fig. 7**) Furthermore, an ongoing analysis is utilizing a metabolomics dataset to supplement our investigations.

Dissemination of the use of weighted gene co-expression networks to analyse high-throughput transcriptomics data has caught the interest of EU regulatory agencies. This led to two separate projects funded by the European Food Safety Agency (EFSA) to apply this technology in a regulatory setting for assessing toxicodynamics whilst accounting for human variability upon chemical exposures.

Additionally, the Omics WG anticipates participating in the evaluation of the ASPA framework developed by the Risk Assessment WG."

One of the challenges facing the OWG is that our primary data source for the steatotic case study relies on transcriptomics microarray datasets. However, acquiring sufficient data from newer transcriptomics platforms has proven challenging. Moreover, securing a comprehensive dose range for each steatotic compound on our list—where a steatotic response is anticipated—for both human and rodent models, across *in vivo* and *in vitro* conditions, presents a formidable challenge. Although collaborations with other ASPIS WG have commenced to address these challenges, the magnitude of the task at this stage appears considerable.

Aims for 2024

Significant progress has been made in the steatosis case study by three distinct sub-teams employing varied analysis strategies: machine learning techniques, differential expression analysis and congruence analysis. Encouragingly, these approaches have yielded promising results, paving the way for our intention to publish these findings within the upcoming year. The OWG is experiencing growth, notably with the inclusion of young scientists from the ASPIS academy. This expansion may lead to an increase in the number of sub-teams in the near future.

Dissemination Activities

- EUROTOX 2023 Session "Toxicogenomics: breaking barriers for regulatory implementation", Chair Bob van de Water (RISK-HUNT*3R*)
- Presentation in the OpenTox 2023 conference, Florian Caiment (ONTOX), “R-ODAF: Omics Data Analysis Framework for regulatory applications”
- Presentation on “Toxicogenomics data and analysis workflows” in Continuing Education Course at EUROTOX 2023, Giulia Callegaro (RISK-HUNT*3R*)

Quantitative Adverse Outcome Pathway Working Group (qAOPWG)

Co-chairs: Huan Yang (ONTOX), Pu Xia (PrecisionTox) and Mark Cronin (RISK-HUNT*3R*)

Background

The scope of the quantitative Adverse Outcome Pathway Working Group (qAOPWG) is to support the development of qAOPs across ASPIS. Specifically, this WG aims to investigate models that quantify Molecular Initiating Events (MIEs) or Key Event Relationships (KERs) within existing AOPs using non-confidential data, as well as identifying and sharing good practice. Specific activities involve bringing added

value to qAOP development across ASPIS by developing common ideas. This would include jointly finding solutions to problems and being able to share knowledge of dose-responses, data and models across the three consortia. The qAOPWG aims is to develop one or more common qAOPs, including those from linear and network AOPs that are of interest to all partners, such that data and expertise can be combined. The WG will facilitate integration of qAOPs with MIE and PBPK modelling to enable Quantitative Systems Toxicology approaches. In terms of application, the qAOPWG will identify how risk assessors can apply qAOPs, with an emphasis on regulatory use. As part of regulatory use, the degree of confidence risk assessors need to use qAOPs in risk assessment will be considered, along with obstacles/ concerns for assessors to use qAOPs in risk assessment.

Successes and Opportunities

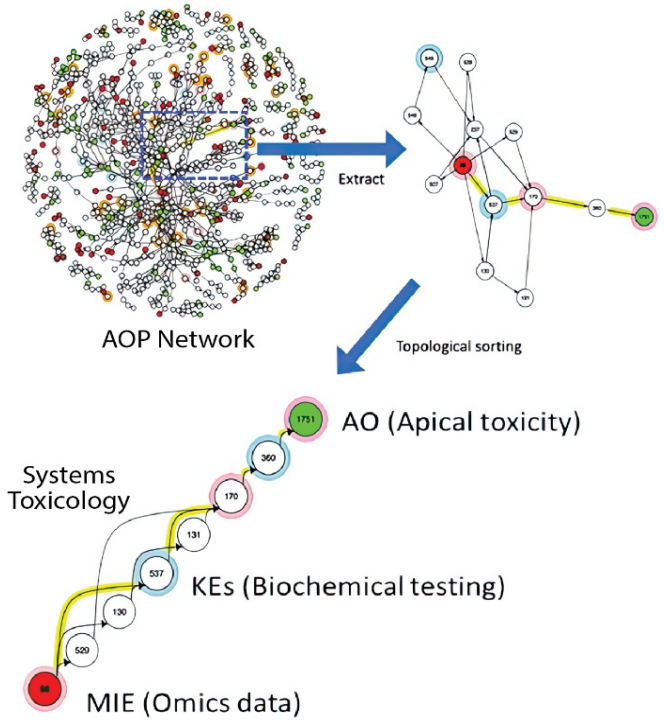


Figure 8. AOP Network and Omics-Based qAOP Investigation

The qAOPWG is actively developing qAOPs for steatosis and developmental neurotoxicity through data and model sharing. It has explored systems modelling approaches to simulate steatotic KEs and have prototyped a QST model to integrate steatosis qAOP and (toxico)kinetics. Additionally, it has established a framework to assess the validity of qAOPs. Working with the CAWG the qAOPWG provides the opportunity to build and present models for MIEs and coordination of qAOP model development (**Fig. 8**). Ultimately, these models will provide input into regulatory decisions and ASPA.

There remain several issues that are of concern to the qAOPWG. First is a lack of understanding different types of qAOP models. There is an inherent uncertainty in all of the qAOP models. Additionally, a firm plan to move qAOPWG activities from a modelling exercise to practical tools with uptake and acceptance by the regulatory community needs to be developed. To address this issue, the qAOPWG intends to develop a tangible product that will directly support interaction with the RAWG.

Aims for 2024

There will be two main collaborative projects in 2024. The first is to identify and use cases to apply qAOPs, e.g. those developed for steatosis and DNT. This will be undertaken within the context of ASPA. Demonstrating the applicability of qAOPs in ASPA will assist in the development and implementation of the ASPA framework. The second collaborative project is to finalise the framework for the established the validity of a qAOPs while engaging with our external stakeholders. The framework is intended to promote the use of qAOPs and create greater understanding and confidence. The overarching aim of the activities of the qAOPWG is to demonstrate the possibility of using qAOPs to support risk assessment.

Dissemination Activities

- Chaired and oral presentation in the session "Progress in Quantifying Adverse Outcome Pathways to Support Next Generation Risk Assessment" at the 12th World Congress on Alternatives to Animal Testing (Niagara Falls, Canada, August 2023).
- Chaired and oral presentation in the session "Towards quantitative Adverse Outcome Pathway Networks" at the 57th Congress of the European Societies of Toxicology (EuroTox 2023) (Ljubljana, Slovenia, September 2023).
- Oral and poster presentations at the 20th International Workshop on (Q)SAR in Environmental and Health Sciences (Copenhagen, Denmark, June 2023).

Computational Approaches Working Group (CAWG)

Co-Chairs: Emilio Benfenati (ONTOX), Gerhard Ecker (RISK-HUNT*3R*) and Nate Keith (PrecisionTox)

Background

The Computational Approaches Working Group (CAWG) is organising the activities related to the computational methods used in ASPIS. These activities are focused in the following areas: (1) development of specific *in silico* models and read-across tools to evaluate properties of interest, (2) methodological studies to improve *in silico* techniques that can be applied within ASPIS - a certain method may ideally be exported from one project to another and (3) supporting the activities within other WGs. The activities focused on liver toxicity in 2023, mainly in relation to cholestasis and steatosis. These activities addressed the sharing of information, data, and tools. A work plan has been prepared to define the best way to optimize ASPIS synergies (**Fig. 9**). The components of the CAWG are aware that there is not a single, best model/approach to solve the issue related to the predictions of the adverse property of a substance. Indeed, each approach is proceeding along a specific perspective, able to capture a relevant component of the phenomenon under evaluation. The information regarding steatosis has been shared, also regarding the availability of the data. A deeper exercise addressed cholestasis. Different groups explained their methodology and results.

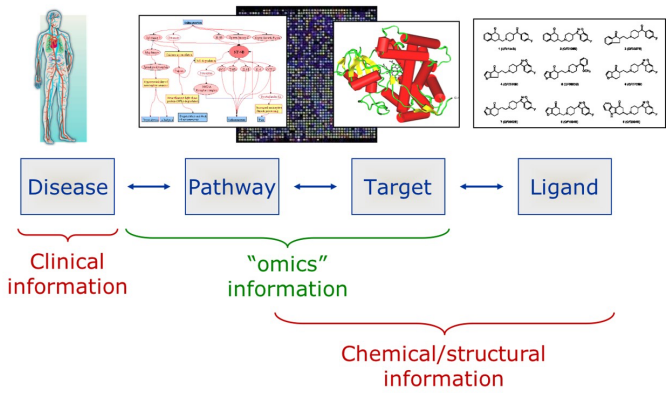


Figure 9. The CAWG Modelling Approach

Successes and Opportunities

The combined use of orthogonal tools can improve the success of the modelling activities. The different tools will provide stronger evidence about a certain effect. Ideally, they will reduce the uncertainty of the assessment, offering multiple lines of evidence. The independence of the approaches will increase the confidence of the results. The fact that some tools are more related to the statistical methodology, while others are closer to the

mechanistic approach is also reinforcing the use of the results for different purposes, related to the statistical robustness of the results, or to reasoning, and possible explanation of the biochemical process associated to the effect.

The complexity of the multiple tools requires a deeper understanding of specific situations addressed within each tool. A detailed analysis of the applicability domain of each model is necessary, since the different models are providing pieces of information which partially are overlapping and partially may be not focused on the same direction.

Aims for 2024

We will proceed investigating the domain of each approach and the ways to integrate the results of the different models achieving a more sophisticated representation of the multiple factors involved in cholestasis, and hopefully better performance. Thus, the CAWG will explore the architectural schemes for the integration and/or interactions between the different tools. This will be useful in proceeding with other endpoints, continuing the activity on steatosis and later on addressing DNT. The CAWG will liaise with the RAWG to contribute to the ASPA for NGRA.

Dissemination activities

- Lecture at the Europin Summer School on Drug Design, Sept 2023, Vienna: Claudia Trivisani, Exploring structure-based methods for the *in-silico* prediction of steatosis.

Risk Assessment Working Group (RAWG)

Co-chairs: Mirjam Luijten (RISK-HUNT*3R*), Erwin L. Roggen (ONTOX) and Stefan Scholz (PrecisionTox)

Background

The three ASPIS consortia have complementary approaches on how to use NAMs for the hazard and risk assessment of chemicals including prioritisation, grouping/read-across and hazard characterization. The ASPIS risk assessment working group (RAWG) intends to share and link different approaches, coordinate joint activities and critically review ASPIS research in comparison to previous activities for promoting NAMs with reference to the EU-ToxRisk project. The RAWG also aims to identify gaps, limitations and advantages of chosen approaches. It will compare approaches and results to those outside of ASPIS, particularly with a global view to identify targets for hazard and risk assessment to plan for joint/coordinated activities. Furthermore, it aims to connect research activities in ASPIS to form joint case studies. Together with other WGs, it will facilitate the requirements for hazard and risk assessment by end-users and stakeholders. Finally, the RAWG ensures complementarity of its activities with PARC to support the European Union and national chemical risk assessment and risk management bodies with new data, knowledge, methods, networks and skills. This will facilitate the transition to next generation evidence-based chemical risk assessment.

Successes and Opportunities

The RAWG established regularly scheduled inter-project meetings. They supported discussions, development and presentations of ASPA (**Fig. 10**). A highlight was the presentation and discussion of ASPA with members of the PARERE network at the JRC in March 2023. With the ongoing development of ASPA, the RAWG will strengthen its interactions with the other WGs to develop future case studies and begin collaborating with other European projects. Additionally, the RAWG will map case studies to the goals and mission of other WG and draw from activities conducted in EU-ToxRisk and under auspices of OECD. To remain agile, the RAWG plans to begin smaller focused meetings, define internal deliverables and carefully consider future collaborations.

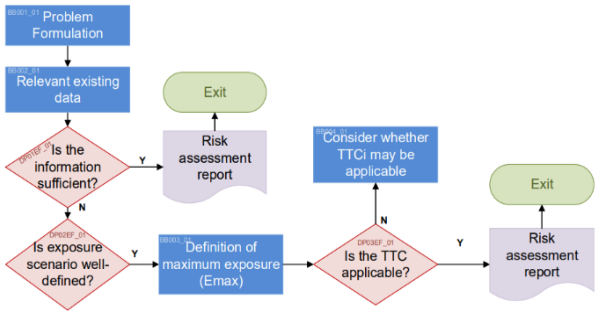


Figure 10. NGRA Framework (cut-out)

Aims for 2024

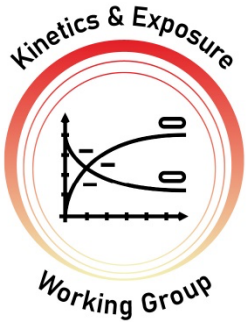
In 2024, the RAWG will evaluate the ASPA workflow against ASPIS case studies to assess its flexibility and identify gaps. The RAWG will identify new ways to engage regulatory authorities at national and international levels. Following the selection of test chemicals for demonstrating and improving ASPA and agreed definition of appropriate problem formulations for the case studies, we will stress-test the ASPA workflow and identify areas that need further improvement. Lessons learned will be discussed both within ASPIS and with external stakeholders, such as the PARERE network.

How Probabilistic Risk Assessment (the ONTOX flagship) and results from having evolutionary knowledge of the mechanisms of toxicity (a key PrecisionTox contribution) will be incorporated remains unclear. In 2024, the first case studies involving Probabilistic Risk Assessment will be performed by ONTOX in collaboration with stakeholders from industry and regulatory environment. It is anticipated that these case studies, some involving ASPIS chemicals and fitting into the ASPIS case study repertoire, others involving chemicals of interest for industry stakeholders and stakeholders from the regulatory environment.

Dissemination activities

- PARERE workshop: Presentation of Risk Assessment WG activities; Presentation an ASPA
- December 2023: ASPIS Academy, presentation of the WG activities
- EUROTOX 2023: Posters on computational methods

Kinetics & Exposure Working Group (KEWG)



Co-chairs: Sylvia Escher (RISK-HUNT*3R*), Beate Escher (PrecisionTox) and Nynke Kramer (ONTOX)

Background

The Kinetics & Exposure Working Group (KEWG) consists of investigators from each of the three consortia that work on defining chemical exposure levels in the environment, human populations, target organs, and *in vitro* assays. Complementary NAMs, namely (1) aggregated exposure assessment tools, (2) physiologically based kinetic modelling and (3) *in vitro* distribution kinetics models, are being developed in each of the three consortia that will benefit from being integrated into a common, pragmatic guideline for risk assessors to perform quantitative *in vitro-in vivo* extrapolation (QIVIVE) and next generation risk assessment (NGRA) (**Fig. 11**). The aim of this working group is to publish on the ASPIS website a guideline detailing a tiered approach to exposure and kinetics assessment using the tools used and developed in each of the three consortia and illustrate the approach with ASPIS case studies by September 1, 2026, when the individual projects will have ended.

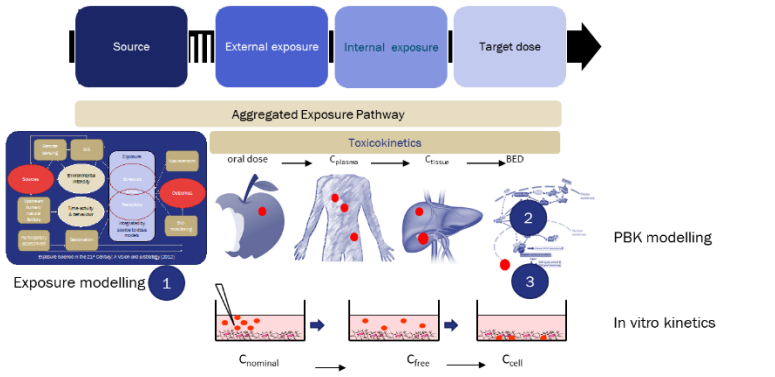


Figure 11. The integration of NAMs in the exposure continuum of an NGRA workflow.
C = concentration, BED = biologically effective dose.

Successes and Opportunities

Regular online meetings with consortia members with an interest in the subject matter are organised on a monthly basis and chaired by one of the three co-chairs. Prof. Beate Escher from UFZ has joined as a co-chair to allow equal representation of the three ASPIS project in the working group leadership. During each meeting, an update of the work done by individual partners on the ASPIS case study is given.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Chemical name	SMILES	ProtoPhyschem _LogKow_experimental	ProtoPhyschem _LogKow_prediction	ProtoPhyschem_ LogKow_AD	ProtoPhyschem_ LogD_experimental	ProtoPhyschem _LogD_prediction	ProtoPhyschem_ LogD_AD	VEGA_LogP_ ALOGP_prediction	VEGA_LogP_ ALOGP_experimental	VEGA_LogP_ ALOGP_ADI	VEGA_LogP_ MEYLAN_prediction	VEGA_LogP_ MEYLAN_experimental	VEGA_LogP_ MEYLAN_ADI	VEGA_LogP_ MLOGP_prediction	VEGA_LogP_ MLOGP_experimental	VEGA_LogP_ MLOGP_ADI	EPISUITE_LogP _KOWWIN_prediction	OPERA_LogP _prediction
2	Propiconazole	CCCC1COC(O1)(CN2C=NC=N2)C3=C(C	3,72	3,72	Inside (Tanimoto)	3,5	2,88	Inside (Tanimoto)	3,8	3.72	1	4,13	3.72	1	3,9	3.72	1	4,1272	3,72
3	Difenoconazole	CC1COC(O1)(CN2C=NC=N2)C3=C(C=C	4,3	4,47	Inside (Euclidean distance)		3,43	Inside (Euclidean c	4,38	4.3	1	5,2	4.3	0,85	4,34	4.3	1	5,2013	4,3
4	Prothioconazole	C1CC1(C(CC2=CC=CC=C2O)(CN3C(=S)N=CN3)O)Cl		3,23	Inside (Euclidean distance)		2,36	Inside (Euclidean c	3,29	-	0,85	1,7	-	0,75	3,36	-	0,85	3,0917	2,89
5	Fenbuconazole	C1=CC=C(C=C1)C(CCC2=CC=C(C=C2)Cl	3,23	4,01	Inside (Euclidean distance)		2,84	Inside (Euclidean c	4,3	3.23	0,75	4,23	3.23	0,75	4,4	3.23	0,75	4,23	3,23
6	Epoxiconazole	C1=CC=C(C=C1)C2C(O2)(CN3C=NC=N	3,44	3,71	Inside (Euclidean distance)		2,72	Inside (Euclidean c	3,43	3.44	1	3,47	3.44	1	4,4	3.44	0,85	3,4748	3,44
7	Itraconazole	CCC(C)N1C(=O)N(C=N1)C2=CC=C(C=C2)N3CCN(CC3)C4=		5,48	Inside (Tanimoto)	5,79	4,87	Inside (Tanimoto)	6,43	5.66	0,85	6,52	5.66	0,85	5,27	5.66	1	6,164	5,66
8	Cyproconazole	CC(C1CC1)C(CN2C=NC=N2)(C3=CC=C(	2,9	3,05	Inside (Tanimoto)		2,17	Inside (Euclidean c	2,92	2.9	1	2,22	2.9	0,85	3,77	2.9	0,85	3,2493	2,9
9	Ketoconazole	CC(=O)N1CCN(CC1)C2=CC=C(C=C2)OC[C@H]3CO[C@]((		3,8	Inside (Tanimoto)	4,33	3,85	Inside (Tanimoto)	3,61	4.35	0,85	4,45	4.35	1	2,73	4.35	0,75	4,4454	4,35
10	Voriconazole	C[C@@H]([C1=NC=NC=C1F)[C@](CN2C=NC=N2)(C3=C(C		2,43	Inside (Euclidean distance)		1,75	Inside (Euclidean c	2,07	-	0,85	1,57	-	0,75	3,68	-	0,75	1,5736	2,2
11	Fluconazole	C1=CC(=C(C=C1F)F)C(CN2C=NC=N2)((	0,5	0,93	Inside (Tanimoto)	0,5	0,21	Inside (Tanimoto)	0,75	-	0,75	0,25	-	0,75	3,41	-	0,75	0,2538	0,5
12	Sulfamethoxazole	CC1=CC(=NO1)NS(=O)(=O)C2=CC=C(C	0,89	0,94	Inside (Tanimoto)		0,33	Inside (Euclidean c	1,19	0.89	1	0,81	0.89	1	0,97	0.89	1	0,484	0,89
13	Posaconazole	CC[C@@H]([C@H](CO)N1C(=O)N(C=N1)C2=CC=C(C=C2		4,49	Inside (Euclidean distance)		3,85	Inside (Euclidean c	5,09	-	0,85	5,13	-	0,85	5,03	-	0,75	4,768	5,5
14	Tebuconazole	CC(C)(C)C(CCC1=CC=C(C=C1)Cl)(CN2C	3,7	3,51	Inside (Euclidean distance)		2,34	Inside (Euclidean c	3,63	3.7	1	3,89	3.7	1	4,01	3.7	1	3,8887	3,7
15	Flusilazole	C[Si](CN1C=NC=N1)(C2=CC=C(C=C2)F	3,7	1,93	Inside (Tanimoto)		1,78	Inside (Euclidean c	5,09	3.7	0,75	4,89	3.7	0,75	5,39	3.7	0,75	3,8856	3,7

Table 1. Excerpt of database for NAM-based physicochemical and toxicokinetic values for parameters in *in vitro* distribution kinetics and PBK models.

Overarching activity for ASPIS in 2023 is a case study on steatosis. Within this working group, the triazole fungicides were chosen as case study chemicals. Exposure to these chemicals is associated with liver steatosis in humans, they are included in the chemical lists of the three

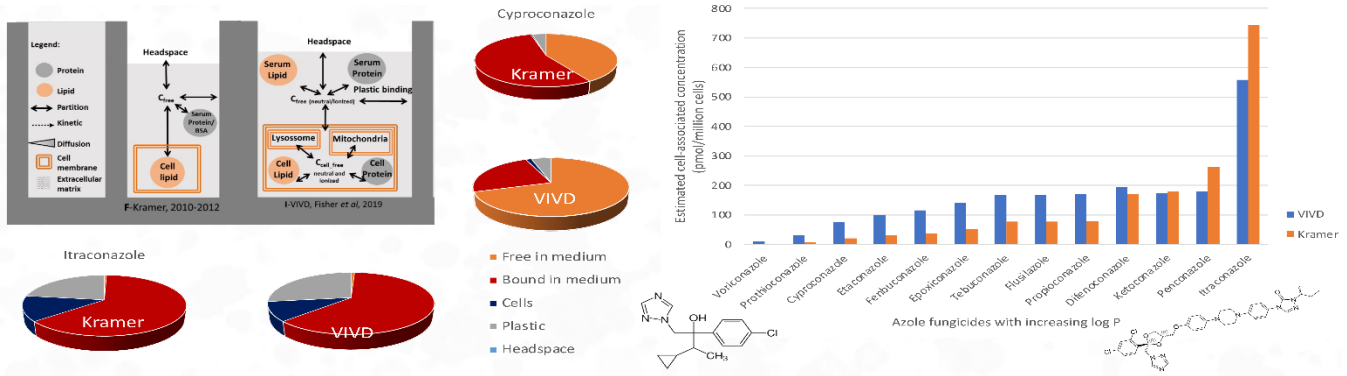


Figure 12. Comparison of free exposure medium concentrations
Comparisons of the steatosis screening assays were made using the Kramer (ONTOX) and VIVD (RISK-HUNT*3R*) in vitro distribution kinetics models.

consortia, and there is internal and external exposure data available for this group. The case study should allow the working group to define the applicability domain of and assign weights-of-evidence to individual exposure assessment tools used and developed in the three consortia. The case study is currently divided into four activities: (1) Collate database of physiochemical and toxicokinetic (absorption, distribution, metabolism, excretion, ADME) values for a selection of triazoles to compare experimental, *in silico* and *in vitro* derived values. This activity has been completed and an excerpt of the database is found in **Table 1**.

(2) Compare free and cell-associated triazole concentrations in *in vitro* ADME and steatosis systems estimated by *in vitro* distribution kinetics models. A comparison of free exposure medium concentrations in a steatosis screening assay with HepG2 cells using the model in ONTOX and RISK-HUNT*3R* has been prepared and is summarised in **Fig. 12**. For 2024, the aim is to include the model estimates of the Fischer et al. (2017) model, which is used in PrecisionTox. Currently, the levels in medium, plastic and cells of a selection of six triazole chemicals in the ONTOX steatosis assay with HepaRG are being assessed at Wageningen University. The plan is to finalise the experiments by the end of 2024 to compare experimental with model estimates. As a deliverable, a manuscript for publication is planned for 1 June 2025.

Compound Name	LOAEL (mmol/kg/day) from rodent studies	Unbound C_{max} in plasma in humans corresponding to LOAEL (µM)
Difenoconazole	0.012	0.058
Propiconazole	0.027	0.46
Epoxiconazole	0.002	0.068
Cyproconazole	0.001	0.044
Prothioconazole	0.01	0.35
Fenbuconazole	0.004	0.04
Itraconazole	0.001	0.00085
Ketoconazole	0.002	0.082

Table 2. Estimated human plasma concentrations of triazoles using Simcyp™ associated with LOAEL in rodent studies.

(3) Predict human oral dosing regimens, and plasma and liver concentrations of triazole fungicide exposure associated with steatosis using PBK modelling. **Table 2** lists the plasma concentrations of triazoles in humans associated with lowest observed adverse effect levels (LOAEL) from rodent studies estimated using PBK modelling. Currently, *in vitro* intrinsic clearance assays using primary human hepatocytes (RISK-HUNT*3R*) and HepaRG (ONTOX) are being measured experimentally for input into PBK modelling, to finalise by the end of 2024. PBK model estimates of maximum plasma concentrations and repeated external (oral) doses in rats and humans using the PK-Sim and

Simcyp™ model will be compared after experimental data becomes available. These comparisons are to be presented at the ASPIS 2024 annual meeting.

Aims for 2024

As aforementioned, during the coming year, the KEWG will compare *in vitro* biokinetic model estimates with experimentally obtained levels in the ONTOX steatosis assay. Similarly, ONTOX and RISK-HUNT*3R* PBK model estimates of triazoles plasma concentrations associated with LOAELs in rodents will be compared once experimental *in vitro* clearance values become available. The ASPA workflow will be updated to include the exposure and toxicokinetics tools available within APSIS for each decision node. Lastly, a new case study chemical associated with developmental neurotoxicity will be selected to study the applicability of exposure assessment tools in ASPIS.

Exposure assessments are prohibitively laborious and performing exposure assessments for all new, ASPIS-specific chemicals is therefore infeasible. The development of the tiered testing and assessment strategy does, however, partly overlap with the individual goals of the three projects so that no major obstacles are foreseen. It is uncertain if sufficient exposure and kinetic information is/will be available to quantify uncertainty and variability of *in vitro* and *in silico* ADME tools.

Dissemination activities

- Oral presentation at the 12th World Congress on Alternatives and Animal Use in the Life Sciences (WC12) by Sylvia Escher.

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