1 Introduction

Safety assessment of chemicals from all industrial sectors has, until recently, almost entirely relied on animal experimentation. As a result, the definition of adversity has been mostly based on so-called apical endpoints that are directly observable (e.g., histopathological changes, morphological defects, and behavioral changes). Shortcomings of this black-box, phenotype-oriented approach include the lack of mechanistic information, problems with interspecies extrapolation due to physiological and genetic differences between animals and humans, and difficulties in detecting pathologies significant to humans but hard to assess in animals. Another disadvantage of an animal-based risk assessment is the high false-positive rate (Hartung and Leist, 2008). This consideration means that the development of valuable candidate drugs and chemicals may be stopped because of toxicity findings in animals that are not relevant to humans. This is a severe drawback for industry and society (Leist and Hartung, 2013; Ennever and Lave, 2003; Busquet et al., 2020). In situations of time limitation (e.g., for candidate COVID drugs), the lengthiness of animal
The Horizon 2020 EU-ToxRisk project, for example, has significantly advanced scientific confidence in the practical application of integrating NAM data into read-across approaches for risk assessment (Escher et al., 2019; Moné et al., 2020). Case studies incorporating NAMs were applied and subsequently positively reviewed by international peers and programs, including the OECD IATA Case Study Project. The Horizon 2020 RISK-HUNT3R(1) project builds on the outcomes of previous large EU projects. The next major step is towards an NGRA of compounds with no safety information (so-called ab initio approach).

Scientific advances in individual NAMs have paved the way for their successful application and integration in chemical safety testing, e.g., for complex endpoints such as skin sensitization. Yet despite the successful integration of various NAMs in the pre-marketing safety assessment of chemicals, remaining challenges for a broad-ranging and successful implementation of animal-free approaches to risk assessment have become more evident. Current limitations have several reasons. Firstly, individual NAMs will not allow a complete hazard assessment for complex endpoints. Although this notion is scientifically evident, a mindset of one-on-one test replacement is still frequently encountered. It has proven difficult to introduce hazard predictions based on a combination of NAM data. Secondly, more and better concepts are needed to set thresholds for human exposure (like acceptable daily intake or tolerable workplace exposure) based on NAM-derived data. Thirdly, while tests have been extensively applied to model acute effects, in vitro tests for chronic effects are still challenging due to limited culturing times. Thus, a systematic evaluation of the validity of NAMs for chronic human health impacts is lacking.

Past projects have contributed to developing strategies to solve the above problems. The Horizon 2020 EU-ToxRisk project, for example, has significantly advanced scientific confidence in the practical application of integrating NAM data into read-across approaches for risk assessment (Escher et al., 2019; Moné et al., 2020). Case studies incorporating NAMs were applied and subsequently positively reviewed by international peers and programs, including the OECD IATA Case Study Project. The Horizon 2020 RISK-HUNT3R project builds on the outcomes of previously funded large EU projects. The next major step is towards an NGRA of compounds with no safety information (so-called ab initio approach).

Through systematic and iterative evaluation of a characterized NAM toolbox and the use of case studies that address various real-life problems, the RISK-HUNT3R consortium aims to optimize the strategy to assess exposure and toxicokinetics, as well as toxicodynamics of a broad range of chemicals. The project plans to use human-relevant NAMs, some of which use novel technologies to investigate mechanisms of toxicity, to validate and implement integrated approaches to NGRA.

### 2 Implementing core principles of NGRA

Key characteristics of an NGRA strategy have been described repeatedly. Dent et al. (2018) gave a good summary for the cosmetics area, and RISK-HUNT3R follows nine similar principles. They may be grouped as describing the objectives, procedures,
and documentation required for an NGRA approach (Fig. 1).

RISK-HUNT3R will implement these principles to build its testing strategy toward animal-free NGRA with a focus on three toxicological areas: specific target organ toxicity (STOT), developmental neurotoxicity (DNT), and non-genotoxic carcinogenicity (NGC). The relevance of the different test systems concerning human physiology is considered essential to provide confidence in the NGRA. The RISK-HUNT3R project addresses this notion on different levels. NGRA case studies will use already characterized state-of-the-art NAMs, which will be further optimized to model human (patho-)physiology. Additionally, using bioinformatics approaches, RISK-HUNT3R will draw from human disease-related transcriptome data in the public domain, establishing human tissue-specific gene network models overlapping those from test systems. Preservation of individual gene networks will be defined between in vitro test systems and human in vivo data, providing scientific confidence in the human relevance of in vitro gene networks. This knowledge will allow a direct translation of in vitro gene network activation to potential human adverse outcomes and will strengthen the underpinning of individual gene networks as biomarkers for key events (KEs) in AOP networks.

2.1 The RISK-HUNT3R strategy is exposure-led
Different approaches will be used to estimate exposure levels. One approach, e.g., is the use of human biomonitoring data from exposome projects and occupational data. An important strategy is the development of a human exposure framework based on internal exposure levels. This requires the application of methods that allow conversion of human external exposure data via several routes (dermal, pulmonary, oral) to concentration-time profiles within blood and organs. For this purpose, the project will, e.g., systematically determine human-relevant biotransformation and clearance capacities in in vitro test systems. RISK-HUNT3R will make predictions both for parent compounds and toxicologically relevant metabolites of chemicals. Finally, toxicokinetics and toxicodynamics data derived from NAMs will be integrated into predictive systems toxicology models. A special application of the exposure framework is exposure-based waiving, analogous to the thresholds for toxicological concern (TTC) waiving concept (Hartung, 2017), but also for identifying relevant concentration ranges for testing and for defining margins of exposure.

2.2 Testing is designed to distinguish adverse effects from epiphenomenal biological perturbations.
Definition of adversity will be hypothesis-driven.
The project will make use of the adverse outcome pathway (AOP) framework to implement procedures to differentiate between NAM-based predictors of adverse effects and non-relevant early adaptive and reversible changes. This is essential for hazard predictions to determine relevant point-of-departure (PoD) values. The RISK-HUNT3R project will thus make use of the extensive general knowledge of toxicity mechanisms and their link to adverse outcomes using AOPs. The project will use more than 200 AOPs currently available as models that describe the sequence of molecular and cellular events required to produce a toxic effect when an organism is exposed to a substance. A particular focus of RISK-HUNT3R is the use of reporter assays and high-throughput omics approaches to drive hypothesis formulation for AOP activation through bioinformatics analysis.

2.3 RISK-HUNT3R will integrate robust and relevant methods and include uncertainty characterization and transparent and explicit reporting
Most test systems in the RISK-HUNT3R toolbox have been extensively documented in the EU-ToxRisk project (Krebs et al.,

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**Box 1. Different types of uncertainties in risk assessment**

As in all risk assessments, uncertainty will also be associated with an integrated NGRA framework, primarily when multiple layers of information and various data domains are used. RISK-HUNT3R will fully embrace the new scientific discipline of uncertainty assessment, addressing both aleatoic and epistemic uncertainty in NGRA.

- **Aleatoric uncertainty** (from the Latin word alea, meaning “dice”) arises from the inherent stochasticity of the biological and physical world, i.e., the “noise level” of test procedures. It is typically called “variability” in risk and safety assessments (see guidances by Food and Agriculture Organization, US EPA, 2011; 2014).

- **Epistemic uncertainty** (from ancient Greek epistēmē, meaning ‘knowledge’) instead originates from imperfect knowledge. For NGRA this may refer to an insufficiently developed AOP used for an IATA.

The recent guidelines developed by EFSA on the analysis (EFSA, 2018) and the communication (EFSA, 2019) of uncertainty highlight the necessity to distinguish epistemic uncertainty from aleatoric uncertainty. Identification, characterization, and communication of uncertainties will be central to all NGRA case studies.
2.4 RISK-HUNT3R will use a tiered and iterative approach

The purpose and the scope of a risk assessment, as well as the depth of the analysis and thus the resources (data, tools, procedures) required for completing a risk assessment, are defined in its problem formulation phase (US EPA, 2014; Luijten et al., 2020; Solomon et al., 2016). The number of resources allocated to conducting a risk assessment should, to some extent, be determined by the level of concern, e.g., preliminary data on specific hazards or knowledge of widespread and high exposure will trigger prioritization or focused testing.

RISK-HUNT3R proposes an NGRA framework composed of logical assessment elements, essential for adequately addressing the risk associated with exposure to a target chemical. Modules cover different aspects, from identifying the exposure situations (Modules 1 and 2, green boxes) to hazard characterization (Modules 3 and 4, blue boxes). Each module is an independent element from which outcomes (data/information) are processed in Module 5 (black box) and transferred to the next module. This process may be reiterated until enough data/information is collected to perform an adequate integrated risk assessment (outward red arrow). qAOP, quantitative adverse outcome pathway; MPS, microphysiological system; PoD, point of departure; IVIVE, in-vitro-to-in-vivo extrapolation; RA, risk assessment; ADME, absorption, distribution, metabolism and excretion; WoE, weight of evidence.
ule is transmitted to the overall risk assessment module for further evaluation (Module 5). In turn, Module 5 may request additional input if there is a remaining information requirement, thus allowing iterative optimization as necessary (Fig. 2).

3 The testing framework of the RISK-HUNT3R NGRA strategy

The RISK-HUNT3R project has structured its overall strategy into two sequential stages. Initially, the available methods will be tested for robustness and performance. Each element of the project toolbox will be scrutinized within relevant work packages. For this purpose, data-rich chemicals will be used so that the assay outcomes can be compared to legacy knowledge in databases. The methods that prove suitable, either directly or following optimization, will be used in the main phase of the project.

During the main stage of RISK-HUNT3R, chemicals with little or no data will be investigated in an iterative and flexible testing strategy (Fig. 3). The toxicity of a particular substance will be examined in light of a specific problem formulation. This step is crucial to determine the mandatory assessment elements, the scope of the testing strategy (e.g., the required modules), and the overall acceptable uncertainty.

The first overarching module group is focused on the determination of absorption, distribution, metabolism, and excretion (ADME) information. In Module 1, human exposure and bioavailability of the substance are evaluated after passing the barriers of first contact (skin, lung, intestine). In vitro models of these barrier organs will be used to establish realistic parameters for bioavailability estimation. Data will then be integrated into physiologically based kinetics (PBK) models. The possibility of metabolism leading to the formation of active and potentially toxic metabolites is often not considered in traditional in vitro approaches. Relevant in vitro models will be developed and applied in case studies to identify and quantify tissue-specific formation and the general release of these metabolites into the systemic circulation, e.g., from the liver, lung, or kidney. When necessary, toxicologically relevant stable metabolites will be synthesized and used for NAM testing. In Module 2, the RISK-HUNT3R toolbox will be applied to evaluate the metabolism, distribution, and excretion of chemicals following their systemic uptake. After each step, an exposure-based waiving principle can be used. If no relevant exposure is measured, the assessment may conclude since no further data is necessary to assess risk to human health. Alternatively, risk assessment will continue by identifying the optimal follow-up strategy: read-across, NAM-based read-across, or hazard identification and quantification steps, allowing a final integrated NGRA.

The second overarching module group applied in the latter scenario is hazard assessment (Fig. 3). The RISK-HUNT3R consortium has assembled a broad and diverse battery of screening and follow-up assays, including high-throughput in silico and in vitro technologies, to identify adverse effects of test compounds and assign them to AOP KEs. In silico approaches may first indicate whether chemicals will interact with cellular targets and initiate subsequent KEs. In vitro assays can map critical candidate molecular initiating events (MIEs) and potential biological responses that are indicative of severe cell injury or loss of pivotal cell functions. At the next level of cell biological perturbation, high-content imaging phenotypic assays based on cell painting approaches (Delp et al., 2018; Nyffeler et al., 2020) will be implemented. For adverse cellular responses, in vitro multicolour reporters established in the EU-ToxRisk project that represent critical cellular stress response pathways, including oxidative stress, unfolded protein response, DNA damage response, and inflammatory signaling (Wink et al., 2014), will be used.

These data will be mapped to toxicological area-specific AOP networks to provide first indications of AOP modulation. The modular safety testing approach of RISK-HUNT3R is designed to provide alerts for most, if not all, potentially relevant adverse effects concerning STOT, DNT, or NGC. It allows for some areas to take steps from qualitative to quantitative AOPs. The alert collection is assembled by capturing at least one node from each possible trajectory of the network of established qualitative AOPs (AOP-Wiki) related to these outcomes. In many cases, high-throughput omics tests will identify relevant MIEs or KEs. Complementary to this, several assays are available to capture phenotypic changes that reflect adverse outcomes. The latter strategy will be used where AOP coverage is limited but in vitro correlates of adverse outcomes have been identified.

A typical example is DNT, where only a few AOPs are known but good tests exist to identify disturbed neural differentiation or network function (Sachana et al., 2021). Similarly, crucial elements for an integrated approach to testing and assessment (IATA) for NGC have been identified, but the overview of AOPs relevant for NGC is not yet complete (Jacobs et al., 2020; Heusinkveld et al., 2020). Subsequently, for Module 4, once alerts for adversity have been generated or even when relevant AOPs have been identified by screening data from Module 3, a large panel of more advanced test systems is available. It provides further quantitative mechanistic information and, eventually, will provide evidence for the relevance and adversity of NAM data in the qAOP assessment.

Finally, in Module 5, the workflow anticipates that a complete IATA-based risk assessment is performed. This process integrates all data and quantifies overall uncertainty.

4 Current challenges for NGRA and RISK-HUNT3R innovations

RISK-HUNT3R will address several challenges towards successfully implementing NAMs in regulatory safety assessment frameworks.

Challenge 1: Integration of human-relevant biotransformation and clearance capacities into in vitro test systems

The ADME modules will systematically integrate data on human-relevant biotransformation and clearance capacities into in vitro test systems. Pre-systemic metabolic stability, formation, and quantification of (potentially toxic) metabolites and estimation of their release into the systemic circulation will be investi-
Challenge 3: Establishment of qAOPs
RISK-HUNT3R will focus on transforming descriptive AOPs into qAOPs and using these as the basis for identifying safe chemical concentrations and acceptable daily intakes. RISK-HUNT3R aims to establish a framework for parameterization of qAOP networks incorporating NAM and legacy data, an essential step toward quantitative risk assessment and moving beyond mere hazard assessment.

Challenge 4: Integration of NAM data in predictive systems toxicology models
Toxicokinetics and toxicodynamics NAM data and partial computational models will be integrated into predictive quantitative systems toxicology (QST) models. QST models complement quantitative systems pharmacology models, incorporating toxicokinetics with mechanistic models of physiological, pharma-
ological, and toxicological mechanisms. QST models for predicting adverse outcomes in humans will integrate multi-tiered biological information, including quantitative knowledge of toxicokinetics, physiology, and baseline function at the level of the cell/tissue/organ system, toxicity mechanisms, and dynamics of injury biomarkers and endpoints (Bloomingdale et al., 2017).

**Challenge 5: Quantification of uncertainties in overall risk assessment**

The qualification and quantification of uncertainties will be addressed in the project concerning all input levels of systems toxicology models to provide confidence in a robust risk assessment setting. The transparent weighting of and reporting on uncertainties will be key in guiding the final assessment.

**5 Impact of RISK-HUNT3R**

The RISK-HUNT3R consortium has put together a multi-stakeholder and multi-expert team. Input from the different perspectives of academia, industry, and regulatory agencies will be used to build confidence and trust that the new strategies are fit-for-purpose and collect essential input. Additionally, a structured dissemination plan will assure a continuous dialogue with the stakeholder community. The impact on non-animal chemical risk assessment will be enhanced by RISK-HUNT3R participation in the ASPIS² (Animal-free safety assessment of chemicals: project cluster for implementation of novel strategies) cluster, teaming up with the two adjacent and complimentary Horizon 2020 projects ONTOX³ and PrecisionTOX⁴. The goal is to ensure optimal conditions, in terms of regulatory and commercial objectives, for sustainable use of *in vitro* and *in silico* methods and non-animal safety assessment strategies. NAM-based NGRA will apply to multiple regulatory contexts across industry sectors. Its goal is to protect different population subgroups (distinguished by, e.g., exposure situation, age, or gender). The broader scope also includes the more vulnerable groups, such as children, pregnant women, and the elderly. Toxicokinetic modelling and the consideration of biological factors affecting hazard (e.g., via gene expression variations) will include the relevant variables and their input factors.

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Conflict of interest
The authors declare that they have no conflicts of interest.

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