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The Precision Toxicology initiative

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ABSTRACT

The goal of *PrecisionTox* is to overcome conceptual barriers to replacing traditional mammalian chemical safety testing by accelerating the discovery of evolutionarily conserved toxicity pathways that are shared by descent among humans and more distantly related animals. An international consortium is systematically testing the toxicological effects of a diverse set of chemicals on a suite of five model species comprising fruit flies, nematodes, water fleas, and embryos of clawed frogs and zebrafish along with human cell lines. Multiple forms of omics and comparative toxicology data are integrated to map the evolutionary origins of biomolecular interactions that are predictive of adverse health effects, to major branches of the animal phylogeny. These conserved elements of adverse outcome pathways (AOPs) and their biomarkers are expected to provide mechanistic insight useful for regulating groups of chemicals based on their shared modes of action. *PrecisionTox* also aims to quantify risk variation within populations by recognizing susceptibility as a heritable trait that varies with genetic diversity. This initiative incorporates legal experts and collaborates with risk managers to address specific needs within European chemicals legislation, including the uptake of new approach methodologies (NAMs) for setting precise regulatory limits on toxic chemicals.

1. Background

Alternative methods to traditional animal testing are preferred by scientists, regulatory agencies, industry, and the public for assessing chemical safety (Hartung, 2009). In addition to the slow pace, high cost, and ethical concerns of animal testing, the traditional approach offers little guidance for converting test outcomes using mammalian species to evidence-based interventions that protect human health, as laboratory animals are known to be imperfect predictors of human toxicological response (Bailey et al., 2014). With more than 350,000 registered chemicals and chemical mixtures in use world-wide (Wang et al., 2020) and a growing demand for evidence-based safety regulations of hazardous substances, there is an urgent need for high-throughput and human-relevant approaches to evaluate chemical safety.

Automation in toxicity testing using human cell lines has delivered valuable new data, particularly through the contributions of the Tox21 and ToxCast initiatives, which examine cellular toxicity pathways rather than solely relying on adversity endpoints such as reproductive failure and death (Collins et al., 2008). More recently, these *in vitro* approaches are providing data that enable screening of chemicals for specific hazards based on knowledge of cellular key events within adverse outcome pathways (AOPs) (Noyes et al., 2019). However, understanding the systemic nature of toxicity requires the study of whole organisms to observe pathway-based health-relevant changes involving interactions among different cell, tissue, and organ systems. For example, toxicity response can involve the endocrine and/or nervous system (Kiyama and Wada-Kiyama, 2015; Smirnova et al., 2014), or even the microbiome (Koontz et al., 2019). Medical and pharmacological research communities make use of model organisms (Ankeny and Leonelli, 2011) that allow for high-throughput screening to reveal disease pathways and to assess the efficacy and toxicity of potential treatments (Giacomotto and Segalat, 2010). These efforts have been supported through genome characterization of biomedical model species including *Drosophila melanogaster* and *Caenorhabditis elegans* by initiatives such as modENCODE (Brown and Celniker, 2015). Within the toxicology field, however, utilization of such approaches has been hindered by regulatory caution in accepting model organisms such as flies and worms as valid human surrogates. Moreover, there have been few large-scale attempts to compare human responses to chemicals with responses of non-mammalian species. By combining evolutionary theory, quantitative genetics, and data science with comparative toxicology experiments, *PrecisionTox* aims to close this research gap by demonstrating that the mechanisms of chemical toxicity can fundamentally correspond among distantly related animal species due to our shared biology by evolutionary descent.

This concept of 'toxicity by descent' (Colbourne et al., 2022) is underpinned by a century of comparative physiology research that exploits the functional diversity among animal species for understanding biological questions, especially those that are not easily answered by the study of humans (Jørgensen, 2001), while also recognizing the potential pitfalls of modeling the human condition based on experimental findings from a single species. Although there are many useful model organisms to investigate specific physiological and biomedical problems (Little et al., 2021), every species has its own unique biology and its own natural history that can hinder translational research (McGonigle and Ruggeri, 2014). The challenge of choosing the best non-mammalian models is particularly delicate for toxicology; key events of AOPs can be especially difficult to assess and to compare between species. Toxicological traits are often highly plastic and dependent on dose, exposure time, ADME (absorption, distribution, metabolism, and excretion), tissue and organ-specific functions, life-history and developmental stages, and ecological and general physiological conditions, among other

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variable aspects of basic biology including genetics. The difficulty of assessing the relevant biological similarities to enable one species to reliably serve as a surrogate model for another in regulatory toxicology is a main barrier to the use of non-mammalian alternative test systems for the protection of human health.

One approach to resolving the problem of cross-species extrapolation in toxicology is to interpret comparative experimental results by using evolutionary principles. This approach can guide the discovery of toxicity pathways that are deeply rooted in the evolutionary history that humans share with other animals. This search for conserved pathways necessitates a departure from the experimental use of a single, presumably adequate, surrogate model organism towards instead obtaining experimental results from a diverse suite of exemplary models (Bolker, 2009) that together represent major branches of the animal phylogeny. By interpreting results from a systematic investigation of toxicological responses across species in light of a known phylogenetic tree, the conserved elements of AOPs are revealed. Depending on which species share these AOP elements, these observed pathways can be used to extrapolate the chemical toxicity response to all species that evolved after that phylogeny diverged. If the AOP elements are shared by both vertebrates and invertebrates, the response can be extrapolated to all species, including humans, as the shared traits have been conserved from their common ancestry (Wiley et al., 1991). At present, evolutionary principles have been applied to provide ecotoxicological insights into patterns of sensitivity among species (Buchwalter et al., 2008; Hammond et al., 2012) to hypothesize origins of developmental toxicity (Leung et al., 2017) and human genetic diseases (Benton et al., 2021). Indeed, comparative genomics investigations are finding that many classes of genes related to human disease are ancient (Colbourne et al., 2022; Domazet-Loso and Tautz, 2008), suggesting that translational research could be carried out effectively by considering the homology of toxicological traits.

The primary goal of the Precision Toxicology initiative is to demonstrate this fundamental approach to understanding how toxic chemicals cause harm to humans: considering our shared genetic ancestry with distantly related animals and investigating how chemicals disrupt biological processes that are fundamental to health across the animal kingdom. This initiative also extends its comparative toxicology approach from the study of intraspecific variation to interspecific genetic variation that accounts for individual differences in susceptibility to the toxicological effects of chemicals. *PrecisionTox* will provide databases and computational tools that allow scientists, risk managers, and chemical safety regulators to utilize new approach methodologies (NAMs) for promoting discoveries and setting precise regulatory controls on toxic chemicals.

2. Approach

In December 2019, the EU Green Deal articulated a promise to provide a toxic-free environment by 2050 (European Commission, 2019). In the following year, the European Chemicals Strategy for Sustainability set out a route to meet this promise (European Commission, 2020). Delivery on the strategy demands better and quicker identification of endocrine disrupters and highly persistent chemicals such as per- and polyfluoroalkyl substances, as well as a better understanding of the effects of chemical mixtures. New Approach Methodologies (NAMs), which collectively refer to alternatives to traditional animal testing, will have a role to play in the delivery of the strategy by employing mechanistic biomarkers to assist in grouping of chemicals for regulatory scrutiny.

The *PrecisionTox* initiative (PrecisionTox, 2021) was launched in February 2021 in response to the call for NAMs in chemical safety testing by the European Commission under its Horizon 2020 program. A definition of NAMs is “any technology, methodology, approach, or combination that can provide information on chemical hazard and risk assessment to avoid the use of animal testing” (USEPA, 2020). *PrecisionTox* is achieving this goal by identifying molecular key event biomarkers, shared across species, that signal disease progression within AOPs associated with exposure to toxic chemicals. If successful, this initiative offers new regulatory options informed by observable mechanistic processes leading to toxicity that bridge the divide between human and environmental toxicology and are consistent with the accepted framework of integrated approaches for chemical safety testing and assessment (OECD, 2021a). To support this development, *PrecisionTox* simultaneously deploys high-throughput testing across an evolutionarily diverse suite of five well-established biomedical and toxicological model species: *Drosophila melanogaster*, *Caenorhabditis elegans*, *Daphnia magna*, and embryos of *Xenopus laevis* and *Danio rerio*.

Xenopus laevis (African tree frog) is a well-established toxicological model whose draft genome sequence has been published (Session et al., 2016). *Xenopus* shares 85% of human homologous gene families, allowing results from *Xenopus* assays to be extrapolated, in many cases, directly to humans. Biotransformation of compounds employs homologous pathways in *Xenopus* and mammals (Fini et al., 2009; Fini et al., 2007; Fini et al., 2012). ***Danio rerio*** (Zebrafish) embryos share common principles of cellular and organism physiology with humans (Ablain and Zon, 2013; Flinn et al., 2009; Lieschke and Currie, 2007; McGrath and Li, 2008) and have therefore been widely used to unravel the mechanisms of many inherited diseases (Cui et al., 2011; Lieschke and Currie, 2007). Their drug metabolism and detoxification pathways are shown to be like those of humans (Vliegenthart et al., 2014). ***Drosophila melanogaster*** (a dipteran insect) is a tool-rich genetic model whose striking conservation of human disease genes makes it an important model for neurological diseases, cancers, heart disease, metabolic diseases and diabetes, and responses to infection by human pathogens. Drosophilidae have a brain, a beating heart, a tubular network analogous to lungs, an osmoregulatory/excretory system analogous to kidneys, a functioning microbiome, and all the other aspects of physiology and homeostasis relevant to *PrecisionTox*. ***Daphnia magna*** (a branchiopod crustacean) is the most-used system for ecotoxicological testing worldwide (Denslow et al., 2007; Shaw et al., 2007). The first sequenced crustacean genome demonstrated that Daphniidae share more genes with humans than any other sequenced invertebrate (Colbourne et al., 2011). ***Caenorhabditis elegans*** (a nematode worm) is a long-used model system for cellular, developmental, and molecular aspects of the effects of toxicants on growth and development as well as gene expression (Leung et al., 2008). An exceptionally detailed database has been compiled on its genome, cell, and developmental biology (Harris et al., 2020), and all somatic cells in the living organism can be observed (Altun et al., 2021). Several studies have demonstrated that changes in *C. elegans* following chemical exposure appear to be predictive of developmental shifts or neurological damage seen in laboratory studies using rodents (Leung et al., 2008).

Within *PrecisionTox*, the high volume and varied data from exposure studies of these species employing phenotyping, metabolomics, and transcriptomics are integrated with existing knowledge of adversity and disease etiology using explainable artificial intelligence algorithms (X-AI). X-AI enables the extraction of meaning from large, complex, highly dimensional data (Wellawatte et al., 2023) to identify relevant biomolecular networks to support the discovery of conserved toxicity pathways and to understand their significance to human health. Complementary investigations with human cell lines further strengthen the predictive power of evolutionary-derived functional genomics and metabolomics networks to exposure-related AOPs in humans.

In addition to this phylogenetic approach, which we term *phylotoxicology*, *PrecisionTox* comprises two other key concepts: *variation in susceptibility* and *embedded regulatory translation*. Methods associated with these three concepts are described below. *Variation in susceptibility* pursues precision in evaluating risk and setting safety thresholds by recognizing that individual susceptibility is a heritable trait that varies with genetic diversity. This approach would replace the presently arbitrary safety adjustment factors (Dorne and Renwick, 2005), which aim to set safe chemical exposure levels for humans by lowering benchmark

dose estimations of toxicity from animal experiments by a factor of 10, and to account for inter-individual variability by an additional factor of 10. Instead, systematic evaluation of DNA variation for susceptibility across genetically diverse populations of both model species and humans will be used to determine safety factors. The expected outcome is a more accurate determination of exposure thresholds that aims to be protective for even the most vulnerable members of society. *Embedded regulatory translation* acknowledges that science alone cannot engineer change. To ensure that the initiative's outputs can be meaningfully applied to health protection, *PrecisionTox* collaborates via external agreements with key stakeholders in the EU, UK, and U.S. in project planning, execution, and dissemination. The initiative also introduces a practical framework, considered below, for progressively unlocking regulatory instruments as evidence accumulates of the causal links between exposure to chemicals and adverse outcomes.

PrecisionTox is constructed from six work packages (Fig. 1) linked by a workflow from stakeholder integration (WP1) and early-stage comparative toxicology data generation (WP2) through to molecular data production (WP3) and phenomic, transcriptomic, and metabolomic data integration for the discovery of novel mechanisms of toxicity that will be cast into a framework of AOPs and molecular key event biomarkers (WP5). These biomarkers in turn form the basis of a NAM Toolbox for regulatory use. Confirmation of these predictions for human relevance will be performed using powerful genetic model systems and human cell lines (WP4). Quantitative genetics and single-cell gene expression profiling will be used to map the underlying variation that determines an individual's and tissue-specific levels of susceptibility to the toxicological effects of chemicals. This genetic variability knowledge flows into a new method for benchmark dose estimation that strengthens the application of NAMs in regulatory decision-making to include setting rational, data-derived human safety standards. The NAMs will be codified in the publication of case studies and guidance documents that will chart where formal legal responsibility for chemical assessment resides to locate the precise extent of legal authority in the accommodation of NAMs in chemical safety reporting by industry (WP6).

In addition to incorporating expertise in molecular profiling and computational integration of highly dimensional data, the consortium leadership features legal and governance experts that are working at the intersection of science and policy (Table 1). Expertise in the five model species is distributed across the University of Birmingham (UK), Indiana University Bloomington (U.S.), Clemson University (U.S.), Karlsruhe

Table 1
Consortium members of *PrecisionTox*, their key complementing skills and contributions to the six initiative work packages.

Partner	Key complementing skills	Work Package involvement
University of Birmingham	PhyloToxicology; Metabolomics; Model species *Daphnia* and *Danio*; Governance, Management	WP1; WP3; WP6
Ruprecht-Karls-Universitaet Heidelberg	Protein evolution; Biomolecular pathways and networks; Data Commons	WP5
Indiana University	Model species *Caenorhabditis* and *Drosophila*	WP2
Karlsruher Institut Fuer Technologie	High-throughput screening, Model species *Danio* and *in vitro* endpoints	WP2
Centre de Regulacio Genomica	Bioinformatics; Systems Biology; Computational data integration; Modeling	WP4; WP5
WatchFrog	Model species *Xenopus*; Endocrine disruption; Thyroid physiology	WP2
Clemson University	*Drosophila* genetics, quantitative genetics, systems biology	WP4
University of Oxford	FAIR data; Data standards; Data publication; Ontology	WP3; WP5
Helmholtz-Zentrum fuer Umweltforschung	Model species *Danio* and *in vitro* endpoints; Toxicokinetics; Analytical chemistry	WP2
Altertox	Promotion of the 3Rs; Dissemination; Communication; Networking; Management	WP1
Cambridge Cell Networks	Commercial toxicology software and database development	WP5; WP6
Michabo Health Science	Regulatory science; NAMs; Explainable AI; Multi-omics; Training; Stakeholder engagement	WP6
Acondicionamiento Tarrasense Associacion	*Daphnia* toxicology, Human and rat cell lines verification, complex cell models (commercial organ on chip models); Technology transfer	WP2; WP4
Misvik Biology	Cell based high throughput screening	WP4
Latvia MGI Tech	Next Gen Sequencing, Omics workflow	WP3

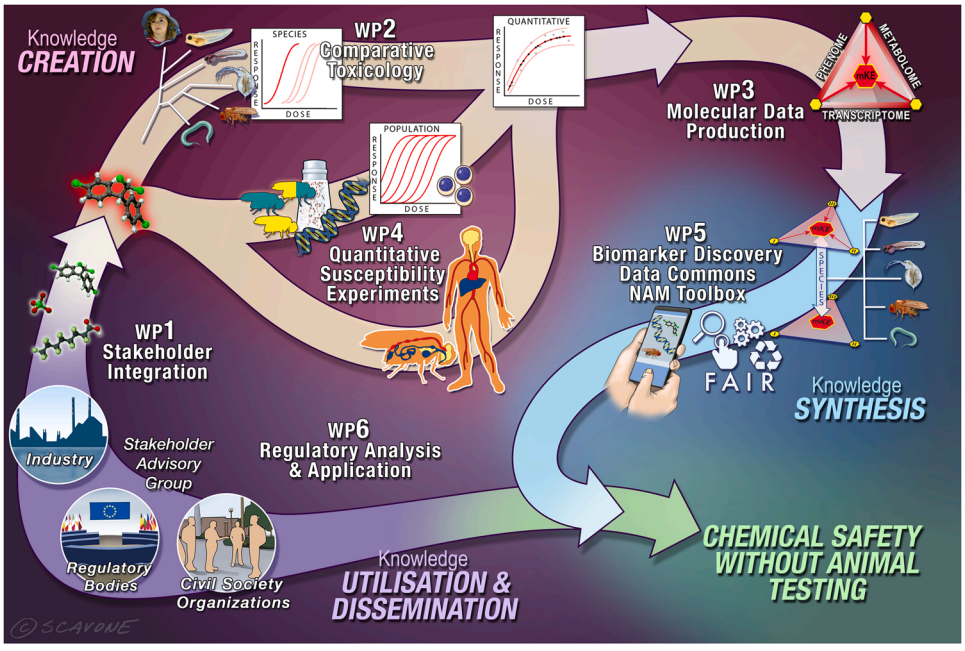

Fig. 1. Construction of *PrecisionTox* from six work packages (WP). WP1 and WP6 inform paths for knowledge creation, utilization/dissemination, and synthesis by the co-production of chemical selection and case studies that result in measurable improvements to chemical safety assessment. Molecular data, transcriptomes, and metabolomes are produced in WP3 from the experimental results and samples created by WP2 and WP4. Combined with measurements of adverse toxicological outcomes (phenome) using explainable artificial intelligence and machine learning, conserved biomarkers across diverse animal species including humans are discovered and synthesized within the Data Commons. The data are integrated within adverse outcome pathways and provided with computational tools that are required for effective chemical safety regulation (WP5). Table 1 maps the consortium members to the work packages.

Institute of Technology (KIT, Germany), Helmholtz Center for Environmental Research (UFZ, Germany), Watchfrog SA (France), and Leitat Technological Center (Spain). Human cell-based screening is conducted by UFZ, KIT, and Misvik Biology (Finland). Expertise in biomolecular pathway evolution comes from the Universities of Heidelberg (Germany) and Birmingham. Molecular data are produced by Latvia MGI Tech (Latvia) and Phenome Centre Birmingham (UK). Computational data integration, including machine learning, is supplied by Michabo Health Science Ltd. (UK) and the Center for Genomic Regulation (Spain). Data standardization, enrichment, and utilization are driven by the University of Oxford (UK) and Cell Networks (Germany). Policy guidance and coordination with regulatory bodies, drawing on expertise across the consortium and particularly at the University of Birmingham, Indiana University, and Michabo Health Science, is further facilitated by Altertox (Belgium).

PrecisionTox joins two other EU consortia in forming the ASPIS cluster (ASPIS, 2021): *ONTOX* (Horizon2020, 2021a) and *RISK-HUNT3R* (Horizon2020, 2021b). The cluster collectively carries €60 million of Horizon 2020 funding to collaboratively enhance chemical safety, without resorting to animal testing, by better employing NAMs. Attached to ASPIS is a regulatory forum of EU regulators and international experts in the science and governance of risk management led by the European Commission's Joint Research Centre, which is tasked with steering the regulatory relevance of the funded research toward fulfilling the goal of a toxin-free environment in line with the aspirations of the European Green Deal.

2.1. Prioritisation of NAMs for regulating chemical toxicity

Within the European Union, both the Green Deal and the Chemicals Strategy for Sustainability seek to drive towards a pollution-free environment in which EU citizens are protected from the hazardous effects of chemical pollution (van Dijk et al., 2021). This goal necessitates prompt and reliable toxicity assessments of the rapidly expanding set of chemical substances coming onto the EU single market. *PrecisionTox* offers NAMs for chemical safety assessment derived from knowledge of the conserved molecular response pathways underpinning toxicity. EU regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has written into its legal text the need to move away from animal testing to alternative methods in a manner already achieved by the EU Cosmetics Regulation (EC No 1223/2009), which bans such testing. These regulatory instruments sit alongside Directive 2010/63/EU on the protection of animals used for scientific purposes, which actively promotes '3Rs' principles to replace, reduce, and refine the use of animals used for scientific purposes (Russell and Burch, 1959).

By reinvigorating a comparative approach to toxicology using a suite of model organisms that are currently classified as non-sentient, *PrecisionTox* will provide information on toxicity pathways that will further reduce the need for animal testing in the future by contributing to a mechanistic understanding of chemical impacts on human health. This initiative is timely because the main plank of EU chemicals regulation (REACH) is periodically reviewed and revised, including in 2022-23. Moreover, REACH has yet to grapple with thousands of data-poor chemicals, each of which are manufactured or imported at a volume of between 1 and 10 tonnes per year. The *PrecisionTox* approach to NAMs is particularly suited to assist with the safety assessment of such substances produced by small- and medium-sized enterprises, including informing the grouping of chemicals based on their modes of action.

2.2. Data-rich workflows

PrecisionTox's untargeted multi-omics approach is designed to uncover both known and currently unknown homologous molecular interactions and pathways of toxicity that can reliably be extrapolated across species. Earlier projects have shown that molecular data are inherently noisy when measuring omics signatures independently (de Jong et al., 2019). This noise has constrained the initial promise of a technologically led revolution in toxicology based on measuring changes occurring in all genes (Jacobs, 2009). However, when molecular observations are made between transcriptomics and metabolomics, experimentally relevant signatures are extracted and combined into co-responsive networks of genes and metabolites that show reproducible correlative structure across many samples and test conditions (Weighill et al., 2019). This clarity is driven by the fact that individual omics modalities have distinct sources of noise and bias, which are largely or completely uncorrelated between modalities. Within *PrecisionTox,* the combined application of phenomics, metabolomics, and transcriptomics modalities serves to triangulate true signals in data of specific pathways and processes (Streich et al., 2020). Comparative analyses across distantly related species are expected to reveal evolutionarily conserved true signals (as well as lineage-specific signals) of molecular processes that specifically respond to the chemical exposures. These discoveries can serve to fill regulatory and policy gaps by expanding the catalog of molecular key events placed within human-relevant AOPs.

2.3. Phylotoxicology

We demonstrate 'toxicity by descent' (see Graphical Abstract) by comparing molecular, cellular, multi-organ, metabolic, physiological, and behavioral outcomes across our chosen suite of evolutionarily diverse animal models exposed to a range of test chemicals that include organ-specific toxicants (heart, liver, kidney, nervous system) and chemical classes (endocrine disruptors, pesticides, pharmaceuticals). Additionally, test chemicals include not only compounds present in the human exposome and the natural environment but also chemicals with known modes or action and chemicals with unknown modes of action. Seven toxicological endpoints including cellular effects are assayed (Table 2). *Danio* embryos are used to identify embryotoxic and teratogenic effects due to this model's concordance with human developmental toxicity. These assays have recently been improved by including deep learning algorithms for the detection of various body structures (small or curved body, shortened tail or fins, cyclopia, pigmentation, edema, necrosis, changes in organ structure) (Shen and Zuo, 2020). Both *Xenopus* and *Danio* embryos are used to elucidate chemically induced changes in the development and function of various endocrine glands. Hormone systems such as the thyroid or glucocorticoid hormonal axis

Table 2
Comparative toxicology assays and endpoints for each of the model test systems.

System	Assay read-outs	Endpoint with human relevance
Xenopus	Embryo thyroid assay	Reproductive toxicity
Danio	Glucocorticoid responsive *in vivo* luciferase activity	Reproductive toxicity
	Automated microscopy	Embryotoxicity & teratology
	Larvae electrocardiography	Cardiotoxicity
	Video recording of heart	Cardiotoxicity
	Embryo photomotor response	Neurotoxicity
Drosophila	Analysis of motility patterns, survival	Neurotoxicity
Daphnia	Motion analysis, reproduction, survival	Neurotoxicity & reproductive toxicity
Caenorhabditis	Motion analysis	Neurotoxicity
2D Human cell cultures	Immunofluorescence microscopy	DNA damage
	High throughput Western blotting	DNA damage
	Reactive oxygen species	Oxidative stress
	CellTiter Glo and Hoechst staining	Cell viability/death
	Stress response	Reporter gene assays for activation of glucocorticoid and antioxidant (nrf-ARE) response elements

are highly related to those in the human body and are functional at late embryonic stages (Ankley and Johnson, 2004; Dang, 2022). Cardiac toxicity including arrhythmias is assayed using *Danio* embryos. Cardiac toxicity is often manifested in a form of QT prolongation (extended intervals between the heart contracting and relaxing), which can predispose people to a sudden cardiac arrest. Unlike in the more commonly used mice, zebrafish eleutheroembryos have a cardiac electrophysiology similar to that of humans and are also sensitive to compounds causing QT prolongation in humans (Dhillon et al., 2013). Locomotory behavioural measurements are taken using *Danio* embryos, *Caenorhabditis*, *Daphnia,* and *Drosophila,* which are highly sensitive assays of toxicological effects on their central nervous systems, particularly for compounds with neurodevelopmental or neurofunctional modes of action. Omics data are sampled for each test system over similar exposure time intervals and effective concentrations for each chemical, thereby improving the likelihood of observing molecular response networks anchored to shared adverse outcomes across species.

This experimental design builds on knowledge that many gene regulatory networks involved in stress response to environmental conditions are highly interconnected with developmental pathways throughout the course of animal evolution, leading to exposure-related effects on homologous structures or functions among species via molecular toxicological responses that are conserved among animals (Leung et al., 2017). By centrally applying automated technologies to simultaneously extract metabolites and total RNA from the same sampled materials (including biological replicates and controls over time), this experiment provides up to 21,000 samples for omics interrogation, yielding a molecular dataset of more than 500 million measurements of gene and metabolic products. Triangulating phenotypes, metabolomics, and transcriptomics analyses using X-AI strategies forming co-response networks (Murdoch et al., 2019) enables the identification of core biomarkers of animal toxicity pathways. Because these investigations concern observable adverse effects, molecular key event biomarkers are identified to signal progression along the discovered AOPs. This approach is expected to reveal fundamental biological mechanisms by which organisms respond to toxicological perturbation, enabling the prediction of adverse outcomes based on observed biomolecular processes rather than extrapolating from adversity endpoints such as reproductive failure and death.

Single-cell RNA sequencing of the model test species is also employed to specifically challenge the unfounded notion that toxicity pathways predictive of human organ damage are restricted to mammalian species. Molecular responses leading to specific tissue-relevant toxicity in humans (for example neurotoxicants, hepatotoxicants, nephrotoxicants, and cardiotoxicants) are expected to emerge from homologous organs or cell types, which can represent proto-organs in distantly related species or early developing organs in other vertebrates, despite their morphological and anatomical differences. For example, liver function and liver-specific molecular markers in humans are similarly expressed in the fat body of *Drosophila* (Sondergaard, 1993), in the intestine and epidermis of *Caenorhabditis* (O'Reilly et al., 2014), and in the liver of *Danio* larvae (Shehwana and Konu, 2019). Where toxicological AOPs are evident throughout relevant branches of the animal kingdom, humans can be expected to share these responses. Moreover, having identified the biomolecular processes by which these responses occur, *PrecisionTox* will determine the appropriate human cell line and 3D organ models for further investigation and perform empirically targeted *in vitro* research.

Human cell lines are selected to reflect responses seen in the whole-organism models. While *in vivo* models address the complexity of multicellular organisms, the human cell lines provide a comparative link to humans at the cellular level. To enable massive numbers of analyses and modelling towards existing toxicity, transcriptomics, and metabolomics data, the *in vitro* models are further qualified by originating from normal tissues or closely mimicking their normal counterpart *in vivo*. This approach supports high-volume data generation through extensive application in toxicological testing and mechanistic studies, enabling comparison to other high volume toxicogenomics projects (see review of available resources (Trapotsi et al., 2022)).

The *PrecisionTox* human cell systems will be complemented by three reporter gene assays that have already been used for *in vitro*-based chemical safety screening by Tox21 (Attene-Ramos et al., 2013). The reporter gene assays serve to define the concentration range for the testing of more complex cell systems, anchor the results with respect to baseline toxicity (Escher et al., 2019), and indicate the stress response likely to be encountered *in vivo* (Simmons et al., 2009). The cellular model systems include the HepG2 cell line, which exhibits liver specific functions and metabolic competency and is suitable to predict drug induced liver injury (DILI) and genotoxicity in humans (Albrecht et al., 2019; O'Brien et al., 2006; Westerink et al., 2010) and the BEAS-2B lung epithelium cell line, which retains normal epithelial features and is able to undergo differentiation in response to serum or high-density culture typical of normal, non-transformed bronchial epithelial cells (Garcia--Canton et al., 2013; Ke et al., 1988). Depending on the comparative results from *phylotoxicology* and single-cell RNA sequencing, additional cell models relevant for the affected target organ(s) will be considered for analysis.

2.4. Variation in susceptibility

In addition to developing biomarkers to improve confidence in cross-species extrapolation of systemic chemical toxicity, *PrecisionTox* also aims to improve methods for determining exposure thresholds that can rationally account for genetic variation in susceptibility within populations by studying the general principles that underlie the relationship between genetic variation and susceptibility to being harmed by chemical exposure. *PrecisionTox* introduces two model systems of genetic diversity to experimentally identify genetic targets (QTLs: quantitative trait loci) controlling the shape of the dose-response relationships that vary among individuals of a population. The *Drosophila* Genetic Reference Panel (DGRP) is co-developed by T. Mackay and R. Anholt of *PrecisionTox* as a community resource that is housed and distributed worldwide through the Bloomington *Drosophila* Stock Centre (BDSC, 2021). The panel consists of 192 inbred strains that were derived from a single outbred population (Huang et al., 2014). Therefore, the panel is representative of natural genetic variation. The panel is fully characterized for mapping of QTLs with complete euchromatic sequence information and a fine-grained recombination map, making it ideal for localizing causal variants. The Human Genome Diversity Panel (HGDP) is a resource of lymphoblastoid cell lines (LCL) distributed to the scientific community since 2002 through the Coriell Institute biobank (NIGMS, 2021). This panel is composed of LCL and DNA of 1050 individuals from 51 populations of diverse heritages and from locations throughout the world. The power of HGDP for this investigation is in the genomic and demographic data that are freely available from previous studies such as the 1000 genomes project (Bergstrom et al., 2020; Genomes Project et al., 2012) that can be used to map genotype and phenotype interactions. The HGDP links variant transcriptional responses to genomic difference amongst human cell lines without the need for further genotyping.

The DGRP and HGDP have independently made significant contributions to the understanding of genetic diversity and human variation (Bergstrom et al., 2020). However, they have only recently been used in the context of toxicology to identify genetic variants of adverse outcomes (Abdo et al., 2015a; Abdo et al., 2015b; Zhou et al., 2016). Each resource offers distinctive advantages. While *in vitro* systems sample actual genetic variation within human populations, they yield only a partial view of the total biological variability and susceptibility to chemical hazards (Zeise et al., 2013). By contrast, variability at the molecular, cellular, and tissue levels is integrated in DGRP population studies. To our knowledge, *PrecisionTox* is the first initiative to combine *in vivo* and *in vitro* approaches to generate a human model for the health

impact of chemicals by determining susceptibility given a known genetic background. This approach will focus on identifying the conserved genetic determinants of molecular key event variation between an invertebrate model species from the phylotoxicology panel and humans that determines the shape of the dose-response relationship.

Examining toxicity outcomes across these genetically diverse fruit fly populations and human lymphoblastoid cell lines allows for the mapping of QTLs, which are regions of DNA associated with the expression of toxicological response to a given compound or class of compounds. QTLs are understood to influence phenotypes by regulating gene expression (Nica and Dermitzakis, 2013). Variation in transcript abundance is therefore a quantitative trait and, importantly, one that can be measured accurately with a sufficiently large volume of data. These qualities make it advantageous to identify expression QTLs (eQTLs) controlling variation in mRNA levels (e.g., exposure-related mRNA expression). The defining feature of exposure-related eQTLs is that the effects of exposure will differ depending on the genotype. This identification is important given evidence that genetic variation is central in many disease phenotypes (Albert and Kruglyak, 2015). The identification of conserved QTLs among flies and human cells that regulate gene co-expression networks that are responsive to genetic variation associated with a toxicological response allows the application of molecular key event biomarkers in risk assessment and setting safe exposure limits. Reverse genetic engineering tools (gene knock-in and knock-out (Sander and Joung, 2014)) allow for verification of the predictive power of QTLs and associated biomarkers. Additionally, 3D human organ tissues (liver, heart, brain) are also used to confirm cross-species extrapolation for organ-specific QTLs.

2.5. Embedded translation

The impact of *PrecisionTox*'s findings on protecting human health will ultimately depend on their utility in chemical risk management. Therefore, integrated into *PrecisionTox's* scientific activities is analysis by consortium experts directly engaging with policy makers and EU, UK, and U.S. regulators and other end users in project activities via the ASPIS regulatory forum (described above) and collaborative agreements. The *PrecisionTox* Stakeholder Advisory Group, which provides feedback and input on the initiative, incorporates representatives from the European Commission's Joint Research Centre, the U.S. National Institutes of Health and U.S. Environmental Protection Agency, and the UK's Department for Environment, Food, & Rural Affairs, along with private and nonprofit organizations working to implement NAMs. These stakeholders play a central role in selecting *PrecisionTox* priority compounds for testing, exploring avenues for innovation and exploitation, identifying socio-technical barriers to uptake, and advising on case studies for regulatory implementation. To facilitate these translational activities, a key contribution of the initiative is its learning programmes at the interface of science and governance, and the introduction of its Progressive Precision Regulatory Framework. This framework links discrete stages of molecular biomarker identification (labeled informative, indicative, relational, probative, and predictive) with corresponding regulatory possibilities beginning with grouping and read-across of compound classes and progressing through hazard identification, characterization, and assessment to ultimately offer data-driven estimation of safety factors.

Regulatory instruments that may be informed by *PrecisionTox* findings can take a number of different forms. REACH is highly detailed with almost 150 Articles and 17 technical annexes, yet it is not prescriptive with regard to testing. This regulation demands that testing be reliable, while explicitly promoting the use of alternative test methods so that testing on animals constitutes a last resort (Cihák, 2009). *PrecisionTox* is designed to satisfy these REACH requirements for reliable alternative methods. In addition to this formal regulation, there are a host of test guidelines and protocols for toxicity testing and assessment recognized by bodies such as the Organisation for Economic Co-operation and Development (OECD, 2021b). These policies, while historically centered on *in vivo* testing in accordance with past practices, are increasingly demanding chemical assessment that does not resort to animal testing. These guidelines are poised to adapt to accommodate Integrated Approaches to Testing and Assessment (OECD, 2021a), allowing for a more pragmatic approach to a science-led hazard characterisation by adopting iterative judgements based on approaches such as grouping and read-across. *PrecisionTox* findings can apply (for example) in the risk characterisation provisions of Annex 12 of REACH, which seeks to isolate downstream risks of substances in use across their lifetime such that these can be adequately controlled.

2.6. FAIR data integration and dissemination

The *PrecisionTox* standardized multi-omics dataset, biomarker information, and compound classification will be publicly shared through a dedicated Data Commons following FAIR Principles of findability, accessibility, interoperability, and reusability (Wilkinson et al., 2016). This Data Commons will also support data integration with other ongoing large-scale toxicology and cellular biology initiatives (Trapotsi et al., 2022) including ToxCast and Tox21 (Thomas et al., 2019), LINCS (LINCS, 2021), and SEURAT-1, as well as the ASPIS projects *ONTOX* and *RISK-HUNT3R*. To further facilitate uptake of data, particularly by regulators, *PrecisionTox* will also deliver a combined database and computational system (the "NAM Toolbox") to support the use of biomarker-based assays for chemical safety assessment. In addition to providing standardized data and metadata formats for biomarkers of toxicity pathways, the NAM Toolbox will supply reporting software compatible with existing regulatory processes and requirements (Harrill et al., 2021).

2.7. PrecisionTox limitations

Potential limitations of the initiative include some that have already been identified during the development and execution of the ToxCast and Tox21 initiatives (Tice et al., 2013), including inadequate coverage of biomolecular pathways perturbed by chemicals, which can occur for a variety of reasons. For example, there may be too few test chemicals, or the diversity of their modes of action may be sub-optimal to achieve *PrecisionTox* objectives. For these reasons, the chemical selection process begins with data mining of existing knowledge and is designed to be inclusive and iterative. Further, the initiative incorporates the participation of key stakeholders in chemical selection in three progressive phases of *PrecisionTox*.

Although the initiative aims to address some known limitations of *in vitro* test systems in toxicology for translating perturbations at the molecular level to possible tissue-, organ-, and organism-level effects, its cross-species extrapolations of the *in vivo* toxicological effects of compounds are restricted to the acquired knowledge of the chemicals' modes of action from conserved biomolecular processes and the observed phenotypes in a small set of non-human test systems and over distinct life stages. Although these observations are made in light of a known phylogeny, there is a risk that the taxonomic coverage of the phylotoxicology panel of model organisms is insufficient to determine the degree to which these conserved biomolecular responses are functionally the same and causally linked to adverse health outcomes in humans. For this reason, reverse genetic engineering (gene knock-in and knock-out experiments) will be applied to determine the essentiality of the discovered molecular key events within an AOP (Hruscha and Schmid, 2015). Additionally, single-cell sequencing of transcriptomes that measure tissue- and organ-specific variation in gene expression will be cross-referenced using 3D human organ tissues. The results of these combined analyses will aid in confirming mechanistic hypotheses relevant to humans and identifying sources of variation that, when considered, increase certainty in the causal associations between exposure to chemicals and pathways to adversity.

2.8. Broader contributions

By filling knowledge gaps regarding the interactions of gene-related disease pathways (Barabasi et al., 2011) and harmful chemicals in the environment, *PrecisionTox* complements the broader Precision Health campaign by the U.S. National Academy of Sciences for a new, molecularly informed taxonomy to define diseases based on molecular endotypes rather than traditional clinical symptoms (i.e. phenotypes). It is well known that phenotypes, including disease phenotypes, are the result of genes by environment interactions. As such, reaching the goals of precision health requires environmental factors, including chemicals, to be considered. Pinpointing the biochemical reactions through which toxicity pathways are initiated also contributes to chemo-informatics modeling efforts to interpret the relationship between chemical structure and biological response. By elucidating the evolutionary origins and functional conservation of toxicity responses with empirical evidence, *PrecisionTox* contributes to evolutionary genetics, building on earlier work indicating that genes coding for health and disease states appear to be among the most evolutionarily conserved (Colbourne et al., 2022; Domazet-Loso and Tautz, 2008).

PrecisionTox also aligns with the One Health initiative that recognizes the interdependence of humans and other natural systems by bridging the artificial divisions between human, animal, and environmental health (Lerner and Berg, 2015; Rivetti et al., 2020). Just as *Drosophila* has been critical to the elucidation of human stem cell biology, it is also a powerful model for the susceptibility of honeybees to low-dose pesticide exposure (Tasman et al., 2021). *Caenorhabditis elegans* is not only a meaningful model for neurobiology; nematodes also play a vital role in the maintenance of healthy soil for food and fuel security (Sochova et al., 2006). *Daphnia* are keystone species that provide insight into the robustness of freshwater ecosystems (Miner et al., 2012). *Xenopus* and *Danio* together constitute an early-warning system for the impacts of endocrine-disrupting compounds and climate change (Brown et al., 2015; Mann et al., 2009). While *PrecisionTox* is explicitly focused on building the tools required to better safeguard human health through NAMs focused on the 3Rs (Russell and Burch, 1959), the initiative also works at the human-animal-environment interface to elucidate and benefit from these intrinsic linkages.

Although the motivation for selecting this diverse suite of five organisms is based on their combined scientific value as human surrogates, these species also represent five major animal clades; by demonstrating their underlying genetic unity with respect to chemical toxicity response, *PrecisionTox* also introduces a model cohort for addressing ecological and climate issues. As genomic data are accumulating for a greater global diversity of species during the next ten years via the Earth Biogenome Project and similar initiatives (Lewin et al., 2018), these *phylotoxicology* methods can support extrapolation to other species regarding genetic susceptibility to toxicity (species read-across), allowing for estimations of the effects of environmental toxicity on ecosystems that indirectly influence human health. Therefore, while the anticipated impact of *PrecisionTox* is a regulatory paradigm that is centered on grouping and classifying chemicals based on their modes of action while avoiding mammalian animal testing, this initiative may also help identify new sentinel species and build utilities to monitor and safeguard vital ecosystems.

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