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Chemical testing using new approach methodologies (NAMs)

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Chemical testing using new approach methodologies (NAMs)

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Editorial: Chemical testing using new approach methodologies (NAMs)

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Editorial on the Research Topic
Chemical testing using new approach methodologies (NAMs)

In 2007, the U.S. National Research Council (NRC) introduced a novel approach to assure the safety of chemicals based on human-relevant mechanisms of toxicity in their landmark publication "Toxicity Testing in the 21st Century: A Vision and a Strategy" (NRC, 2007). As written by Andersen and Krewski (2009), member and chair of the NRC Committee on Toxicity Testing and Assessment of Environmental Agents, the vision detailed "a not-so-distant future in which virtually all routine testing would be conducted in human cells or lines *in vitro*". This Research Topic in Frontiers in Toxicology's section on "*In Vitro* Toxicology" is devoted to highlighting research and methods that continue to advance this vision and to informing readers of the regulatory frameworks where these technologies may be used to inform regulatory filings and decisions.

Although no unanimous definition of new approach methodologies (NAMs) exists, NAMs are often defined as any technology, methodology, approach, or combination thereof that can provide information on chemical hazard and risk assessment without the use of animals, including *in silico*, *in chemico*, *in vitro*, and *ex vivo* approaches (ECHA, 2016; EPA, 2018; Stucki et al.). NAMs are not necessarily newly developed methods, rather, it is their application to regulatory decision making or replacement of a conventional testing requirement that is new. In this Research Topic, we are pleased to present on contributions to NAMs development and go a step further by bridging the science behind NAMs with the regulatory frameworks under which NAMs may be used, and in some cases, the regulatory impediments for their use.

Three original research articles on respiratory toxicity provide NAMs for evaluating specific regions of the respiratory tract, such as an adverse outcome pathway (AOP) for impairment of mucociliary clearance in the tracheobronchial region (Luettich et al.) that can be used to design in vitro testing strategies. Sengupta et al. present a lung-on-a-chip with a newly established cell line from epithelial cells from the alveolar regions. Zhang et

al. introduce approaches for performing in vitro to in vivo extrapolations (IVIVE) for inhaled mixtures. These NAMs may be combined to provide a more complete understanding of the potential for inhaled chemical substances to cause adverse effects along the respiratory tract, and importantly, for deriving in vivo equivalent concentrations for quantitative risk assessments.

The original research articles also include investigations on the utility of cardiomyocytes derived from induced pluripotent stem cells as a screening tool for potential cardiotoxicity of new tobacco and other nicotine-containing products (Simms et al.), as well as investigations on potentially expanding the applicability domain of the Organisation for Economic Co-operation and Development's (OECD)'s Guideline 497 on Defined Approaches for Skin Sensitisation from individual substances to pesticide formulations (Strickland et al.).

Beyond the original research articles, this Research Topic includes a methods article that advances our understanding of mitochondrial dysfunction and oxidative stress, and its role in acute and "low noise" chronic toxic effects (Loric and Conti).

Few would argue that the research and methods development on NAMs that has taken place since the NRC published its 2007 vision is nothing short of impressive. However, regulatory acceptance is a fundamental requirement for advancing the use and application of NAMs. This Research Topic provides three contributions that aid with understanding various regulatory framework where NAMs may be used and how NAMs were applied already (Miller-Holt et al. and Stucki et al.), as well as perspectives from Canadian regulators on their approaches for utilizing NAMs (Bhuller et al.).

Collectively, these articles showcase available test methods, how to design testing strategies using defined approaches or AOPs, or the current opportunities to use NAMs and proposals to help further advance their use and regulatory acceptance. The contributions in this Research Topic highlight the promise of answering "Yes" to the question "Is Animal Testing Overrated?", a question posed by the U.S. Environmental Protection Agency nearly 4 decades ago (EPA, 1984). Realizing the full potential of NAMs will only be achieved, however, through continued publications, discussion, and transparency about new testing approaches as well as continued cross-sector partnerships and educational opportunities for stakeholders to gain confidence in NAMs (van der Zalm, 2022). Ultimately, these activities are leading to improved toxicology testing paradigms that better align with the NRC's 2007 vision of harnessing modern technology and our current understanding of human biology to protect human health, while avoiding testing on animals.

Author contributions

All authors wrote, edited, reviewed, and agreed on final version. All authors were involved in conceiving and managing the Research Topic.

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Conflict of interest

CH was employed by JT International SA. TS was employed by Bergeson & Campbell PC. CT was employed by Corteva Agriscience. CH and CT declare competing interests as they are employed by companies using the technologies discussed in this Research Topic for toxicological evaluation purposes and for the determination of product safety, respectively. AS is a co-inventor listed on a patent of one of the methods described in this Research Topic.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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PERSPECTIVE
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Canadian Regulatory Perspective on Next Generation Risk Assessments for Pest Control Products and Industrial Chemicals

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In 2012, the Council of Canadian Academies published the expert panel on integrated testing of pesticide's report titled: Integrating emerging technologies into chemical safety assessment. This report was prepared for the Government of Canada in response to a request from the Minister of Health and on behalf of the Pest Management Regulatory Agency. It examined the scientific status of the use of integrated testing strategies for the regulatory health risk assessment of pesticides while noting the data-rich/poor dichotomy that exists when comparing pesticide formulations to most industrial chemicals. It also noted that the adoption of integrated approaches to testing and assessment (IATA) strategies may refine and streamline testing of chemicals, as well as improve results in the future. Moreover, the experts expected to see an increase in the use of integrated testing strategies over the next decade, resulting in improved evidence-based decision-making. Subsequent to this report, there has been great advancements in IATA strategies, which includes the incorporation of adverse outcome pathways (AOPs) and new approach methodologies (NAMs). This perspective provides the first Canadian regulatory update on how Health Canada is also advancing the incorporation of alternative, non-animal strategies, using a weight of evidence approach, for the evaluation of pest control products and industrial chemicals. It will include specific initiatives and describe how this work is leading to the creation of next generation risk assessments. It also reflects Health Canada's commitment towards implementing the 3Rs of animal testing: reduce, refine and replace the need for animal studies, whenever possible.

Keywords: next generation risk assessment, integrated approach to testing and assessment, adverse outcome pathways, new approach methodologies, weight of evidence

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INTRODUCTION

Evidence-based decision-making, rooted in robust scientific risk assessments, is paramount for the initial market-approval and subsequent evaluations of registered pest control products and industrial chemicals in Canada. The federal regulatory frameworks governing the life-cycle management of these products provides sufficient flexibility for the responsible regulatory authority to evaluate scientific studies from a wide variety of published and unpublished sources. It also provides an agile approach to considering alternative strategies to health risk assessments and incorporating

non-animal technologies, when applicable, for hazard identification. The health risk assessment process itself, a function of both hazard and exposure, is well described in several documents and is aligned with international approaches. These include technical documents, describing program-specific decision-making frameworks (Health Canada, 2021a), and non-technical ones, such as Health Canada's primer on scientific risk assessment (Saner, 2010). Further, in the area of industrial chemicals assessment, efforts have been made to advance the development and implementation of novel scientific assessment approaches through the publication of science approach documents (Health Canada, 2021b). Health Canada has also progressively introduced new methods to effectively identify and address substances of varying concern and continues to update their data requirements (Health Canada, 2013a) thereby enabling them to be well positioned to transition to next generation risk assessments (Krewski et al., 2014).

In 2012, the Council of Canadian Academies (CCA) published the expert panel report on integrating emerging technologies into chemical safety assessment (CCA, 2012). This report was prepared for the Government of Canada in response to a 2009 request from the Minister of Health and on behalf of the Pest Management Regulatory Agency (PMRA). It was the first Canadian report that provided the scientific status on integrated strategies and identified the potential paradigm shift for a more inclusive approach where integrated approaches to testing and assessment (IATA) go beyond using them just for data-poor chemicals (e.g., pesticide formulants and industrial chemicals). The report also included a 10-year vision for the evolution of IATA within the regulatory context and a foundational starting point that included these elements: using a common vocabulary, data platforms and standards, digitization of legacy data, international coordination, stakeholder communication, and functional collaboration. The CCA and other international reports, such as the National Research Council's report (NRC 2007), have been pivotal in establishing the Canadian regulatory approach for identifying, exploring, and implementing IATAs. Some IATAs utilize adverse outcome pathways (AOPs) and more recently new approach methodologies (NAMs). Publications, such as the 2020 article on toxicity testing in the 21st century (Krewski et al., 2020), provide insights on the advances in biological sciences and how these have led to this ongoing paradigm shift. Future perspectives on the continued evolution of toxicity testing to strengthen regulatory risk assessment are also noted, which includes ensuring that any alternative approach adheres to the established health and safety standards required for these products.

This article now provides the first Canadian regulatory update on how the regulatory authorities responsible for pest control products and industrial chemicals are advancing the incorporation of alternative and non-animal strategies. It demonstrates how these program areas have successfully positioned themselves for the next generation of risk assessments by elaborating on early conceptual frameworks. References to recent and key publications are provided along with insights on how these areas have been contributing to this paradigm shift through the establishment and successful maintenance of a strong, multi-stakeholder collaborative approach.

REGULATION OF PEST CONTROL PRODUCTS AND INDUSTRIAL CHEMICALS

Chemical substances, which includes pest control products and industrial chemicals, are stringently regulated in Canada to protect human health and the environment (Health Canada, 2017a). While Health Canada is the responsible federal department for the market approval and subsequent oversight of pest control products and industrial chemicals, there are two program areas that are accountable for this work. Specifically, Health Canada's PMRA is responsible for pesticide regulation in Canada while, in part, the Healthy Environments and Consumer Safety Branch (HECSB) in collaboration with Environment and Climate Change Canada is responsible for industrial chemicals.

Under authority of the Pest Control Products Act, Health Canada registers pesticides after a stringent, science-based risk assessment, re-evaluates pesticides on the market on a cyclical basis, and is actively involved in national and international science-policy initiatives. As noted in the 2019–2020 annual report, PMRA continues to evaluate pesticides in cooperation with other jurisdictions and over the last 2 years, the Agency's focus has been on a major transformation of its pesticides program (Health Canada, 2021c). The latter is exploring a further integration of the pre- and post-market activities, which includes incorporation of next generation approaches to risk assessment.

The Canadian Environmental Protection Act, 1999 (CEPA, 2019; CEPA) provides the legislative framework for industrial chemicals, including new chemical substances (domestic and imports) as well as substances that are currently on the Canadian market (i.e., existing substances). Leading the world in chemicals management, Canada was the first to systematically categorize or prioritize the 23,000 substances on the Domestic Substances List (DSL) for risk assessment, initiating the Chemicals Management Plan (CMP) in 2006 (Health Canada, 2016a). Risk assessments of the approximate 4,300 priority chemicals were conducted over three phases (2006–2021) and required the development of new methodologies and scientific approaches to continue to effectively deliver an evolving risk assessment program. For industrial chemicals, there is a range of toxicity data available, from data-rich to data-poor, and an ongoing need to prioritize, assess and manage diverse and increasingly complex substances and mixtures. The Government of Canada is also building on the successes of the CMP to renew its approach to chemicals management including follow-up considerations on the report from the House of Commons Standing Committee on Environment and Sustainable Development on the statutory review of CEPA (Environment and Climate Change Canada, 2018).

MODERNIZING APPROACHES TO RISK ASSESSMENT

In comparison to industrial chemicals, pesticides and pest control products are considered data-rich chemicals. The regulatory submissions rely on a prescribed list of data requirements that

include several animal studies and often comprise *in silico* (quantitative-structure activity relationship (QSAR)), *in vitro* assays, and more recently NAMs (e.g., defined approaches for skin sensitization (OECD, 2021a). Similarly, when considered equally or better suited to measure toxicity, alternate approaches, such as *in vitro* data, read-across using surrogate data, weight-of-evidence (WoE) for substance classes, and QSAR data from internationally accepted models, are examples of frequently accepted NAMs for industrial chemicals.

In contrast, there are no prescribed data requirements for existing substances, under CEPA, and assessments make use of best available data. Accordingly, the program has progressively advanced the use of NAMs from computational modelling, read-across and category approaches to more complex evidence integration approaches to identify and address emerging priority substances. Typically, a WoE approach is relied upon by evaluating the results from the alternative approaches along with the totality of evidence, which includes published information. Enriching evidence integration for WoE assessment has been supported through the development of IATA methodologies; endocrine activity has been one area of focus in this respect for the existing substances program at Health Canada. Workflows to assimilate data collected from traditional and NAM sources to generate predictions regarding potential endocrine disruption activity for a subset of chemicals of regulatory interest has illustrated that NAMs can be a protective approach for human health risk assessment (Webster et al., 2019).

The year 2022 marks a decade since the release of the CCA report and significant progress has been made on a variety of NAMs, which includes *in silico* based approaches. The latter has found the most widespread use and acceptance in regulatory data submission and assessment. To address existing substances in Canada, efforts have focused on validation exercises to increase confidence in the application of a suite of models for the DSL chemical space (Kulkarni and Barton-Maclaren 2014; Kulkarni et al., 2016) as well as contributing to imperative steps forward to promote international harmonization. Key developments have included progress on standardized *in silico* toxicology (IST) protocols (Myatt et al., 2018; Hasselgren et al., 2019), endorsement of OECD guidance for defined approaches to testing and assessment (OECD 2016a), and grouping of chemicals and read across (OECD 2017). Evolving these approaches further, cheminformatics-based methods for read-across of point of departures (PODs) are being explored to build confidence in quantitative read-across to specific endpoints (Yang et al., 2021).

Notably, *in vitro* and omics-based approaches are also being explored quite broadly across Health Canada. Specifically, transcriptomics data is currently used in a WoE to better understand chemical mode of action, justify read-across groupings, and fill data gaps (Yauk et al., 2019). Health Canada's CMP phthalate assessment demonstrated that gene expression patterns could be used to support category development and the selection of specific compounds for cumulative risk assessment (Health Canada 2015). Transcriptomics also holds promise in the selection of PODs for prioritization and quantitative risk assessments. Results from recent case studies focused on flame retardants demonstrated that *in vitro* transcriptomics data, coupled with *in vitro* to *in vivo* extrapolation (IVIVE), provide PODs that are protective of human health and allow for potency ranking (Gannon et al., 2019; Rowan-Carroll et al., 2021). Similarly, quantitative high-throughput screening assays, that provide mechanistic and quantitative data across a broad toxicological space, also have established utility in the assessment of potential for human health risk. Specifically, a multi-agency retrospective case study conducted under the Accelerating the Pace of Chemical Risk Assessment (APCRA) initiative demonstrated that *in vitro* data from the ToxCast program, comprising nearly 1400 toxicological endpoints, could be used to derive points of departure for risk assessment activities (Paul Friedman et al., 2020). Building on the approach and learnings from the collaborative case study, Health Canada published a science approach document providing a rationale and guidance for how to apply the approach as an early screen of potential for risk in the context of the CMP (Health Canada, 2021d).

IMPORTANCE OF MULTI-STAKEHOLDER COLLABORATION

As an OECD member, Health Canada is involved in several initiatives related to IATA, NAMs, and ongoing developments of several technical guidelines. An underlying reason for this international collaboration continues to be rooted in the 3R principles: reduce, refine and replace animal studies, when possible. However, another aspect is the mutual acceptance of data whereby harmonizing requirements provides a common basis for all authorities (OECD, 2021b).

To allow for broader acceptance of IATAs, NAMs, and no longer routinely requiring specific animal assays for toxicity testing, Health Canada continues to rely on the North American Free Trade Agreement (NAFTA) Technical Working Group on Pesticides (TWG) and the Canada-United States Regulatory Cooperation Council (Health Canada, 2016b; 2020; RCC). This cooperation has resulted in successful collaboration with stakeholders and global experts from all areas including Industry, Academia, and Non-Governmental Organizations. Health Canada's participation also provides an opportunity to provide guidance so that outputs are fit-for-regulatory purpose and build regulatory, public, societal, and scientific confidence in NAMs. This is consistent with the 2018 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) strategic roadmap for establishing new approaches to evaluate the safety of chemicals and medical products in the United States (ICCVAM, 2018). Individual project plans are also built upon the strategy noted in the CCA report by first focusing on retro-analysis and less complicated assays such as the acute toxicity studies (Health Canada, 2017b; Linke et al., 2017; Allen et al., 2021). The NAFTA TWG has also been used to develop science-policies, which are then brought for broader acceptance through OECD. For example, built on the NAFTA QSAR document (NAFTA TWG, 2012), which was primarily focused on pesticides, the

OECD guidance document expanded to cover industrial chemicals with added focus on mechanistic considerations (OECD, 2015). Similarly, the NAFTA developmental neurotoxicity study guidance (NAFTA TWG, 2016) as well as PMRA's guidance document for waiving or bridging of mammalian acute toxicity tests (Health Canada, 2013b) were also used as the foundational pieces for completed (OECD, 2016b) and/or ongoing OECD technical guidelines.

With parallel goals in mind, industrial chemicals have the additional pressures of lack of data, aggressive priority setting and assessment mandates. In turn, RCC has also played a role in advancing assessment methods for Health Canada's industrial chemicals programs (Health Canada, 2017c), as has the OECD Hazard Assessment Programme related to the improvement and acceptance of approaches intended to minimize the need for animal testing. Foundational work upon which HECSB continues to build include concepts, guidance and lessons learned related to IATA (OECD, 2020; OECD, 2021c) and guidance on physiologically based kinetic models for regulatory purposes (OECD, 2021d). Considerable momentum for regulatory application of NAMs has been gained through research-regulatory partnerships, nationally and internationally, including regulatory, academic, and stakeholder communities. The APCRA network, co-led by the US EPA, Health Canada and the European Chemicals Agency (ECHA), is another example of a successful collaboration between international and intergovernmental bodies (Kavlock et al., 2018). The Friedman et al. and Health Canada work highlighted above are examples of complete progression from collaboration to development of a Canadian-specific approach. It is important to also note that partnerships between risk assessment and research experts to achieve the goal of demonstrating robustness, reliability and readiness of non-animal based approaches in regulatory applications is also a model of interest beyond the chemicals assessment community (Chauhan et al., 2021).

MOBILIZING TEAMS AND ESTABLISHING THE REGULATORY PIVOT

The transition from exclusively relying upon conventional testing approaches to inclusion of NAMs requires a high level of engagement and collaboration given the pivot required to consider incorporating such approaches in regulatory decision-making. Specifically, some complex issues to address include validation, interpretation and application frameworks, guidelines for NAMs or other disruptive technologies, and ethical considerations for using big data (Mittelstadt and Floridi, 2016). There are also legal considerations along with how the public and society will view this transition. While these areas are beyond the scope of this perspective, they continue to be part of ongoing discussions. This section will now focus on the approaches used to mobilize Health Canada scientists.

The model used to engage regulatory scientists and establish the pivot for exploring non-animal testing strategies has relied upon an adaptation of the design-thinking approach (**Figure 1**). Briefly, a top-down approach that relies on the user experience (UX) with conventional assays required for regulatory purposes is the starting point. This insight is then incorporated from concept through to application using a process that understands the data gaps/uncertainties, explores approaches through collaboration, and materializes by learning from successes and failures from the UX perspective. The implementation is then achieved through publication to allow for broader distribution and potentially acceptance of the alternative approach.

Translating case study findings into applications, using a framework that incorporates both innovation and acceleration, has also been extremely useful in the exploration and implementation of NAMs (**Figure 2**). Through the use of practical case studies designed to address specific regulatory needs, methods can be informed by proof of concept research and lessons learned to develop best practices and guidance for the application of fit-for-purpose approaches. Consistent with focused efforts internationally, Health Canada has as an objective to enhance innovation and risk assessment modernization to maintain a world-class chemicals management program. The overarching program and risk assessment principles that have been key for success to date must be reinforced and incorporated to effectively provision the proposed path toward modernization. A multi-pillar approach is envisioned for the transition to modernization of some elements of the program through the accelerated development and acceptance of new methods, taking into consideration a wide range of use and decision contexts. Importantly, the aim is to bring all of these elements together in order to use the most relevant data for the protection of human health and the environment.

DISCUSSION AND NEXT STEPS

This perspective provides the first Canadian regulatory update on how Health Canada is advancing the incorporation of alternative, non-animal strategies for the evaluation of pest control products and industrial chemicals. It includes specific, multi-stakeholder initiatives that are aligned with the Department's commitment towards implementing the 3Rs of animal testing, whenever possible. While beyond the scope this paper, it notes that the incorporation of alternative approaches includes critical discussions around challenges for regulatory implementation. Building upon best practices, such as communication of NAMs through standard regulatory platforms (e.g., guidance documents) along with publications in peer-reviewed journals, presentations at conferences, and more recently through social media, will also continue to be pivotal for advancing this work.

Decades of international efforts have gone into developing legal frameworks and data requirements. While NAMs are largely in the early phases, conventional strategies such as the development of OECD guidelines, defined approaches, IATA case studies and reporting formats will continue to play a key role. Many regulators are also currently relying on testing conducted by governmental or academic research groups to develop proof of concept case studies related to the incorporation of NAMs. With established methods and acceptance criteria, broad scale testing will ultimately require

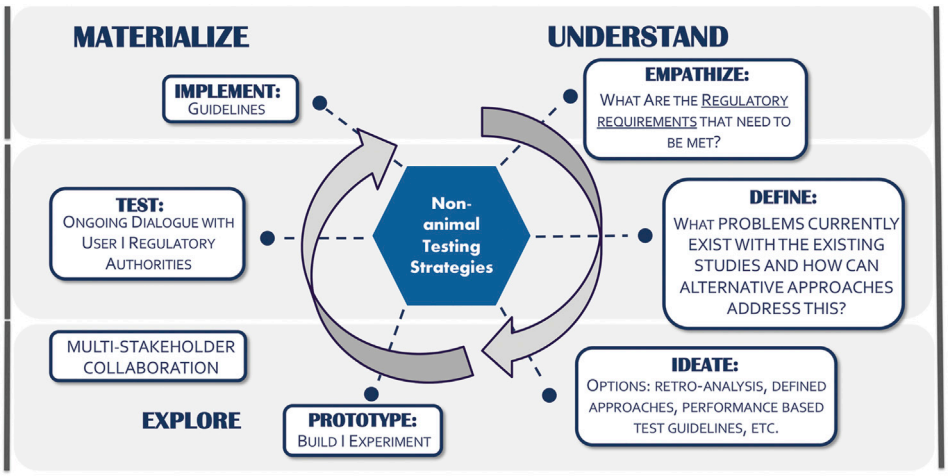

FIGURE 1 | Non-animal testing approaches: Using design thinking.

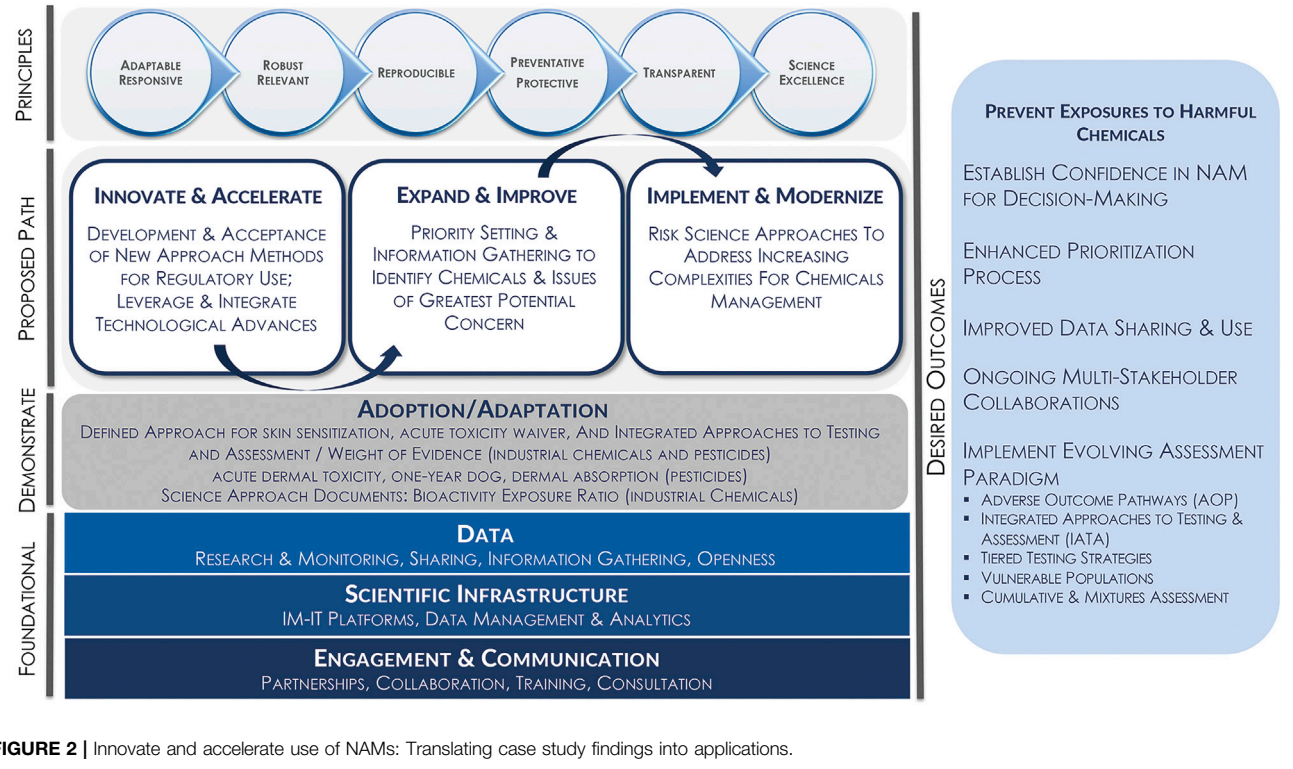

FIGURE 2 | Innovate and accelerate use of NAMs: Translating case study findings into applications.

industry uptake (similar to what is currently in place with traditional testing methods).

Multi-stakeholder collaboration will also continue to be important in the broader acceptance of NAMs and in enabling a better understanding of what is required for regulatory purposes. This includes initiatives led at the national level by regulatory authorities along with ensuring that the regulatory bodies continue to be engaged in key activities led by organizations such as, but not limited to the Health and Environmental Sciences Institute (HESI), PETA Science Consortium International (PSCI), and NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). There are also several academic-led initiatives along with research and consulting firms that are immersed in developing models, which includes open source. This includes the Canadian Centre for Alternatives to Animal Methods (CCAAM) and the Canadian Centre for the

Validation of Alternative Methods (CaCVAM), which aims to develop, validate, and promote non-animal, human biology-based platforms in biomedical research, education, and chemical safety testing.

There is also a need to bring all of this work together for regulatory risk assessments and decision-making. This is where frameworks, such as the Next Generation Risk Assessment as described by Krewski et al., 2014, and the recently enacted HESI committee that is responsible for the project titled Transforming the Evaluation of Agrochemicals will play a key role, in addition to other ongoing IATA and NAM-related activities at the national and global level.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YB and TSB-M designed the concept of the manuscript, the formulation of figures and wrote the manuscript. All authors contributed important intellectual content and helped in the writing and revisions of the article. All authors read and approved the final manuscript.

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An Adverse Outcome Pathway for Decreased Lung Function Focusing on Mechanisms of Impaired Mucociliary Clearance Following Inhalation Exposure

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Adverse outcome pathways (AOPs) help to organize available mechanistic information related to an adverse outcome into key events (KEs) spanning all organizational levels of a biological system(s). AOPs, therefore, aid in the biological understanding of a particular pathogenesis and also help with linking exposures to eventual toxic effects. In the regulatory context, knowledge of disease mechanisms can help design testing strategies using *in vitro* methods that can measure or predict KEs relevant to the biological effect of interest. The AOP described here evaluates the major processes known to be involved in regulating efficient mucociliary clearance (MCC) following exposures causing oxidative stress. MCC is a key aspect of the innate immune defense against airborne pathogens and inhaled chemicals and is governed by the concerted action of its functional components, the cilia and airway surface liquid (ASL). The AOP network described here consists of sequences of KEs that culminate in the modulation of ciliary beat frequency and ASL height as well as mucus viscosity and hence, impairment of MCC, which in turn leads to decreased lung function.

Keywords: adverse outcome pathway, AOP, mucociliary clearance, ciliary beat frequency, lung function, new approach methodologies, NAMs, inhalation toxicity

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1 INTRODUCTION

Regulatory frameworks are moving towards risk assessment approaches that better protect human health and are not reliant on testing in animals. Therefore, 21st century science is incorporating the use of human-relevant methods that are ethical, scientifically sound, and can accurately predict the toxicity of chemicals. *In silico* models that consider human-relevant parameters as well as *in vitro* methods that vary in complexity—spanning from mono-to co-culture systems—are already being used to predict human outcomes. For example, the Organisation for Economic Co-operation and Development (OECD) (OECD, 2021) uses combined information from several sources (e.g. *in silico* predictions, *in chemico*, *in vitro* data) to predict pathological outcomes in humans in response to chemical exposure. Anchored to known mechanisms of human toxicity such mechanism-based

approaches enable us to understand whether a chemical will be toxic and through which pathway(s) it may act to cause the adverse outcome (AO) (Clippinger et al., 2018).

Adverse outcome pathways (AOP) are a means to organize known information related to a pathological outcome and understand the mechanism leading to the adverse effect. Starting with a molecular initiating event (MIE) and ending in an AO, AOPs are a sequence of causally linked key events (KE) that span different levels of biological organization—from the molecular to the whole organism level (Ankley et al., 2010). An AOP may not necessarily include every single event that contributes to the development of the AO, but it does include all KEs that are critical for its development (OECD, 2017). *In vitro* and *in silico* assays that measure each of the KEs of an AOP can be used to design testing approaches that closely predict human responses and replace the need for *in vivo* data in order to derive benchmark values for determining the potential adverse health impacts of chemicals. When combined with existing data and physicochemical information related to the test substance, AOP-based testing can help develop integrated approaches that can predict human responses (OECD, 2021). Given the usefulness of AOPs in the risk assessment of chemicals, the OECD launched a program for the development of AOPs in 2012; called the AOP-Wiki, this program is overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Several online resources, including the OECD AOP users' handbook, are available to aid developers in compiling AOPs on the AOPwiki (OECD, 2018). There are currently more than 300 AOPs online, at various stages of completion. One of these is AOP148 [EGFR Activation Leading to Decreased Lung Function, https://aopwiki.org/aops/148], which is extended and complemented by the AOP network described here.

With a surface area of ~100 m^2 and ventilated by 10,000 to 20,000 L of air per day (National Research Council, 1988; Frohlich et al., 2016), the lungs are a major barrier that protect the body from a host of external factors that enter the respiratory system and may cause lung pathologies. Mucociliary clearance (MCC) is a key aspect of the innate immune defense against airborne pathogens and inhaled particles. MCC is governed by the concerted action of its functional components, the cilia and the airway surface liquid (ASL), where the latter comprises mucus and the periciliary layer (PCL) (Bustamante-Marin and Ostrowski, 2017). Healthy subjects produce >10 ml airway secretions daily (King, 2006), which are continuously transported by the mucociliary escalator. Disturbances in any of the processes that regulate ASL volume, mucus production, mucus viscoelastic properties, or ciliary function can cause MCC dysfunction and are linked to airway diseases such as chronic obstructive pulmonary disease (COPD) and asthma, both of which bear a significant risk of increased morbidity and mortality. The mechanism by which exposure to inhaled toxicants might lead to mucus hypersecretion and thereby impact pulmonary function has already been mapped in AOP148 on decreased lung function. However, whether an exposure-related decline in lung function is solely related to excessive production of mucus is debatable, particularly in light of the close relationship between mucus, cilia function, and efficient MCC. To date, no single event has been attributed to MCC impairment. This AOP work evaluates the major processes known to be involved in ensuring efficient MCC and consists of sequences of KEs that culminate in the modulation of ASL, ciliary beat frequency (CBF), and mucus viscosity. Together, these processes impair MCC, which—when persistent—leads to decreased lung function. Evidence was gathered from the peer-reviewed literature from multiple sources (e.g., PubMed, Web of Science, Scopus) by keyword searches. No publication date limit was applied. Both empirical and quantitative evidence was captured, consolidated and transferred to the corresponding KE and KER pages on the AOPwiki following the recommendations in the AOP User's Handbook.

2 SUMMARY OF KEY EVENTS AND MECHANISMS

The epithelium of the respiratory tract has a powerful defense mechanism against airborne pollutants, owing to the combined performance of mucus-producing goblet cells and ciliated cells that are covered with microtubular projections called cilia. In response to various irritants and pathogens, goblet cells produce and secrete mucus, and the cilia sweep the mucus upward through coordinated beating motions, thus clearing the airways of these substances—a process which is termed MCC. Optimal MCC is dependent on multiple factors, including cilia number and structure, ASL height[1], and the physical and chemical properties of mucus. Any disturbances in these factors can lead to impaired MCC. A summary schematic of the AOP network delineating processes that lead from oxidative stress to decreased lung function is presented in **Figure 1** and detailed on the AOPwiki (https://aopwiki.org/aops/411, https://aopwiki.org/aops/424, https://aopwiki.org/aops/425).The MIE for this network of AOP is oxidative stress. Oxidative stress is generally regarded as a redox imbalance characterized by the increased production of oxidative species and concurrent depletion of antioxidant defenses. Thus, the overall redox balance of the cell/tissue is tipped in favor of oxidation. Various highly reactive species, collectively referred to as "reactive oxygen species" (ROS) or "reactive nitrogen species" (RNS), are formed continuously at relatively low concentrations during the normal biochemical functioning of cells and tissues. They are highly unstable because they contain unpaired electrons capable of initiating oxidation reactions and include free radicals such as hydroxyl radicals, superoxide anions, oxygen radicals, nitric oxide, and non-free radicals, such as hydrogen peroxide, peroxynitrite and hypochlorous acid (Rahman et al., 2006). However, upon exposure to certain xenobiotics or in the presence of pathogens, cells may form excessive ROS/RNS,

[1]The literature interchangeably refers to ASL height, ASL volume and ASL depth. The data presented in original research articles indicate that these terms are synonymous. To simplify the presentation of evidence, we will refer to "ASL height" here.

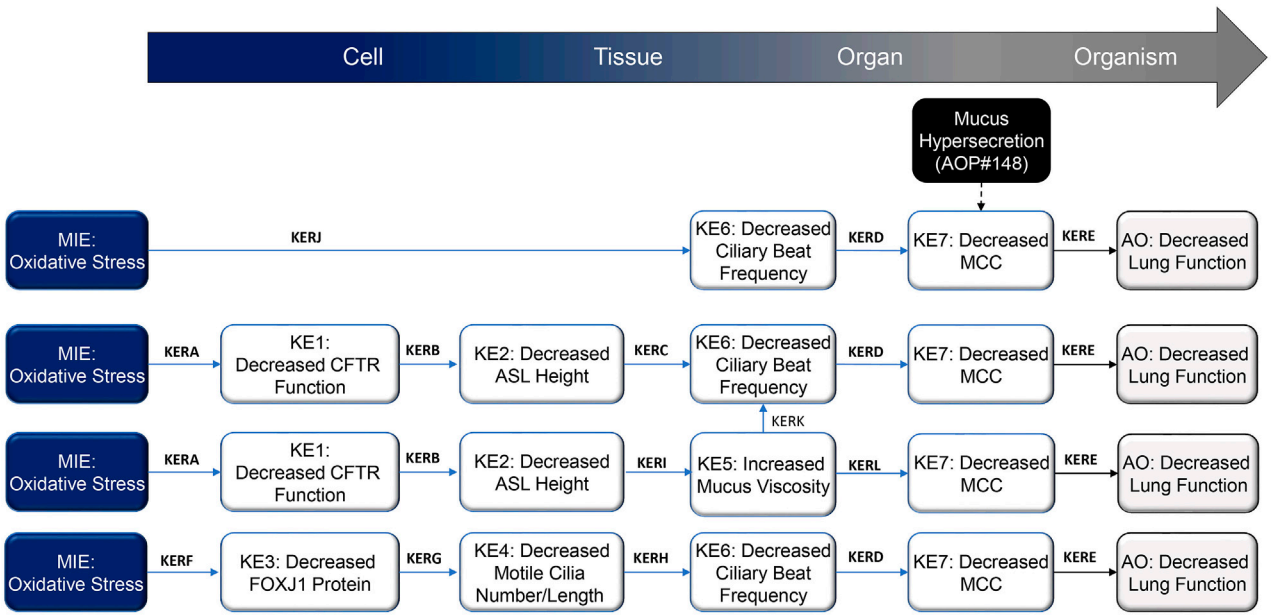

FIGURE 1 | Schematic representation of the proposed decreased lung function adverse outcome pathways (AOP), focusing on mechanisms that result in impaired mucociliary clearance (MCC) following inhalation exposure. Abbreviations: AO, adverse outcome; ASL, airway surface liquid; CFTR, cystic fibrosis transmembrane regulator; FOXJ1, forkhead box protein J1; KE, key event; KER; key event relationship; MCC, mucociliary clearance; MIE, molecular initiating event.

which may react with cellular components such as proteins, lipids and nuclear material, leading to the dysfunction of these components and, ultimately, cell death and disease manifestation (Halliwell and Aruoma, 1991; Berlett and Stadtman, 1997). Protective enzymes such as catalase, glutathione peroxidase, superoxide dismutase, and thioredoxin—in combination with radical scavengers such as glutathione, ascorbic acid, uric acid and vitamin E—work in concert to maintain ROS/RNS levels that are not overly damaging to cells and cellular systems (Rahman et al., 2006).

In the lungs, free radical species may be endogenously produced or introduced following exposure to exogenous sources, such as air pollutants, inhaled chemicals/therapeutics, and cigarette smoke (Church and Pryor, 1985). The main cellular sources of reactive species in the lungs include neutrophils, eosinophils, alveolar macrophages, alveolar epithelial cells, bronchial epithelial cells, and endothelial cells (Holland et al., 1990; Kinnula et al., 1992; Kinnula et al., 1995); these cells may increase their ROS/RNS production in response to infection or tissue damage. ROS/RNS generally inflict their effects by remodeling extracellular matrix and stimulating mucus secretion and repair responses (Poli and Parola, 1997). Oxidative stress can lead to a variety of respiratory diseases, such as asthma, acute respiratory distress syndrome and COPD (Rahman and MacNee, 1996; Chabot et al., 1998).With respect to this specific AOP, localized oxidative stress in the airways as a result of cigarette smoke exposure, for example, can cause damage to various proteins linked to the regulation of cilia function. Reduced expression of the *CFTR* (cystic fibrosis transmembrane conductance regulator) transcript, diminished CFTR protein levels, and altered chloride (Cl^-) channel gating lead to acquired CFTR dysfunction (Clunes et al., 2012; Braun, 2014), which perturbs the height of the ASL and facilitates cilia collapse. Furthermore, oxidative damage has been reported to decrease the FOXJ1 (forkhead box protein J1) gene and protein expression, a critical protein involved in the assembly of motile cilia (Milara et al., 2012; Brekman et al., 2014; Garcia-Arcos et al., 2016; Valencia-Gattas et al., 2016; Ishikawa and Ito, 2017). Collectively, these perturbations result in decreased MCC from the upper airways.

CFTR is a multi-domain membrane protein belonging to the large family of adenine nucleotide-binding cassette transporters (Riordan, 2008). It is an integral membrane glycoprotein which functions as cyclic adenosine monophosphate (cAMP)-activated and phosphorylation-regulated Cl^- channel at the apical membrane of epithelial cells (Farinha et al., 2013). In respiratory epithelia, CFTR mediates fluid and electrolyte transport, and its function is critical to ASL homeostasis. Exposure to inhaled oxidants leads to decreased CFTR gene and protein expression as well as CFTR internalization, which reduces protein presentation at the membrane and reduces or abolishes short-circuit currents (Cantin et al., 2006a; Cantin et al., 2006b; Clunes et al., 2012; Sloane et al., 2012; Rasmussen et al., 2014). Decreased CFTR expression (KE1) in airway epithelium has been observed in cystic fibrosis and after hypoxia and cigarette smoke exposure, resulting in reduced Cl^- transport and, ultimately, reduced ASL depth (Alexander et al., 2012; Clunes et al., 2012; Rasmussen et al., 2014; Woodworth, 2015; Raju et al., 2016).

The ASL is a liquid layer on the apical side of the respiratory epithelium, reportedly between 5 and 100 µm in depth (Widdicombe and Widdicombe, 1995). It consists of an inner aqueous PCL, which spans the length of the cilia, and an outer gel-like mucus layer. The PCL has a low viscosity and enables ciliary beating, thereby facilitating the movement of the outer

mucus layer toward the glottis and, ultimately, its removal by cough or ingestion (Antunes and Cohen, 2007). Both ASL composition and height are considered critical for its function (Fischer and Widdicombe, 2006). Under physiological conditions, ASL composition and height are regulated through vectorial transport of electrolytes, driven by transepithelial transport and apical secretion of Cl^- by (predominantly) CFTR, which results in passive water secretion and, consequently, increased ASL height. Absorption of sodium ions (Na^+) on the apical side by the epithelial sodium channel (ENaC) and its interaction with the basolateral Na^+/K^+-ATPase leads to net absorption of Na^+, which in turn drives fluid absorption and therefore decreases ASL height (KE2) (Hollenhorst et al., 2011; Althaus, 2013). Impairment of CFTR or ENaC function can lead to the dysfunction of the other ion channel (increased CFTR activity leads to decreased ENaC activity and vice versa) (Hobbs et al., 2013; Munkholm and Mortensen, 2014), resulting in perturbation of ASL height.

The number, structure, and cohesive beating of the motile cilia lining the upper and lower respiratory tract are critical for efficient MCC. Motile cilia are microtubular organelles, 6–7 μm long and 0.2–0.3 μm in diameter (Brooks and Wallingford, 2014; Yaghi and Dolovich, 2016). They protrude from the cell surface and generate directional flow of fluid though coordinated beating. Approximately 50–80% of the human respiratory epithelium is comprised of ciliated cells; each ciliated cell is covered by more than a hundred motile cilia, which move mucus upwards (together with mucus-trapped substances) upward (Yaghi and Dolovich, 2016; Bustamante-Marin and Ostrowski, 2017). Cilia formation is initiated and coordinated by a distinct gene expression program, led by the transcription factor FOXJ1 (Brody et al., 2000; Zhou and Roy, 2015). The multiple motile cilia assembly factors MCIDAS (multiciliate differentiation and DNA synthesis associated cell cycle protein) and GMNC (geminin coiled-coil domain containing) converge in positively regulating FOXJ1 (Stubbs et al., 2012; Arbi et al., 2016; Berta et al., 2016), whereas NOTCH (Notch homolog (Drosophila))-, IL-13 (interleukin-13)- or EGF (epidermal growth factor)-triggered signaling antagonizes FOXJ1-driven multiciliogenesis (Gomperts et al., 2007; Shaykhiev et al., 2013; Gerovac et al., 2014; Gerovac and Fregien, 2016). Although various other factors are involved in multiple motile cilia assembly—including MYB (MYB proto-oncogene), RFX3 (regulatory factor X3), ULK4 (Unc-51 like kinase 4), Wnt signaling, and others—they mostly act upstream or in parallel to FOXJ1 (Tan et al., 2013; Choksi et al., 2014; Liu et al., 2016; Schmid et al., 2017). FOXJ1 appears to be the major factor in multiciliogenesis, whereby its activity is necessary and also sufficient for programming cells to assemble functional motile cilia (Vij et al., 2012; Zhou and Roy, 2015). It is not surprising, therefore, that a decrease in FOXJ1 levels (KE3) inhibits ciliogenesis in multiciliated cells in zebrafish and *Xenopus* (Stubbs et al., 2008), and knockdown of FOXJ1 results in almost complete absence of cilia in mouse epithelial cells (Chen et al., 1998; Brody et al., 2000). FOXJ1 expression also decreases in cigarette smoke extract-treated human airway epithelial cells, leading to suppression of cilia growth, which can be restored by overexpression of the protein (Brekman et al., 2014).

Because ciliated cell density and the multiple motile cilia length and number per cell correlate with CBF—which is routinely used as a predictor of MCC efficiency (King, 2006)—it follows that, if cilia numbers decrease (KE4), CBF decreases (KE6). Cohesive beating of multiple motile cilia with a specific frequency and pattern propels mucus (and trapped particles or pathogens) upwards, creating a continuous movement (Chilvers and O'Callaghan, 2000). CBF is influenced by several factors, including structural modulation in the cilia and the concentrations of the cyclic nucleotides cAMP and cGMP and intracellular calcium (Ca^{2+}) (Rubin, 2002). CBF also depends on the physical and chemical properties of the ASL. If ASL height decreases following, for example, exposure to cigarette smoke, the cilia cannot extend to their full height, and MCC efficiency will drop. In addition, reduced ASL height results in airway dehydration, which increases mucus viscosity (KE5) (Gheber et al., 1998; Lai et al., 2009; Fahy and Dickey, 2010). Increased mucus viscosity, in turn, decreases CBF and slows the transport of mucus on the mucociliary escalator (i.e., decreases MCC; KE7). In chronic inflammatory states, as seen (for example) in the lungs of cystic fibrosis, asthma, or COPD patients, decreased MCC can lead to mucus impaction, resulting in the formation of mucus plugs, which then in turn obstruct the airways and, consequently, lead to decreased lung function (AO) over time (Wanner et al., 1996; Szczesniak et al., 2017; Dunican et al., 2021).

3 EMPIRICAL EVIDENCE FOR KEY EVENT RELATIONSHIPS

Table 1 presents a summary of supporting evidence for each of the KERs in this AOP. KERs are rated as "strong", "moderate", or "weak" on the basis of empirical evidence supporting a change in an upstream KE (KEup) leading to an appropriate change in the immediate downstream KE (KEdown). Other considerations are whether KEups occur at lower doses, earlier time points, and at a higher incidence than KEdowns and if there are any inconsistences in the published data. The experimental evidence for a causal relationship between the KEup and KEdown in this AOP has been provisionally rated as "moderate" or "strong" in most cases.

Exposure to inhaled oxidants, such as cigarette smoke and ozone, leads to decreased CFTR gene and protein expression as well as CFTR internalization (KE1), thereby reducing or abolishing open probabilities, short-circuit currents and subsequently ASL height/volume (KE2) (Kulka et al., 2005; Cantin et al., 2006a; Cantin et al., 2006b; Qu et al., 2009; Clunes et al., 2012; Sloane et al., 2012; Rasmussen et al., 2014). Both reduced mRNA stability (Cantin et al., 2006a) and decreased transcription rates (Bargon et al., 1992a; Bargon et al., 1992b; Rasmussen et al., 2014) reportedly contribute to diminished *CFTR* mRNA expression. CFTR expression was also modulated by STAT1 (Kulka et al., 2005; Qu et al., 2009) and Nrf2 signaling (Zhang et al., 2015). Additionally, on the post-transcriptional level, CFTR function has been shown to be affected by oxidative stress (Clunes et al., 2012) and ischemia (Brézillon et al., 1997; Bodas et al., 2017).

TABLE 1 | Empirical evidence for key event relationships (KER).

KER		
		Defining question: Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses, earlier time points, and higher in incidence than KEdown? Inconsistencies? High (Strong): Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data Moderate: Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors Low (Weak): Limited or no studies reporting dependent change in both events following exposure to a specific stressor, and/or significant inconsistencies in empirical support across taxa and species
KERA Oxidative stress leading to decreased CFTR function	Strong	Inducers of oxidative stress such as cigarette smoke reduced CFTR expression at both the RNA Cantin et al. (2006a); Cantin et al. (2006b); Qu et al. (2009); Rennolds et al. (2010) and protein (Cantin et al. (2006b); Qu et al. (2009); Rennolds et al. (2010); Sloane et al. (2012); Hassan et al. (2014); Rasmussen et al. (2014); Xu et al. (2015) level *in vitro*. CFTR protein expression was lower in the airways of smokers compared to non-smokers Dransfield et al. (2013). In some of these studies, an accompanying decrease in Cl^- conductance was also observed Qu et al. (2009); Rennolds et al. (2010); Sloane et al. (2012). There are many studies that support a direct link between oxidative stress and decreased CFTR function *in vitro*, *ex vivo*, *in vivo* and in human subjects. Human primary epithelial cells and cell lines of respiratory epithelial origin have consistently decreased conductance of Cl^- and other ions following exposure to cigarette smoke and other oxidants (Cantin et al. (2006b); Schwarzer et al. (2008); Raju et al. (2013); Lambert et al. (2014); Schmid et al. (2015); Raju et al. (2016); Chinnapaiyan et al. (2018), which could be reversed upon antioxidant treatment Raju et al. (2013); Lambert et al. (2014); Schmid et al. (2015). Similar observations were made under hypoxic conditions Brézillon et al. (1997); Zhang et al. (2013); Woodworth, (2015). Antioxidants could also increase Cl^- conductance and anion transport in the absence of oxidant treatment or hypoxia induction in human and murine respiratory cells *in vitro* and in *ex vivo* tissues Azbell et al. (2010); Alexander et al. (2011); Conger et al. (2013). Healthy smokers and smokers with COPD have reduced Cl^- conductance Sloane et al. (2012); Dransfield et al. (2013) and increased sweat chloride concentrations Raju et al. (2013); Courville et al. (2014)
KERB Decreased CFTR function leading to decreased ASL height	Strong	As a major Cl^- channel in the respiratory epithelium, CFTR levels and function are vital for maintenance of ASL homeostasis. *In vitro* studies on the effects of cigarette smoke exposure on human lung primary cells and cell lines showed a reduction in ASL height, associated with decreased CFTR levels Hassan et al. (2014); Rasmussen et al. (2014); Xu et al. (2015); Ghosh et al. (2017) and decreased Cl^- current Lambert et al. (2014); Raju et al. (2016). Moreover, pharmaceutical stimulation and inhibition of CFTR function and expression directly increased and decreased ASL height, respectively Song et al. (2009); Van Goor et al. (2009); Van Goor et al. (2011); Tuggle et al. (2014)
KERC Decreased ASL height leading to decreased CBF	Weak	Concurrent ASL height and CBF decreases were noted in human 3D airway epithelial cultures following exposure to cigarette smoke Åstrand et al. (2014); Xu et al. (2015) and following the addition of large dextran molecules, low-melting point agarose or endogenous mucus Button et al. (2012). Treatment of human airway epithelial with an ENaC inhibitor prevented the cigarette smoke effect on ASL height and CBF Åstrand et al. (2014). In addition, treatment of cystic fibrosis airway cultures with a CFTR-modifying drug increased both ASL height and CBF Van Goor et al. (2009)
KERD Decreased CBF leading to decreased MCC	Moderate	A decrease in CBF resulting from sulfur dioxide exposure reduced mucociliary clearance in dogs Yeates et al. (1997) and mucociliary activity in guinea pig tracheas Knorst et al. (1994). In rats, formaldehyde inhalation exposure resulted in lower numbers of ciliated cells, while ciliary activity and mucus flow rates were decreased in a dose and time-dependent manner (Morgan et al. (1986). In humans, CBF positively correlates with nasal mucociliary clearance time Ho et al. (2001), and bronchiectasis patients have lower nasal CBF and slower mucociliary transport (MCT) Rutland and Cole, (1981). Administration of nebulized CBF inhibitors and enhancers quantifiably decreased or increased mucociliary clearance, respectively Boek et al. (1999); Boek et al. (2002). Increased CBF and MCT was also noted in human sinonasal epithelial cell cultures treated with Myrtol®, an essential oil distillate Lai et al. (2014) and in sheep tracheas and human airway epithelial cultures subjected to temperature changes Kilgour et al. (2004); Sears et al. (2015). Exposures of frog palate epithelia to formaldehyde and PM10 reduced MCC and mucociliary transport, but only formaldehyde-treated epithelia showed decreases in CBF Morgan et al. (1984); Macchione et al. (1999); Fló-Neyret et al. (2001) *Ex vivo* treatment of sheep trachea with acetylcholine and epinephrine increased CBF, but only acetylcholine increased surface liquid velocity, while both parameters were decreased upon incubation with platelet-activating factor Seybold et al. (1990)
KERE Decreased MCC leading to decreased lung function	Moderate	Changes in MCC rate are typically paralleled by effects on lung function in several studies where both endpoints have been assessed. In patients with primary ciliary dyskinesia, absence of cilia motion prevents normal MCC and consequently, lung function is reduced Denizoglu Kulli et al. (2020). In cystic fibrosis patients, the ASL is depleted resulting in impaired MCC Boucher, (2004). Although the known CFTR genotypes can result in a variety of phenotypes Derichs, (2013), clinical data indicate that some specific gene defects, such as the p.Phe508del variant, are more frequently associated with decreased lung function indices (e.g. FEV1% predicted, FVC % predicted, FEF25-75) Kerem et al. (1990); Johansen et al. (1991); Schaedel et al. (2002). Both cigarette smoking and occupational exposure to biomass fumes led to slower MCC and reduced FEV1% predicted and FEV1/FVC Ferreira et al. (2018). Nasomucociliary clearance was slower in COPD smokers compared to former smokers with COPD or to nonsmokers Ito et al. (2015). Allergen challenge in asthma patients resulted in both reduced MCC and FEV1, which could be reversed by inhalation of hypertonic saline solution Alexis et al. (2017). In cystic fibrosis patients, treatment with mucolytic agents Laube et al. (1996); McCoy et al. (1996); Quan et al. (2001); Elkins et al. (2006); Amin et al. (2011); Donaldson et al. (2018) or a CFTR potentiator Rowe et al. (2014) improved both MCC and lung function (FEV1, FVC and FEF25-75)
KERF Oxidative stress leading to decreased FOXJ1 protein	Moderate	Cigarette smoke-induced oxidative stress downregulates FOXJ1 expression at both the gene and protein levels in human lung cells *in vitro* Milara et al. (2012); Brekman et al. (2014); Valencia-Gattas et al. (2016); Ishikawa and Ito, (2017). Oxidative stress induced by human respiratory syncytial virus reduces FOXJ1 mRNA levels, which can be restored by treatment with antioxidants or the phosphodiesterase 4 inhibitor roflumilast N-oxide Akaike et al. (1990); Geiler et al. (2010); Mata et al. (2012). In mice, thoracic irradiation results in free radical generation and subsequent reduction in FOXJ1 mRNA expression

(Continued on following page)

TABLE 1 | (*Continued*) Empirical evidence for key event relationships (KER).

		Bernard et al. (2012). Many genes that are transcriptionally regulated by FOXJ1 are also downregulated following exposure to cigarette smoke, which implies a reduction in FOXJ1 transcriptional activity Brekman et al. (2014)
KERG	Strong	Homozygous null mutation of Foxj1 results in complete absence of cilia in mouse respiratory epithelium Chen et al. (1998); Brody et al. (2000). In a previous study, wild-type mice had approximately 20% heavily ciliated cells in the proximal pulmonary epithelium, while explanted Foxj1$^{-/-}$ mouse trachea had no ciliated cells Gomperts et al. (2004). Loss of FOXJ1 orthologs FoxJ1–4 in flatworm *Schmidtea mediterranea* results in loss of ciliation of the ventral epithelium which closely resembles the human airway epithelium Rompolas et al. (2009); Vij et al. (2012). Loss of Foxj1 activity in *Xenopus* and zebrafish—through antisense morpholino oligonucleotides—reduces cilia formation, while, conversely, ectopic Foxj1 overexpression results in formation of multiple motile cilia Stubbs et al. (2008); Yu et al. (2008). There is a strong correlation between FOXJ1 and expression of the FOXJ1 ciliogenesis program genes in zebrafish, *Xenopus* and mouse cells Abedalthagafi et al. (2016)
Decreased FOXJ1 protein leading to decreased motile cilia length/number		Treatment with cigarette smoke extract downregulates FOXJ1 mRNA and protein expression, which is accompanied by a reduction in cilia length and number in human bronchial epithelial cells *in vitro* Milara et al. (2012); Brekman et al. (2014). This can be prevented by overexpression of FOXJ1 Brekman et al. (2014) or treatment with roflumilast N-oxide, which reduces intracellular free radical levels and increases FOXJ1 mRNA and protein expression Milara et al. (2012)
KERH	Moderate	In *Chlamydomonas*, ciliary motion is directly related to the length of the cilia Bottier et al. (2019). Similar observations have been made in zebrafish, where modulation of cilia length and number by FOR20 (centrosomal protein 20) deletion/knockdown directly impairs ciliary motility Xie et al. (2019). There is also a positive correlation between cilia number and CBF in sinusitis patients Joki et al. (1998), while cilia number, length and orientation correlate positively with mucociliary transport rate in patients with recurrent or longstanding respiratory infections Toskala et al. (1995); Joki et al. (1998). Comparisons of strips of normal and disrupted ciliated epithelium have shown that CBF is decreased in the latter Thomas et al. (2009)
Decreased motile cilia length/number leading to decreased CBF		Mathematical models and simulations have shown that periciliary liquid and mucus velocity are directly affected by cilia number and length Lee et al. (2011); Jayathilake et al. (2012); Jayathilake et al. (2015)
KERI Decreased ASL height leading to increased mucus viscosity	Moderate	The phenomenon of ASL volume changes determining mucus viscosity is well described in the cystic fibrosis literature. In patients with this genetic defect, impaired CFTR function results in ASL depletion and mucus hyperviscosity Knowles and Boucher, (2002); Puchelle et al. (2002); Mall et al. (2004); Tarran, (2004). This has been confirmed experimentally in pig and rat models of this disease Birket et al. (2014); Birket et al. (2016); Birket et al. (2018). Studies with transgenic mice overexpressing βENaC in the airways also corroborate the link between ASL dehydration and increased mucus viscosity, evidenced by the increased incidence of airway mucus plugging [129, 195]. In a ferret model of cigarette smoke-induced COPD, ASL depletion was shown to be one of the drivers of increased mucus viscosity and decreased MCC Lin et al. (2020). The same study also showed that mucus from COPD patients, obtained from 3D organotypic airway epithelial cultures from different smoking donors with COPD, is significantly more viscous than that from healthy, non-smoking individuals and smokers without disease Lin et al. (2020)
KERJ Oxidative stress leading to decreased CBF	Strong	Experimental studies *in vitro* have shown that exposure of ciliated respiratory cells directly or indirectly to sources of oxidative stress leads to decreased CBF Burman and Martin, (1986); Wilson et al. (1987); Feldman et al. (1994); Yoshitsugu et al. (1995); Min et al. (1999), which can be reversed by treatment with antioxidants Schmid et al. (2015). Cigarette smoke condensate, a known inducer of oxidative stress, also causes a decrease in CBF *in vitro* Cohen et al. (2009), while, in human subjects exposed to different oxygen levels, oxygen stress causes a decrease in nasal CBF Stanek et al. (1998)
KERK Increased mucus viscosity leading to decreased CBF	Moderate	Several studies have shown that there is an optimal range of viscoelastic mucus properties that facilitates efficient MCC and that changes in mucus viscosity beyond that optimal range impact CBF and alter MCC. Studies in humans, mice, hamsters, horses and frogs have shown that increased mucus viscosity correlates with a decrease in CBF King, (1979); Gheber et al. (1998); Matsui et al. (1998); Andrade et al. (2005); González et al. (2016); Kikuchi et al. (2017); Birket et al. (2018)
KERL Increased mucus viscosity leading to decreased MCC	Moderate	Mucus viscoelastic properties, whether altered by airway dehydration or mucus hypersecretion, directly influence MCC. Studies cystic fibrosis models and those on mimicking changes in mucus viscosity by using (bio)polymers or large molecules such as dextran have indicated a dose-response effect of increasing mucus viscosity on mucociliary transport rates, although these changes are transient in nature in *ex vivo* and *in vitro* systems Birket et al. (2018); Fernandez-Petty et al. (2019). Increased mucus viscosity also has a negative impact on MCC in horses with recurrent airway obstruction Gerber et al. (2000). Conversely, inhalation of hypertonic saline solution decreases mucus viscosity and enhances MCC in cystic fibrosis patients Robinson et al. (1997)

Abbreviations: 3D, three-dimensional; ASL, airway surface liquid; CBF, ciliary beating frequency; CFTR, cystic fibrosis transmembrane regulator; Cl^-, chloride (ion); COPD, chronic obstructive pulmonary disease; ENaC, epithelial sodium channel; FEF25-75, forced expiratory flow between 25 and 75% of FVC; FEV1, forced expiratory volume in 1 s; FOR20, centromere protein 20; FOXJ1, forkhead box J1; FVC, forced vital capacity; MCC, mucociliary clearance; MCT, mucociliary transport.

Serous and glandular secretions of the airway epithelium contribute to the ASL, and epithelial ion channel function is critical to ASL homeostasis. Absorption of liquid to and from the mucus layer serves to maintain ASL depth. The regulation of these reabsorption processes is complex and not fully elucidated (Boucher, 2004). Experimental evidence suggests that the balance between Na^+ absorption and Cl^- secretion—mediated by ENaC and CFTR, respectively—plays a major role in these processed, with the ion channels affecting each other's activity (Boucher, 2003; Boucher, 2004; Schmid et al., 2011). Impaired functioning of the CFTR and ENaC ion channels results in enhanced Na^+ absorption, reduced Cl^- secretion, and consequently, reduced ASL height (KE2). This phenomenon is well known not only from studies in models of cystic fibrosis and acquired CFTR deficiency—even though the exact mechanism of the interaction between these two channels remains to be elucidated (Tarran et al., 2001; Boucher, 2003; Zhang et al., 2013; Hassan et al., 2014; Rasmussen et al., 2014; Woodworth, 2015; Raju et al., 2016)—but also from studies with pharmacological agents that enhance CFTR expression and/or

function or perturb the interaction between CFTR and ENaC (Van Goor et al., 2009; Van Goor et al., 2011; Lambert et al., 2014).

Under physiological conditions, ASL height is adjusted to the appropriate height, which helps maintain the PCL depth at approximately the length of the cilia (Antunes and Cohen, 2007). If the airways become "dehydrated" (i.e., the ASL height decreases; KE2), the cilia collapse and ciliary movement is slowed or inhibited (KE6) (Matsui et al., 1998; Tarran et al., 2001; Knowles and Boucher, 2002; Munkholm and Mortensen, 2014). Decreased ASL height also contributes to increased mucus viscosity (KE5), a phenomenon that is well described in cystic fibrosis, where *CFTR* defect results in decreased ASL height, leading to decreased MCC (KE7) and subsequent mucus plugging (Birket et al., 2014; Birket et al., 2016; Birket et al., 2018).

Free radicals such as super oxides, hydroxyl radicals, and hydrogen peroxides are a common factor in various respiratory diseases, such as acute respiratory distress syndrome, asthma and pneumonia. Oxidative stress (such as that caused by cigarette smoke exposure or irradiation) leads to decreased FOXJ1 gene and protein expression (KE3) as well as to decreased FOXJ1 target gene expression (Milara et al., 2012; Brekman et al., 2014; Garcia-Arcos et al., 2016; Valencia-Gattas et al., 2016; Ishikawa and Ito, 2017). Because FOXJ1 is a key factor of multiple motile cilia assembly in the respiratory airways (Zhou and Roy, 2015), oxidative stress blocks the multiciliogenesis program, which is necessary and also sufficient to program cells to grow functional motile cilia (Hua et al., 2010; Vij et al., 2012). Studies in different model organisms have shown that the loss of FOXJ1 (KE3) results in a loss of multiple motile cilia (KE4) (Chen et al., 1998; Brody et al., 2000; Stubbs et al., 2008; Vij et al., 2012).

Cilia in the respiratory epithelium beat in a coordinated fashion at a frequency of approximately 7–16 Hz, propelling mucus upwards (Joki et al., 1998; Smith et al., 2012; Jing et al., 2017). Many factors have been shown to affect ciliary function, including cilia length, number, structure, orientation, and distribution as well as mucus viscosity, temperature, pH, chemicals, ASL height, and exposure to bacterial and viral pathogens (Kanthakumar et al., 1996; Clary-Meinesz et al., 1998; Joki et al., 1998; Ho et al., 2001; Mall, 2008; Smith et al., 2012; Jing et al., 2017; Snyder et al., 2017). Alterations in normal physiological conditions and healthy cilia number/length/structure (KE4) as well as oxidative stress through exposure to hydrogen peroxide or free radicals typically reduce CBF (KE6) (Burman and Martin, 1986; Clary-Meinesz et al., 1998; Min et al., 1999; Jayathilake et al., 2012).Synchronized ciliary action helps transport mucus from the lungs to the mouth, where it is swallowed or expectorated (Munkholm and Mortensen, 2014). In addition to ASL and mucus properties, the speed of mucus movement—and hence the effectiveness of MCC—is dependent on ciliary amplitude and beat frequency (Rubin, 2002). Aside from genetic defects leading to ciliopathies, there is ample evidence that prolonged exposure to noxious agents, such as cigarette smoke, nitrogen oxide and sulfur dioxide, causes a decrease in CBF (KE6) and, subsequently, MCC (KE7) (Knorst et al., 1994; Yeates et al., 1997; Kakinoki et al., 1998; Cohen et al., 2009; Schmid et al., 2015). CBF also seems to be dependent on mucus viscosity, with the CBF decreasing with increasing viscosity in animal models (Andrade et al., 2005; Kikuchi et al., 2017). This linear correlation between CBF (KE7) and mucus viscosity (KE5) has also been confirmed in mathematical models simulating the two-layer mucociliary transport process (Lee et al., 2011; Sedaghat et al., 2016).

Finally, the link between decreased MCC and decreased lung function (AO) is well established through observations in patients with ciliary defects (e.g., primary ciliary dyskinesia) and cystic fibrosis. Failure to clear mucus from the lungs causes mucus build up, which can lead to mucus plugging in the airways and, consequently, leads to decreased lung function over time (Mossberg et al., 1978; Regnis et al., 1994; Wanner et al., 1996; Robinson and Bye, 2002; Kerem et al., 2014; Szczesniak et al., 2017). Mucus plugging due to decreased MCC is also considered a major cause of airway obstruction and airflow limitation in COPD patients (Okajima et al., 2020; Dunican et al., 2021) and asthmatics (Maxwell, 1985; Kuyper et al., 2003).

4 OVERALL ASSESSMENT OF THE ADVERSE OUTCOME PATHWAYS

4.1 Key Event Essentiality

The definition of essentiality implies that modulation of upstream KEs impacts the downstream KEs in an expected fashion. When blocked or when they fail to occur, the KEs in the current AOP will not necessarily stop the progression to subsequent KEs. Owing to the complex biology of motile cilia formation and function, ASL homeostasis, mucus properties, and MCC, the KEs and AO may be triggered because of alternative pathways or biological redundancies. However, when exacerbated, the KEs promote the occurrence of downstream events that eventually lead to the AO. The causal pathway starting from exposure to oxidants and leading to decreased lung function involves parallel routes with KEs, each of which is sufficient to cause the downstream KE to occur. Different mechanisms—such as oxidant-induced decreases in ASL height due to CFTR function decline or oxidant-induced decrease in cilia number and length as a result of decreased FOXJ1 levels—lead to decreased CBF and decreased MCC. Each of these pathways contributes to the AO, but their relative contributions are difficult to evaluate. We judge the KEs MIE, KE1, KE3, KE4, KE6, and KE7 as highly essential and suggest moderate essentiality for KE2 and KE5 (**Table 1**; AOPwiki, https://aopwiki.org/aops/411, https://aopwiki.org/aops/424, https://aopwiki.org/aops/425).

4.2 Key Event Relationship Biological Plausibility

Mechanistic data on the pathways that contribute to oxidative stress-elicited lung damage have varied coverage in current literature. The AOP network we present here starts with an oxidant exposure or exposure-causing oxidative stress leading to decreased CFTR and FOXJ1 mRNA and protein levels as well as decreased protein function. KERA (oxidative stress leading to

decreased CFTR function) is supported by multiple studies across different species, which suggest their high biological plausibility (for empirical evidence supporting each KER, refer to **Table 1**). For a similar inhibitory role of oxidative stress on FOXJ1, the studies are less ample. However, there is credible evidence that oxidative stress has a deteriorating effect on FOXJ1 transcript and protein levels as well as on the function of this transcription factor. Therefore, we judge the plausibility of KERF (oxidative stress leading to decreased FOXJ1 protein) to be moderate.

The biological functions of CFTR and FOXJ1 are extensively studied and established across different test systems, implying the high biological plausibility of both KERB (decreased CFTR function leading to decreased ASL height) and KERG (decreased FOXJ1 protein leading to decreased motile cilia length/number). Specifically, CFTR contributes to healthy lung function by regulating epithelial ion conductance to support ASL height maintenance (Boucher, 2003; Csanady et al., 2019), and FOXJ1 is an essential factor for functional multiple motile cilia assembly (Vij et al., 2012; Choksi et al., 2014). Both decreased ASL height (KE2) and decreased motile cilia length/number (KE4) lead to decreased CBF (KE6), as outlined in KERC and KERH, respectively. Multiple studies describe the link between decreased ASL height and reduced CBF. However, the causality between these KEs is not well-established, prompting us to judge KERC as weakly supported. As for KERH, higher numbers of motile cilia with a healthy length support efficient ciliary beating, and a decrease in cilia number and/or length results in a proportionate reduction in CBF. This causal relationship is logical but is directly tested only in few studies. Therefore, we rank the biological plausibility of KERH as moderate. ASL height is also linked to the physical properties of mucus, and studies in models of or individuals with cystic fibrosis support the link between ASL depletion and increased mucus viscosity (KERI), though the overall evidence is sparse, and causality is not always proven. Because the dependencies between these two KEs were highlighted in different species *in vitro* and *in vivo*, and the underlying mechanism is well established, we judge the plausibility of KERI as moderate.

Additionally, we propose a direct relationship between oxidative stress and KE6, decreased CBF (KERJ). A variety of oxidants, such as hydrogen peroxide, nitric dioxide, sulfur dioxide, acetaldehyde, ozone, and cigarette smoke decrease CBF in airway epithelial cells in a dose- and time-dependent manner after exposure. This link is demonstrated in several studies in various species, and we judge the plausibility of KERJ as strong. Synchronized ciliary beating helps transport mucus from the distal airways to the mouth, where it is cleared through ingestion or expectoration. *In vivo* studies and observations in patients with ciliopathies, respiratory infections, or allergies, and following exposure to inhaled toxicants that compromise ciliary function demonstrate that absent, decreased, or asynchronous cilia beating results in defective mucus clearance. Pharmacological studies have demonstrated that CBF stimulation typically results in MCC stimulation. While some results support both a dose-dependent response and temporal sequence of decreased CBF (KE6) leading to decreased MCC (KE7), most studies evaluate these KEs in parallel, and no clear causal linkage is affirmed. The same is true for increased mucus viscosity. Mucus viscoelastic properties, whether altered by airway dehydration or mucus hypersecretion, directly influence MCC. In fact, there is an inverse relationship between mucus viscosity and CBF (KERK) and mucus transport/MCC (KERL), as demonstrated in several *in vivo* and *ex vivo* studies. A large proportion of these studies have employed (bio) polymers or other large organic molecules to mimic the mucus layer in the airways and the increase in its viscosity. In addition, some of these studies have shown that decreased mucus viscosity may also result in impairment of MCC. Therefore, a causal link is only tentatively supported. Because cilia function, ASL height, and mucus properties are intricately linked to each other as evidenced by cystic fibrosis studies, we consider the plausibility of KERD, KERK, and KERL as moderate.

Different routes lead to impaired MCC, such as smoking-related oxidative stress, ciliary defects or CFTR mutations. Regardless of the route that leads to a reduction in MCC, individuals with impaired MCC exhibit decreased lung function. Moreover, many pharmacological treatments that enhance MCC also improve lung function. KE7 and the AO are thus closely related; however, as causal evidence is not always available, we judge the biological plausibility of KERE (decreased MCC leading to decreased lung function) as moderate.

The linear AOPs presented here have certain knowledge gaps; however, overall, we consider the biological plausibility of our AOP network as strong, as the network integrates different plausible pathways from the same MIE, leading to a common AO. For example, while oxidative stress leading to decreased lung function through the branch MIE → KE1 → KE2 → KE5 and/or KE6 → KE7 → AO has a weakly supported link represented by KERC (KE2 → KE6, i.e., decreased ASL height leading to decreased CBF), the oxidative stress can lead to the AO also through MIE → KE6 → KE7 → AO or via decreased FOXJ1 protein levels through MIE → KE3 → KE4→ KE5 and/or KE6 → KE7 → AO.

5 DISCUSSION AND CONCLUSION

Here, we have presented an AOP that links oxidative stress resulting from inhalation exposure to toxicants to impaired lung function via a decrease in MCC. Given the individual and public health burden of the consequences of lung function impairment, gaining a greater understanding of the underlying mechanisms of this pathology is extremely important in the risk assessment of inhaled toxic chemicals. There is strong empirical evidence to support several of the KERs in this AOP, particularly at the cellular level (i.e., oxidative stress leading to decreased CFTR function [KERA], decreased CFTR function leading to decreased ASL height [KERB], and oxidative stress leading to decreased CBF [KERJ]). However, additional evidence on causality is required to attribute stronger plausibility to KERs such as that between decreased ASL height and decreased CBF (KERC), which we evaluated as being weak. Future studies, using *in vitro* assays such as those outlined in **Supplementary Table S1**, that directly assess this linkage as well as the KERs we determined to have moderate plausibility (i.e., KERD,

KERE, KERF, KERH, KERI, KERK, and KERL) will help greatly strengthen this AOP overall.

An integrated assessment of substances with the potential to be inhaled, either intentionally or unintentionally, could incorporate inhalation exposure and dosimetry modelling to inform an *in vitro* assessment approach with appropriate exposure techniques and cell systems for assessing the KEs in this AOP (EPA's Office of Chemical Safety and Pollution Prevention, 2019). Standardization and robustness testing of assays against explicit performance criteria using suitable reference materials can greatly increase the level of confidence in their use for KE assessment (Petersen EJ. et al., 2021; Petersen E. J. et al., 2021). Much of the empirical evidence that supports the KERs in the qualitative AOP described here was obtained from *in vitro* studies using well-established methodologies for biological endpoint assessment (**Supplementary Table S1**). Being chemical-agnostic, this AOP can be applied to a variety of substances that share the AO. For example, impaired MCC and decreased lung function have a long-known relationship with smoking, but little is known about the consequences of the long-term use of alternative inhaled nicotine delivery products such as electronic cigarettes and heated tobacco products. This AOP can form the basis of an assessment strategy for evaluating the effects of exposure to aerosol from these products on the basis of the KEs identified here.

AOPs such as this one can play a central role in risk assessment strategies for a wide variety of regulatory purposes by providing mechanistic support to an integrated approach to testing and assessment (IATA; (Clippinger et al., 2018)) or defined approach (DA). IATAs are flexible frameworks that can be adapted to best address the regulatory question or purpose at hand. Unlike the assessment process within IATA that involves some level of expert judgement, DA uses rule-based fixed data interpretation procedure. Both DA and IATAs are a means to integrate existing data on a chemical (e.g., physicochemical properties and ADME [absorption, distribution, metabolism, and excretion] information) with an AOP-based *in vitro* testing strategy to generate data that does not currently exist (Willett, 2019). An important feature of these approaches is that they should also include a measure of uncertainty to facilitate regulatory decision-making. AOPs can be used in an iterative fashion to identify and reduce or resolve, where possible, areas of uncertainty by generating data to fill those knowledge gaps. Furthermore, a quantitative AOP could provide data that would be translated to prediction models for human risk assessment through the application of *in vitro* to *in vivo* extrapolation (IVIVE) approaches.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: At the point of submission, evidence has been gathered from the literature and compiled prior to populating the AOPwiki at https://aopwiki.org/aops/411, https://aopwiki.org/aops/424 and https://aopwiki.org/aops/425. Special care was taken to properly cite all original studies that were evaluated during AOP development and assessment.

ETHICS STATEMENT

Ethical approval was not provided for this study on human participants because the work presented here cites publicly available data (e.g. reports of clinical study results). No new/additional studies in humans was conducted for the purpose of this work. Ethical review and approval was not required for the animal study because this work is based on the review of publicly available data (e.g. scientific articles), some of which came from animal experimentation. No new/additional animal study was conducted for the purpose of the work presented here.

AUTHOR CONTRIBUTIONS

All authors listed have made significant contribution to the work (literature review and evaluation, development of AOP, synthesis of evidence, draft and revision of manuscript) and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2021.750254/full#supplementary-material

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Evaluation of Inhalation Exposures and Potential Health Impacts of Ingredient Mixtures Using *in vitro* to *in vivo* Extrapolation

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In vitro methods offer opportunities to provide mechanistic insight into bioactivity as well as human-relevant toxicological assessments compared to animal testing. One of the challenges for this task is putting *in vitro* bioactivity data in an *in vivo* exposure context, for which *in vitro* to *in vivo* extrapolation (IVIVE) translates *in vitro* bioactivity to clinically relevant exposure metrics using reverse dosimetry. This study applies an IVIVE approach to the toxicity assessment of ingredients and their mixtures in e-cigarette (EC) aerosols as a case study. Reported *in vitro* cytotoxicity data of EC aerosols, as well as *in vitro* high-throughput screening (HTS) data for individual ingredients in EC liquids (e-liquids) are used. Open-source physiologically based pharmacokinetic (PBPK) models are used to calculate the plasma concentrations of individual ingredients, followed by reverse dosimetry to estimate the human equivalent administered doses (EADs) needed to obtain these plasma concentrations for the total e-liquids. Three approaches (single actor approach, additive effect approach, and outcome-oriented ingredient integration approach) are used to predict EADs of e-liquids considering differential contributions to the bioactivity from the ingredients (humectant carriers [propylene glycol and glycerol], flavors, benzoic acid, and nicotine). The results identified critical factors for the EAD estimation, including the ingredients of the mixture considered to be bioactive, *in vitro* assay selection, and the data integration approach for mixtures. Further, we introduced the outcome-oriented ingredient integration approach to consider e-liquid ingredients that may lead to a common toxicity outcome (e.g., cytotoxicity), facilitating a quantitative evaluation of *in vitro* toxicity data in support of human risk assessment.

Keywords: new approach methodologies (NAMs), electronic cigarette, *in vitro* to *in vivo* extrapolation (IVIVE), physiologically based pharmacokinetic (PBPK) model, *in vitro* toxicity mechanism, mixture assessment

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INTRODUCTION

Electronic cigarettes (EC) are gaining popularity among adult smokers who are looking for reduced-risk alternatives. Since EC are noncombustible and have substantially lower levels of harmful and potentially harmful constituents (HPHCs) they hold the promise as potentially reduced-harm alternative to combustible tobacco products. However, its long-term health effects are currently unknown. The e-liquid and the produced aerosol are both complex mixtures, which are typically

composed of flavor ingredients, nicotine, organic acids, and carrier chemicals (propylene glycol [PG] and vegetable glycerol [VG]). PG and VG are commonly used carriers in the EC products and make up a large portion of the e-liquid (e.g., approximately 80% of the total mass and above, National Academies of Sciences, Engineering, and Medicine. 2018). Nicotine is considered the active ingredient in the e-liquid while flavors are added to accommodate user's sensory preference. Hundreds of unique flavor ingredients are identified based on reviews of e-liquids on the market (Hua et al., 2019; Krüsemann et al., 2021; Salam et al., 2020), with acids (such as lactic, benzoic, levulinic, salicyclic, malic, and tartaric acids) in some EC products converting nicotine to nicotine salts (Harvanko et al., 2020). The combinations of these ingredients lead to a myriad of e-liquids in the market. Traditionally, the health impact of EC products is evaluated as ingredients or as a whole product mixture in preclinical *in vitro* and if necessary *in vivo* testing, with carriers at various PG:VG ratios as the control (Merecz-Sadowska et al., 2020). Currently, data from *in vivo* animal testing is often regarded the gold standard for risk assessment, although to test all the EC products is unrealistic and against the current move to 3R's principle (refinement, reduction, and replacement) (Russell and Burch, 1959; FDA, 2019), considering the number of studies and animals needed. In addition, *in vivo* data also have inherent uncertainty of the interspecies extrapolation from animal to human.

New approach methodologies (NAMs) such as human cell-based *in vitro* testing can potentially provide a rapid approach to assess hazards and toxicity potential to support risk assessments, while reducing or eliminating the need for animal testing. *In vitro* testing can provide various stages of mechanistic information about the bioactivity of test compounds without traditional descriptive animal testing. However, most current applications of *in vitro* testing within safety assessment are for screening, prioritization and hazard identification rather than quantitative risk assessment. One of the challenges in using NAMs for chemical risk assessment is to relate *in vitro* bioactivity dose-responses to relevant *in vivo* exposures (Parish et al., 2020). To put the *in vitro* data into *in vivo* context requires the use of *in vitro* to *in vivo* extrapolation (IVIVE) approaches. The IVIVE approach uses physiologically based pharmacokinetic (PBPK) models to generate in silico predictions of exposure metrics in human based on *in vitro* data. Specifically, PBPK models predict the amount of chemical reaching systemic circulation or tissues of interest corresponding to biological responses measured with *in vitro* assays. The IVIVE approach then uses PBPK models to translate *in vitro* bioactivity data to corresponding equivalent administered doses (EADs) *in vivo*, thus estimating an external dose in humans that may induce similar bioactivity reflected by the *in vitro* assay (Bell et al., 2018). Fit-for-purpose IVIVE analyses require consideration of several key factors, including pharmacokinetic modeling, biochemical and biophysical properties of the test article, *in vivo* (human) exposure parameterization, and the selection of *in vitro* assays (Bell et al., 2018; Chang et al., 2021). Several case studies have explored these fit-for-purpose modeling approaches for individual chemicals (Clewell et al., 2008; Yoon et al., 2014; Baltazar et al., 2020; Algharably et al., 2021). However, there are few studies that investigate the application of the IVIVE approach for mixtures.

There are several challenges when conducting computational modeling of mixtures (Raies and Bajic, 2016) including IVIVE analysis. One challenge is that the *in vitro* testing for the mixture is sometimes designed to assess the entirety of the mixture while PBPK model and parameterization is, by default, constructed for specific individual compounds. In e-liquids and EC aerosols, the differential pharmacokinetics of the mixture ingredients may also shift the availability of those ingredients in plasma compared to the exposure condition employed in the *in vitro* testing. In addition, each ingredient in the mixture may have different modes of action (MOA) and may induce perturbations in multiple, disparate biological pathways that may or may not lead to the same *in vitro* or *in vivo* outcomes. Moreover, there may be agonistic and antagonistic interactions among ingredients, as well as phase partitioning in multiphase flows (e.g., tobacco smoke and e-vapor aerosols).

Given that most exposures of interest are as mixtures rather than single chemicals, there have been many efforts trying to address the above challenges through modeling (Desalegn et al., 2019). Expressing the mixture components in a standardized way and treating the exposure additively is one common approach (Delistraty, 1997; Haddad et al., 2001). Other studies that have looked at how to integrate additional information to address the toxicity as well as the absorption, dispersion, metabolism and excretion (ADME) of the mixture (Ruiz et al., 2020). No standardized approaches are currently available for computational modeling of mixtures. Therefore, it is necessary to define and evaluate fit-for-purpose criteria to guide the calculation for the mixture IVIVE modeling.

In a previous case study (Chang et al., 2021), IVIVE analyses were conducted for the exposure and health impacts of nicotine and flavor mixtures in EC products using publicly available *in vitro* cytotoxicity data of the mixtures (Omaiye et al., 2019) and *in vitro* bioactivity for individual ingredients available from the ToxCast/Tox21 inventory. Chang et al. (2021) provide a methodology to predict maximal plasma concentrations (Cmax) for ingredients and to integrate ingredient-level Cmax predictions to generate product-level estimates based on both individual ingredient and mixture bioactivity. As a proof of concept, this simplified methodology assumes minimum interaction between ingredients, equal time to reach the Cmax in the plasma after exposure (Tmax) across ingredients, and additive effects of accounted ingredients in the mixtures. It also assumes that the biological responses of mixtures are attributed only to nicotine and flavors that are identified and quantitated in the e-liquids and aerosols as reported in Omaiye et al. (2019), which did not include other major ingredients (PG, VG and benzoic acid [BA]). However, PG, VG and BA are major ingredients in the tested e-liquids and aerosols and their *in vitro* bioactivity is demonstrated in other studies (Sassano et al., 2018; Ghosh et al., 2021). Understanding how the results are affected by the composition of the test mixture is necessary for applying IVIVE analyses to inform decision making towards risk assessment.

In this work, we expand Chang et al. (2021) case study on the application of IVIVE for mixtures, with the following specific goals: 1) to understand the contribution of the carriers and acid to

the EAD predictions when their *in vitro* activity is considered and 2) to develop and evaluate methods by which the ingredient-level data are integrated to better inform mixture bioactivity. We achieved the two goals in two stages described as below. The first stage predicted EADs using *in vitro* bioactivity data of e-liquid mixtures and evaluated the impact of the contribution of carriers and the acid to the EAD predictions by comparing the results to the previous study (Chang et al., 2021). The second stage focused on integrating ingredient-level *in vitro* bioactivity information to provide insight on the bioactivity of the mixtures and discussed the selection of ingredient-level *in vitro* assays based on cytotoxicity. The ingredient and mixture data were obtained from publicly available sources, and an open-source, generic PBPK model was used to perform the EAD estimations. Methods are developed to facilitate the mixture integration and described.

MATERIALS AND METHODS

E-Liquid Composition

Publicly available data of the e-liquid composition were obtained (Omaiye et al., 2019). Briefly, we extracted the numerical flavor and nicotine concentrations for the eight e-liquids from figures in Omaiye et al. (2019) using WebPlotDigitizer (Rohatgi, 2019) as described in Chang et al., 2021. As carrier ingredients (PG and VG) and the BA were not analytically quantified in Omaiye et al. (2019), we estimated their concentrations using the reported nicotine concentration and PG:VG ratio of 30:70 by mass (https://www.fda.gov/tobacco-products/market-and-distribute-tobacco-product/deemed-new-tobacco-product-applications-lists#list%20of%20deemed; checked on December 01 2021), assuming BA in equal molar concentration to nicotine. Estimated concentrations of all ingredients of the eight e-liquids in this study are presented in **Supplementary Table S1**.

In vitro Bioactivity Data

In vitro cytotoxicity data for the EC aerosols were obtained from the dimethylthiazol diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assay data in Omaiye et al. (Omaiye et al., 2019). Both MTT and NRU assays provided the half-maximal inhibitory concentration (IC50) values as indicators of cellular cytotoxicity (Omaiye et al., 2019); IC50 values are included in the **Supplementary Table S1**.

In vitro bioactivity data for single ingredients were obtained from the curated HTS assays available from the Integrated Chemical Environment (ICE; Bell et al., 2017; Bell et al., 2020), which provides a curated version of the U.S. EPA's invitroDB V3.2 (accessed December 2020) taking into account chemical quality control information. Furthermore, an additional manual review of the concentration-response curves was performed to remove any ambiguous bioactivity calls to improve robustness of the final dataset. Among the 46 ingredients which were identified and quantitated across the eight e-liquids (Omaiye et al., 2019), information was not available for 13 ingredients (not tested) with an additional 12 having no active assay data passing curation. This resulted in 21 out of the 46 ingredients across all e-liquids with data for subsequent analyses. The ingredient AC50 of the 21 ingredients are available in **Supplementary Table S2** with values used for the outcome-oriented integration approach in **Supplementary Table S3**.

PBPK Models

We used an open-source eight-compartment PBPK (physiologically based pharmacokinetic) model for inhalation exposure (Gas_PBTK) from the httk (High-Throughput Toxicokinetics) package to calculate the Cmax resulting from each ingredient (Pearce et al., 2017). A 1 mg/kg single daily dose was used for determining plasma Cmax for conducing the IVIVE analysis. The gas PBPK model uses an inhaled air concentration (uM); therefore, a unit conversion from mg/kg to uM was conducted assuming complete aerosolization of the e-liquid and uniform dispersion in air for an inhaled chemical. The airborne concentration was obtained by dividing the total chemical mass (mg) by the total inhaled air volume over an assumed exposure period (15 min). The total inhaled air volume (L) is calculated by multiplying average human tidal volume (0.6 L/breath), respiratory rate (12 breath/minute) and the 15-min exposure period (Needham et al., 1954; Russo et al., 2017). The air concentration (mg/L) was converted to uM using the molecular weight prior to use as dosing input for Gas_PBTK model. The plasma Cmax at a dose of 1 mg/kg of each ingredient at 2-h dosing intervals was also predicted and the results are available in **Supplementary Table S5**. The impact of the 2-h and 24-h dosing regimens were discussed in the previous study (Chang et al., 2021).

Parameters used in PBPK modeling for each ingredient were obtained from ICE (accessed March 2021). Modeling was limited to the ingredients only and did not consider metabolites or potential byproducts from interactions. When available, measured values for hepatic intrinsic clearance (Clint) and fraction unbound (fu) were used for ingredients. Otherwise, in silico predictions were used. Physiochemical data was obtained from OPERA (v2.6) QSAR models and used to calculate additional parameters using internal functions of the httk package (Pearce et al., 2017). Parameters used in modeling along with the sources are provided in **Supplementary Table S4**. All other parameters used the httk's internal values or calculated valued based on these provided parameters.

Approaches for the Estimation of Human EADs

We applied IVIVE to estimate EADs using three approaches: the single actor approach (for EC aerosols and ingredients), the additive effect approach (for EC aerosols), and the outcome-oriented ingredient integration approach (for ingredients). The first two approaches are described in Chang et al. (2021). In this study, we developed the outcome-oriented ingredient integration approach to allow "combining" or integrating *in vitro* bioactivity data from multiple ingredients in a mixture based on a common biological target or process, in contrast to the single actor approach. In the outcome-oriented ingredient integration approach, we select a representative assay data for each ingredient and estimate the

combined EAD for the targeted biological responses. Data can be integrated from different assays resulting in the same changes at the molecular level or leading to the same biological outcome (e.g., cytotoxicity) creating a better estimate of the additive effect the ingredients may have. The outcome-oriented ingredient integration approach is similar to the additive effect approach used for the e-liquid mixtures but using the individual ingredient *in vitro* (HTS) data as opposed to the mixture *in vitro* (MTT) data. In this study we used *in vitro* cytotoxicity data as the common target for the integration.

Single Actor Approach

The single actor approach assumes the observed *in vitro* activity of a mixture is solely attributable to a single ingredient in the mixture. For each single ingredient that is selected as the "single actor," an EAD for the mixture is estimated based on the *in vitro* activity concentration (AC) of that ingredient. This approach was used for calculating the EAD of the e-liquid from mixture cytotoxicity data as well as from HTS data for individual ingredients.

To calculate the single actor EAD for the EC aerosol, we first calculate the EAD for each ingredient, which is then divided by mass fraction of the ingredient. To calculate the EAD for each ingredient (**Equation 1**), the IC50 for the mixture was first adjusted by the mass fraction of the ingredient to get the IC50 for the ingredient, which was then divided by the maximum plasma concentration (Cmaxi) at a dose of 1 mg/ kg. When considering only single ingredient activity data, the lowest AC50 values of *in vitro* HTS data was used. The Equation 1EAD$_i$**Eq. 1** is calculated by dividing the lowest AC50 by Cmax at a dose of 1 mg/kg and then scaling with the mass fraction of the ingredient in the mixture to get EAD for the mixture.

$$EAD_{mix-i} = \frac{EAD_i}{frac_i} = \frac{\frac{AC_i}{Cmax_i}}{frac_i} = \frac{AC_{total} * \frac{frac_i}{Cmax_i}}{frac_i} = \frac{AC_{total}}{Cmax_i} \quad (1)$$

(Where AC is the activity concentration of a mixture)

$$EAD_{mix-i} = \frac{EAD_i}{frac_i} = \frac{AC_i / Cmax_i}{frac_i} \quad (2)$$

(Where AC is the activity concentration of a single chemical).

In **Eqs 1**, **2**, EAD$_{mix\text{-}i}$ is the equivalent administered dose for the mixture estimated based on *in vitro* activity concentration of chemical *i,* mg/kg; EAD$_i$, is the EAD for chemical *i* corresponding to *in vitro* activity concentration of chemical *i, mglkg;* frac$_i$ is the mass fraction of chemical *i* in the mixture; AC$_i$ is activity concentration for an ingredient from *in vitro* assay. It is IC50 from the *in vitro* cytotoxicity assay of EC aerosol mixture after being adjusted by the mass fraction of chemical *i* (**Eq. 1**) or AC50 from *in vitro* assay of an ingredient (**Eq. 2**); AC$_{total}$ is the activity concentration of *in vitro* cytotoxicity assay of EC aerosol; Cmax$_i$ is the maximum plasma concentration at 1 mg/ kg/ dose of **Eq. 1** chemical *i;* For additional context, EADs were converted to e-liquid pod equivalents by dividing the total mass of a single pod after being scaled up to whole body exposure (**Supplementary Figures S1–S3**).

Additive Effect Approach

The additive effect approach assumes all chemicals in the mixture contribute to the *in vitro* bioactivity of the mixture proportionally to their mass fraction in the mixture. This creates a single point estimate of the EAD-mix representing the integration of the activities. The *in vitro* cytotoxicity data of the mixtures (MTT, Omaiye et al., 2019) were also used in this approach. To compare the impact of including the total ingredients in EC product as contributing to bioactivity, as opposed to previous Chang et al., we considered the IC50 value representing either the bioactivity from the quantified ingredients (nicotine and flavor; Chang et al., 2021) or the total product (nicotine, flavors, BA and carriers).

When only a subset of ingredients is used as in Chang et al. (nicotine and flavors), the equation to calculate the EAD and number of pods is as follows:

$$EAD_{mix} = \frac{AC_{total} * \sum_{i=1}^{m} frac_i}{\sum_{i=1}^{m} (Cmax_i * frac_i)} \quad (3)$$

In **Equation 3**, EAD$_{mix}$ is the equivalent administered dose for the mixture, AC$_{total}$ is the IC50 of *in vitro* cytotoxicity assay of the EC aerosol, Cmax$_i$ is the maximum plasma concentration at 1 mg/kg/dose of ingredient *i*, frac$_i$ is the fraction of individual flavor or nicotine ingredient in the total product, and m is the number of ingredients in the subset.

When all ingredients of the e-liquid (total product) are assumed to contribute to the bioactivity, the equation to calculate the EAD is as follows:

$$EAD_{mix} = \frac{AC_{total}}{\sum_{i=1}^{n} (Cmax_i * frac_i)} \quad (4)$$

Equation 4 uses the same variable names as **Eq. 3** except that n is the total number of ingredients in the e-liquid (total product). As with the single actor analysis, we provided additional context for the EAD predictions by converting them to e-liquid pod equivalents dividing the mass of a single pod (0.7 ml) scaled for body weight (70 kg) (**Supplementary Figures S1, 2**).

Outcome-Oriented Ingredient Integration Approach

The outcome-oriented ingredient integration approach quantitatively integrates individual chemical bioactivity measures from *in vitro* HTS assays to provide estimates of the mixture bioactivity with common biological responses. This approach assumes that chemicals affecting the same targeted outcome contribute in an additive manner and the Cmax from each chemical occurs at the same time (i.e. Tmax is the same). While it is likely different chemicals in a mixture have different Tmax values, this simplification can provide a conservative estimate as it maximizes the total concentration (Cmax). In this study, we selected cytotoxicity or cell viability as the common biological response and used relevant assays from the HTS database, which is parallel to the MTT and NRU assays used in EC mixtures. ICE provided mapping of the HTS assays to mechanistic targets (Bell et al., 2020) and was used to identify assays annotated to cytotoxicity (cell viability) in the integrated analysis (**Supplementary Table S2**). The majority of the ingredients tested in the cell viability assays were not found to

TABLE 1 | Ingredients treated as active in the integration analysis based on at least one active HTS cytotoxicity assay.

CASRN	Ingredient name	Number of cytotoxicity assays	Number active assays	AC50 for integration approach; geometric mean[a] (uM)
104-50-7	4-Octanolide	66	1	383.3755
104-67-6	5-Heptyldihydro-2(3H)-furanone	81	2	380.1397
562-74-3	4-Methyl-1-(propan-2-yl) cyclohex-3-en-1-ol	66	1	390.8753
706-14-9	gamma-Decanolactone	66	1	380.6671
57-55-6	1,2-Propylene glycol	81	1	386.8474
87-25-2	Ethyl anthranilate	68	11	274.1430
99-49-0	dl-Carvone	66	1	389.1948

[a]*Geometric mean of AC50 values was calculated using measured AC50 values and a value of 400 uM for inactive assay results.*

be bioactive (inactive designation). Ingredients with at least one active HTS cell viability assay (7 ingredients, out of total 21) were designated as active for the purposes of this analysis, as most of the cell viability assays for these ingredients were inactive (**Table 1**).

To account for this distribution of active and inactive assays, the geometric mean of AC50 values was calculated to provide a weighted bioactivity estimate considering both active and inactive assays for each ingredient. Inactive assay results were assigned an AC50 value of 400 uM, twice the maximum concentration tested (200 uM) for most ingredients. Ingredients that were tested in HTS cell viability assays but had all inactive results (26 ingredients) were considered inactive in this analysis (designated with 400 uM). It is notable that three major EC ingredients (nicotine, BA, and VG, making up about 30% of total mass) were inactive for Tox21/ToxCast HCS cytotoxicity assays (**Table 1**). When no cytotoxicity data were available for an ingredient (13 ingredients), the geometric mean AC50 value (387.012 uM) across all cell viability assays, calculated considering both the active and inactive (400 uM) response values as a conservative estimate. Full details about the number of assays tested for each ingredient and the AC50's used in the integration analysis are shown in **Supplementary Table S3**. An RNotebook with code describing the analysis is available in **Supplementary File S1**.

The integration approach first predicted plasma concentrations for each ingredient and adjusted them based on the ingredient's mass percent in the mixture. The "Gas_PBTK" httk model was used for Cmax prediction. Then, using AC50 data as a measure of bioactivity for each active or not tested ingredient (**Supplementary Table S3**), individual ingredient plasma concentrations were scaled to "equivalent plasma concentration" of the most sensitive mixture ingredient. These equivalent plasma concentrations were calculated using the ratio of the ingredient AC50 to the lowest AC50 and describe the Cmax of the most sensitive ingredient that is expected to contribute the same bioactivity as the predicted Cmax for the focal ingredient. Finally, these relative plasma concentrations for each mixture ingredient were summed to predict activity from the mixture. A detailed example calculation is provided in **Data Sheet S2** to demonstrate each step of the analysis using a hypothetical mixture.

We compared the EAD for *in vivo* toxicity (based on cytotoxicity *in vitro*) from two different IVIVE scenarios: "total product," and "flavor only." The total product scenario contained all active or not tested ingredients in the mixture, while the flavor only scenario considered only flavor ingredients (**Supplementary Table S3**). Ingredient Cmax and AC50 values of included ingredients were used to estimate EAD for each EC flavor under both scenarios.

RESULTS

EAD Predictions Using the Cytotoxicity Data of EC Aerosols

Both the single actor and additive effect approaches (Chang et al., 2021) were applied to estimate EADs from the MTT data of EC aerosols (Omaiye et al., 2019). With the single actor approach, comparison of EADs from "total product" and "nicotine + flavor" showed that the inclusion of PG, VG, and BA (total product) did not change the upper or lower limits of the EAD range while the median shifted due to different compositions of each EC aerosol (**Figure 1**). The distribution of the EAD estimates varied depending on what ingredient was used as the primary contributor to the bioactivity. With the additive effect approach, the inclusion of PG, VG and BA resulted in an increased to the calculated EAD (**Figure 1**) compared to considering just "nicotine + flavor". This is due to a combination of the ADME properties and the percent composition of the ingredients and thus may change if different carrier ingredients were considered. EAD estimations using the NRU assay data (**Supplementary Table S1**), along with pod exposure estimations can be seen in **Supplementary Figures 1, 2**.

EAD From Single Actor Approach Using Ingredient HTS Data

The single actor approach was used to predict EADs for mixtures using the assay with the lowest AC50 (representing the most sensitive assay) *in vitro* HTS data from the ToxCast and Tox21 programs for each ingredient (**Supplementary Table S2**). This approach considered 21 ingredients out of total 46, which have at

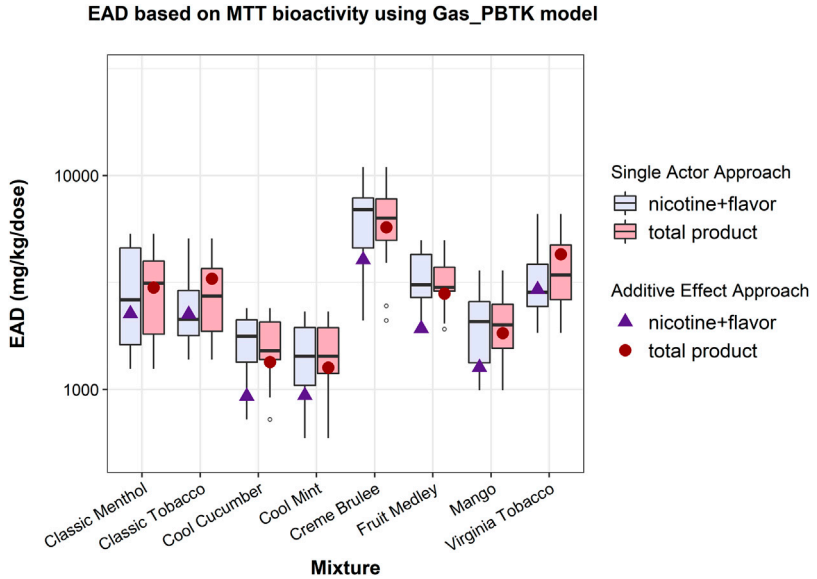

FIGURE 1 | EAD estimates for the bioactivity of e-liquids based on the MTT assay using different assumptions about contributors to bioactivity under both the single actor and additive approaches. "Total product" (shown in red) assumes all e-liquid ingredients (including PG, VG, and BA) contribute to bioactivity, while "nicotine + flavor" (shown in blue) assumes only nicotine and flavors contribute to bioactivity. Box plots show distributions of results across all ingredients using the single actor approach while points (solid triangles and circles) show the point estimate for EAD using the additive effect approach.

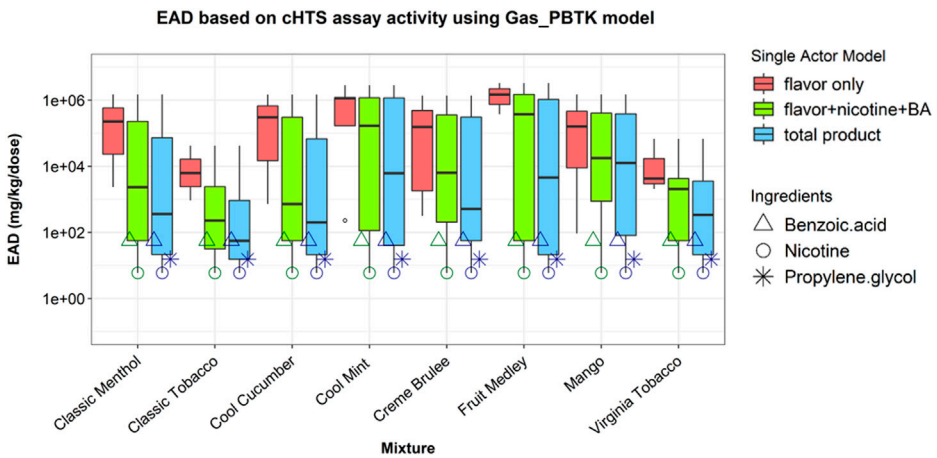

FIGURE 2 | EAD estimated using the HTS data for individual ingredients in the single actor approach. EAD estimates were generated based on each ingredient's most sensitive bioactivity from the HTS assay and adjusted for the ingredient's concentration in the e-liquid flavor. The data were plotted based on each of three combinations: flavor, flavor + nicotine + BA, and total product. Boxplots show the distribution of EAD calculations for all considered ingredients using the single actor approach. Triangle, circle, and asterisk points indicate the EADs predicted when BA, nicotine, and PG were used as active ingredients, respectively.

least one valid active *in vitro* assay in the HTS database. The annotated mechanisms of these *in vitro* assays include cytotoxicity and various early cellular responses, such as receptor modulation and oxidative stress.

The EAD predictions using the Gas_PBTK model are shown in **Figure 2**. Comparisons among "flavor", "flavor + nicotine + BA", and "total product" showed the impact of nicotine's bioactivity on IVIVE outcomes. Nicotine, while making up ~5% of the aerosolized mixture, generated the lowest EAD for combinations where it was included. The AC50 (1.36 μM) used for this estimation is based an *in vitro* assay of which the mechanistic target is annotated as neurotransmission (competitive binding to neuronal acetylcholine receptor subunit α -2). This supports the earlier observation that considering the combination of flavor and nicotine may provide a conservative estimate for bioactivity (Chang et al., 2021). Furthermore, the PG (AC50 of 26.66 μM from a cell viability assay) and BA (AC50 of 7.54 μM from an assay of which the mechanistic target cannot be annotated) consistently produced the second and third

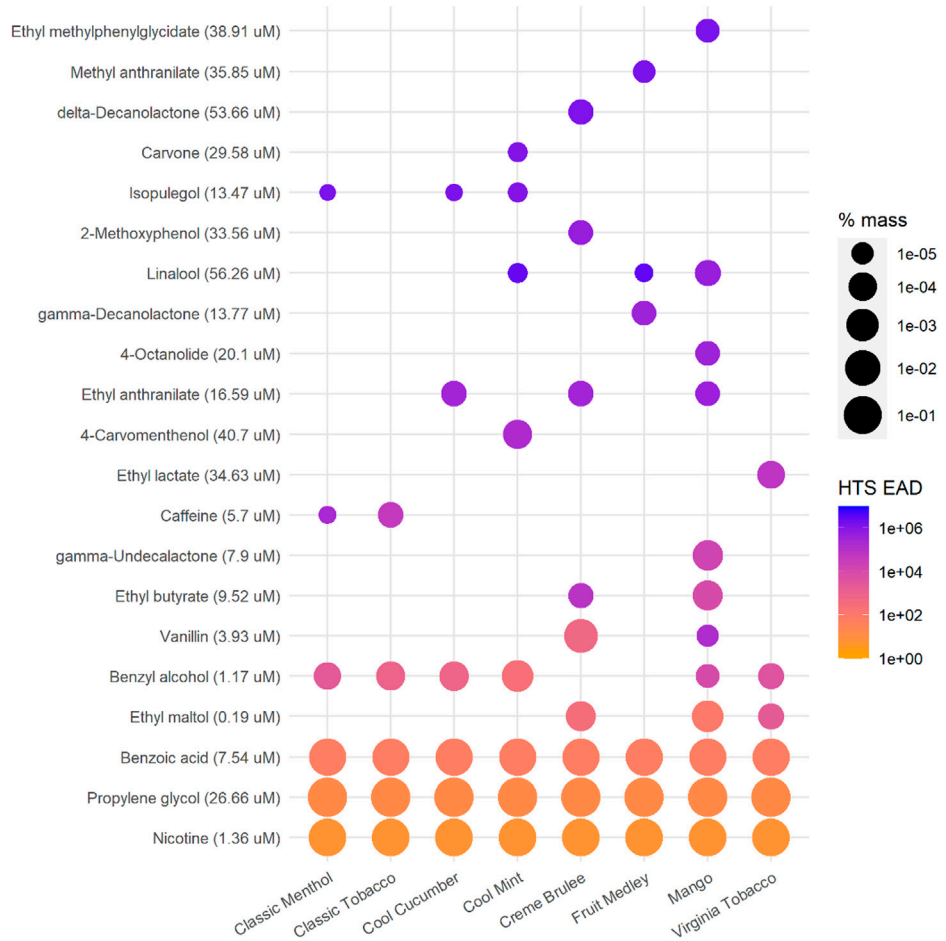

FIGURE 3 | EAD predictions for individual ingredients using the single actor approach and the lowest AC50 values reported in the HTS data sets. Data are ordered based on the minimum EAD for each ingredient across all mixtures. Orange colors indicate lower EAD estimations (more toxic), while blue colors show higher EAD estimations. The size of dots indicates the mass percentage of the e-liquid mixture for each ingredient (log scale). Note that nicotine and BA represent ~5% mass while PG represents ~30% mass (**Supplementary Table S1**). The AC50 for each ingredient used in calculating is shown in parentheses next to the ingredient name.

lowest EAD estimates (**Figure 2**) wherever they were included. The median of "flavor only," "flavor + nicotine + BA" and "total product" ranked from high to low for all eight EC mixtures, indicating increased health risks.

The EAD predictions for each ingredient in the EC mixtures are shown in **Figure 3**. Notably ingredients with lower AC50 values did not necessarily produce the lowest EAD predictions, as the mass fraction as well as the metabolic clearance also played a role in EAD estimation using this approach. For example, the lowest available AC50 for isopulegol is 13.47 uM from an *in vitro* assay that measures estrogen receptor modulation, which is about half of the PG AC50 (26.66 μM), but the estimated EAD based on isopulegol was 10,000-fold higher than that estimated based on PG mainly because the percent mass of isopulegol is much lower than PG (10^{-4} *vs.* 10^{2} in terms of order of magnitude) in the formulations containing both ingredients (**Figure 3**; **Supplementary Table S1**).

EAD From Outcome-Oriented Ingredient Integration Approach Using Ingredient HTS Data

Using the outcome-oriented ingredient integration approach, EAD predictions for the "total product" scenario (**Figure 4**, blue dots) were the lowest, with little difference observed across EC product flavors. This suggests that the EAD estimation was mainly determined by the large mass percentage of PG, a carrier ingredient with cytotoxicity demonstrated in the HTS assays. EAD predictions for the "flavor only" scenario (**Figure 4** red triangle) were substantially higher than the "total product"

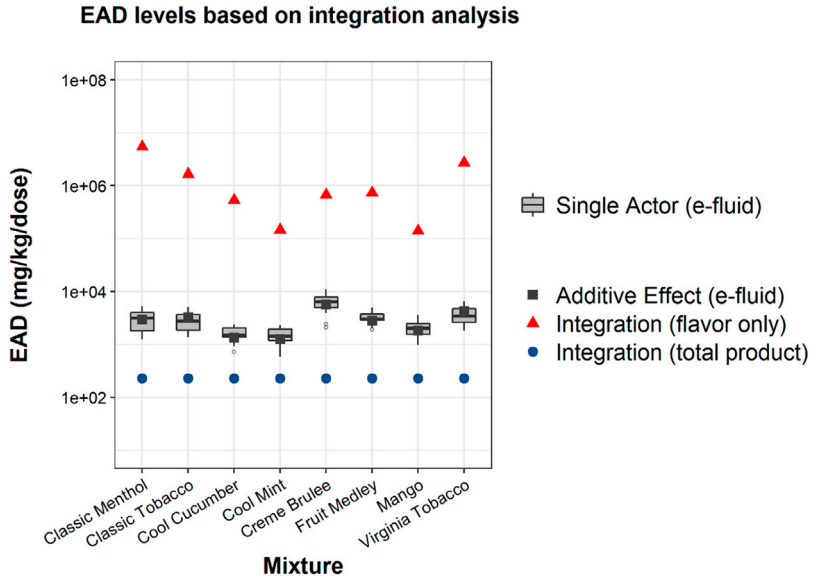

FIGURE 4 | EAD predictions for each flavor using the ingredient integration analysis based on the geometric mean *in vitro* cytotoxicity assay AC50 from the HTS data for individual ingredients. This figure compares integration analysis results under different scenarios (red and blue points) to the e-liquid mixture results presented in **Figure 1** (gray points and boxplots). Blue circular points show predictions for total product, while red triangle points show predictions for flavor ingredients only. Note that for this analysis ingredients that were inactive in cytotoxicity assays were excluded (**Supplementary Table S3**); nicotine, BA, and VG were among the inactive ingredients. The grey boxplots show the results of the single actor analysis for each e-liquid using the MTT assay for comparison, while the grey square points show the additive effect results.

scenario results and showed high variability across the eight EC aerosols. Full sets of EAD predictions for all the combinations of PBPK models and scenarios were provided in **Supplementary Table S5**. The single actor (**Figure 4** grey boxplots) or additive effect e-liquid (**Figure 4** grey squares) results derived from the mixture cytotoxicity data were added to compare with the "total product" results (**Figure 4** blue dots) derived from the ingredient cytotoxicity data. The e-liquid results were approximately a factor of 10 higher than the "total product" results and showed good consistency between different approaches.

DISCUSSION

The Committee on Toxicity Testing and Assessment of Environmental Agents of the National Research Council (NRC, 2007) has envisioned toxicity testing is to transit from whole animal *in vivo* testing toward *in vitro* approaches conducted in human cells. Besides regular *in vitro* assays, a suite of HTS assays targeted to specific biochemical targets has been developed by Tox21 consortium (Dix et al., 2007; Tice et al., 2013) to aid this transition. At the same time, putting the *in vitro* test data into an *in vivo* context requires the IVIVE approach to extrapolate *in vitro* bioactivity to *in vivo* exposures and dosimetry. While conceptually feasible, there are many variables and assumptions before applying IVIVE, especially on mixtures such as EC aerosols, decisions such as what the expected bioactivity is, what components of the mixture contribute to the bioactivity, what *in vitro* assays will be used, and how that data will be integrated need to be determined as all of these influence the interpretation of model predictions. This work investigated these issues by using publicly available *in vitro* cytotoxicity data of EC aerosols and ingredients and comparing EADs estimated with various IVIVE data integration approaches. We evaluated the impact of e-liquid carriers (PG and VG) and BA on the total EC aerosol bioactivity and EAD predictions, albeit limited to available *in vitro* data for EC mixtures and ingredients. We also compared the EAD results from this study with results predicted by previous approaches (Chang et al., 2021).

EAD Predictions for Total Product

Chang et al. (2021) estimated EADs of e-liquids using *in vitro* cytotoxicity and analytical data of EC mixtures reported in Omaiye et al. (2019). However, they did not account for PG, VG, and BA, of which the mass percentage in the e-liquids or aerosols were not available. With the composition information obtained from the FDA's website (2020), we were able to calculate the EAD based on the total e-liquid composition (PG, VG, BA, nicotine, and flavors) using the EC aerosol MTT assay (Omaiye et al., 2019). As shown in **Figure 1**, the EAD predictions using both the single actor approach and the additive approach indicated that the inclusion of PG, VG or BA in IVIVE modeling did not have a significant impact on the human EADs based on the mixture MTT assay (Omaiye et al., 2019), despite that the three ingredients accounted for over 85% of the total mass of e-liquids and aerosols. The results can be explained by the distribution of the mixture bioactivity across all the ingredients and the relatively faster clearance of the carriers, which represent the majority of the mixture mass (**Supplementary Table S1**). The relatively high intrinsic

clearance rate and fraction unbound of the carriers (**Supplementary Table S4**) contributed to their rapid clearance and resulted in a lower Cmax in the dosimetry model and thus a minor impact on the EAD.

This result based on total mixture (EC aerosols) *in vitro* bioactivity (**Figure 1**) seemed to contrast with the result of the single actor approach using individual ingredient *in vitro* HTS data. EAD estimations resulting from the HTS data of individual ingredients (**Figure 2**) indicated that the "total product" EAD might be lower than nicotine + flavors alone. Using the lowest AC50 value across multiple assays from the HTS data set, the ingredient-based single actor approach indicated that PG and BA consistently produced two of the three lowest EAD estimations immediately following nicotine (**Figure 3**). Using the lowest HTS AC50 among all assays is an extremely conservative approach and might have overestimated the bioactivity of these ingredients relative to what was observed in the *in vitro* mixture cytotoxicity testing. This was also partly due to the lack of bioactivity across the assays in general and the lowest measure is not related to cytotoxicity or generally not as robust due to experimental and technical variability. This discrepancy in the results highlights how assay selection and interpretation can impact IVIVE calculations.

Either the single actor or the additive effect approaches did not incorporate the biological mechanisms. When we used the single actor approach with the HTS data of individual ingredients, we selected the most sensitive assay among assays annotated with various mechanistic targets to obtain the most conservative EAD estimations. This has a disadvantage of being overly sensitive and may reflect a spurious interaction as opposed to a biologically relevant measure of ingredient effect on the biological system. Nonetheless, this preliminary approach can be used in screening and prioritizing when the goal is to be conservative to assess potential bioactivity. In contrast, when we used the additive effect approach with the mixture cytotoxicity data, we assumed equal toxic potential for all ingredients of the mixture, as individual ingredient bioactivity for the assay was not available. This assumption likely attributed bioactivity to relatively inert ingredients and could affect the EAD estimates when combined with different PK profiles.

EAD Predictions Using an Outcome-Oriented Ingredient Integration Approach

As an alternative to the above approaches, we explored the mechanism-based IVIVE for the mixtures, an outcome-oriented ingredient integration approach using a common mechanism. This approach has the advantage of consolidating biological effects from ingredients that would contribute to the same toxicity endpoints in a mixture that has not yet been tested in experiments. In this regard, the outcome-oriented ingredient integration approach is similar to the toxicity equivalent factor (TEF) approach applied by the U.S. Environmental Protection Agency to assess the toxicity of structurally similar chemicals that affect the same endpoint (Delistraty, 1997). This approach also assumes additive effects from single ingredients. We selected cellular cytotoxicity to make relevant comparison to the results derived from the mixture cytotoxicity data (Omaiye et al., 2019).

The EAD estimations from the cytotoxicity-oriented ingredient integration approach encompassed a range of approximately 10-fold below to 100-fold above those estimated using the mixture cytotoxicity data, depending on which ingredients are included in the analysis ("total product" or "flavor only") (**Figure 4**). Notably, the "total product" EAD estimation produced conservative results, i.e., the EAD predictions for the total product were the lowest. When comparing the outcome-oriented ingredient integration approach to the single actor approach based on the HTS ingredient data, the "total product" EAD estimations were the lowest (**Figure 2**; **Figure 4**). When comparing to the additive effect approach using mixture cytotoxicity data (**Figure 4**, grey points and boxplots), the results of the outcome-oriented ingredient integration approach are about a factor of 10 lower than the mixture cytotoxicity-based results, which could be explained in part by the inclusion of different ingredients and the varied sensitivity of the *in vitro* assays. Similar to the additive effect approach demonstrated in **Figure 1** (also included in **Figure 4** as gray square points), the outcome-oriented ingredient integration approach modeled the pharmacokinetics of each ingredient separately. The two approaches (the additive approach vs. the outcome-oriented ingredient integration approach) differ, however, in the weight of contribution that is assigned to each ingredient. The additive effect approach assumes that all ingredients contribute to the mixture cytotoxicity and every ingredient is equally cytotoxic, while the outcome-oriented ingredient integration approach assumes that only ingredients of which cytotoxicity is demonstrated ("positive" in Tox21/ToxCast database) and the contribution of a single ingredient is inversely proportional to its AC50 (**Supplementary File**).

Despite increased biological relevance, there are limitations of the outcome-oriented ingredient integration approach, for example, limited *in vitro* data relevant to mechanistic targets of interest. In this case study, there were only seven distinct ingredients with active *in vitro* responses out of the total 46 ingredients (**Table 1**). An additional 13 ingredients were considered active using the median AC50 of cytotoxicity assays (387.012 uM) for the calculation as a conservative estimation based on a lack of *in vitro* testing data. Nicotine and BA were excluded from the outcome-oriented integration modeling as they are both identified as inactive in the Tox21/ToxCast cell viability assays. Although cytotoxicity and cell viability assays were not available for all ingredients, it may be possible to address the data gap by searching the literature or conducting ingredient-specific *in vitro* experiments.

EADs for Preliminary Risk Assessment

The methodologies presented in the work incorporated the bioactivity, ADME and composition of ingredients to enable preliminary chemical risk assessment. The results can be used to prioritize individual ingredients or ingredient groups for further toxicological testing and risk assessment. For EC products, flavors, nicotine (or nicotine salt), and humectant carriers can play different roles in the *in vitro* and *in vivo*

responses and the IVIVE methods provided a modeling approach to estimate the potential contribution of each or groups of ingredients. For example using the HTS data, the EAD predictions for the "flavor only" group are the highest, suggesting flavors (less than 1.2% of the total mass in this case study) are not likely the major toxicity driver in the tested e-liquid and aerosol mixtures. The medians of "flavor-only" EAD predictions are about a factor of 100 higher than the "total product" results when the lowest AC50 data are used (**Figure 2**). The "flavor-only" predictions are about a factor of 10,000 higher than the "total product" results when the selected cytotoxicity data are used for the estimation (**Figure 4**). The difference between the "flavor only" and "total product" scenarios suggests the contribution of the flavor and non-flavor ingredients to the potential *in vivo* toxicity of the mixture. In addition, the IVIVE results can help to identify priority ingredients that may drive the bioactivity. Considering their bioactivity and levels, it is not surprising that nicotine and BA significantly contribute to the bioactivity of the mixture as shown in **Figure 2**. Among flavors, benzyl alcohol, identified as a flavor compound in six out of eight EC products, and ethyl maltol, identified in three products, could contribute to lower EADs among flavor ingredients (non-PG, VG, BA, or nicotine ingredients) across the EC products (**Figure 3**). Additional toxicological assessment (e.g., *in vitro* responses in various combinations of these ingredients) could be of interest to further elucidate their contributions to mixture bioactivity and to evaluate their use level in the products. For the EC aerosols as a whole, the lowest EAD predictions for the total product (>100 mg/ kg body weight, i.e. > 7,000 mg/ day based on 70-kg body weight, about ten pods per person per day) (**Figure 4**) were still regarded substantially higher than typical consumer daily uses (e.g., approximately two pods per person per day (0.7 ml of e-liquid per pod https://www.juul.com/resources/what-is-the-size-of-a-juulpod).

Despite many potential applications, it is important to acknowledge the limitations of the current IVIVE approaches for the EAD predictions. Firstly, although cytotoxicity was selected as the surrogate biological response in this study, *in vitro* assays that are more mechanistically relevant and specific to the exposure would be desirable for the EAD prediction and risk assessment. For example, increased vascular and lung oxidative stress level was reported to be associated with e-vapor aerosol exposure (Taylor et al., 2016; Kuntic et al., 2019) and may lead to potential lung injury. Secondly, the *in vitro* values used did not account for any potential interaction with endogenous ligands for the targeted receptors. Additionally, the metabolic saturation was not considered in the generic PBPK model used in this study. In future studies, the PBPK model may be expanded by adding key metabolic information (e.g. saturable metabolic pathways and resulting metabolites). Target tissue concentrations can also be considered for the EAD calculation in addition to the systemic plasma concentration. Finally, for EAD calculation of mixtures, we used the simple assumptions on the interaction of ingredients (additive as opposed to synergistic, for instance). For a simple mixture system such as a binary system, the interactions between chemicals could possibly be incorporated in the model and verified by experiments. Considering these limitations and data gap, uncertainty factors can also be applied to EAD calculation depending on the application (Raies and Bajic, 2016).

CONCLUSION

IVIVE modeling for mixtures can be complex as it must consider both the bioactivity measure and the pharmacokinetics of each ingredient in the mixture. This work investigated the application of IVIVE as a NAM to use *in vitro* data toxicity assessment of EC ingredients separately and as part of total product in the context of human *in vivo* exposure. Specifically, we illustrated the impact of the inclusion or exclusion of carrier chemicals (PG and VG) and BA in e-liquids and EC aerosols on EAD prediction using various IVIVE approaches. The single actor analysis demonstrated in this work can be informative for prioritization of testing for ingredients or in sets of mixtures. By considering bioactivity, mass fraction, and toxicokinetic properties, the IVIVE results can identify which ingredients in a set of mixtures are most likely to contribute to toxic effects, potentially supporting usage limit (**Figure 3**). We also demonstrated that the outcome-oriented ingredient integration approach as introduced in this work can provide conservative screening estimates of mixture bioactivity for selected bioactivity targets when experimental mixture data are not available. The EAD estimations generated by these approaches have the potential to inform risk assessment and decision making through a margin of exposure approach that compares expected exposures with the equivalent exposures to match *in vitro* results. As different assumptions are embedded in each approach, it is necessary to define the purpose of the study and the assumptions to develop fit-for-purpose methodologies. In conclusion, IVIVE is a useful tool for interpreting *in vitro* data in the context of *in vivo* human exposure and can be applied to mixtures assessment for hypothesis generation and preliminary risk assessment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JZ, KL, and SB designed the study. AK acquired, curated and analyzed data from the HTS database. DH and XC ran the modeling. TH did literature search. JZ, KL, DH, and SB interpreted the data and draft the work. All individual authors reviewed the manuscript and provided critical comments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2021.787756/full#supplementary-material

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METHODS
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Versatile Functional Energy Metabolism Platform Working From Research to Patient: An Integrated View of Cell Bioenergetics

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Mitochondrial dysfunctions that were not discovered during preclinical and clinical testing have been responsible for at least restriction of use as far as withdrawal of many drugs. To solve mitochondrial machinery complexity, integrative methodologies combining different data, coupled or not to mathematic modelling into systems biology, could represent a strategic way but are still very hard to implement. These technologies should be accurate and precise to avoid accumulation of errors that can lead to misinterpretations, and then alter prediction efficiency. To address such issue, we have developed a versatile functional energy metabolism platform that can measure quantitatively, in parallel, with a very high precision and accuracy, a high number of biological parameters like substrates or enzyme cascade activities in essential metabolism units (glycolysis, respiratory chain ATP production, oxidative stress...) Its versatility (our platform works on either cell lines or small animals and human samples) allows cell metabolism pathways fine tuning comparison from preclinical to clinical studies. Applied here to OXPHOS and/or oxidative stress as an example, it allows discriminating compounds with acute toxic effects but, most importantly, those inducing low noise chronic ones.

Keywords: functional metabolism, cell energetics, oxphos, oxidative stress, automatisation

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INTRODUCTION

During the last decade, in the field of pharmaco-toxicology, early discovery impairments of adverse effects such as mitochondrial dysfunctions have generated late drug attrition (Nadanaciva and Will, 2011a). As understanding mitochondrial behaviour remains extremely complex and because mitochondrial insults can occur through still unknown mechanisms, it is not surprising that mitochondrial metabolism has often been found to be a target of drug induced toxicity (Nadanaciva and Will, 2011b). Mitochondria produce most of the ATP used by mammalian cells through oxidative phosphorylation (OXPHOS). OXPHOS represents a functional unit located in the inner mitochondrial membrane combining electron transport chain (ETC) through its four complexes (I-IV) and ATP synthesis (Hüttemann et al., 2007). TCA cycle NADH and FADH2 electrons are accepted by ETC complex I or II then transferred to complexes III and IV and later to oxygen. This electron transfer along ETC is coupled with proton transport across mitochondrial inner membrane, establishing the electrochemical gradient that allows ATP generation through complex V (Sherratt, 1991). Mitochondria continuously function to metabolize oxygen and generate superoxide radical and subsequently other reactive oxygen species (ROS) mostly at complex I and III (Chance et al., 1979; Richter et al., 1988; Murphy,

2009). It has been measured that 1–4% of oxygen reacting with ETC is incompletely reduced to ROS (Barja, 1999). This ROS overproduction damages mitochondrial proteins, alters mitochondrial membrane permeability, disrupts mitochondrial calcium homeostasis, induces mitochondrial DNA mutations, leading to interruption of mitochondrial essential function such as ATP production (James and Murphy, 2002; Endlicher et al., 2009). Thus, global free radical production level is likely to guide mitochondria metabolic efficiency (Kramer and Nowak, 1988; Krähenbühl et al., 1994). These ROS deleterious effects are normally counterbalanced by efficient mitochondrial antioxidant defence driven by either enzymatic or non-enzymatic mechanisms (Krähenbühl et al., 1996). When ROS/antioxidant balance is disrupted, every cellular biological system will suffer oxidative stress (OS) that overwhelm mitochondria and damage other cellular components (lipids, proteins, DNA...) thus generating global functional impairment leading to cellular dysfunction then cytotoxicity (Dröge, 2002). This intricate metabolism between ROS generation and ETC function in mitochondria explains why most of mitochondrial dysfunctions (a common term that includes alteration of different metabolic pathways and damage to mitochondrial components) and impaired bioenergetics are strongly implicated in the aging process (Finkel et al., 2000; Short et al., 2005) and the pathogenesis of many chronic illnesses [from metabolic diseases (Bhatti et al., 2017) to various disorders including cancer (Yin et al., 2012; Iommarini et al., 2017), insulin resistance (Houstis et al., 2006; Rocha et al., 2013) and diabetes (Parish and Petersen, 2005; Blake and Trounce, 2014), atherosclerosis (Madamanchi and Runge, 2007), cardiac (Ide et al., 1999; Le Chen and Knowlton, 2011; Ong et al., 2013) and neurodegenerative illnesses (Reddy, 2006; Reddy, 2008; Itoh et al., 2013; Misrani et al., 2021)]. In most cases, there are only subtle metabolic or enzymatic variations that will be indicative of mitochondrial dysfunction. Therefore, it makes sense to combine sensitive ETC analysis with OS evaluation to get an overview of mitochondrial function especially in mitochondrial-dependent diseases.

To address such intricate issues, only an integrative approach combining different data could represent a strategic way to solve mitochondrial machinery complexity. Such integrative methods are very hard to develop. The technological development of large-scale analysis of genes (genomics), transcripts (transcriptomics), proteins (proteomics) and more recently small metabolites (metabolomics), all referred to the neologism "omics" has been thought to be an interesting tool to understand complex cellular mechanisms and thus find out innovative and active drugs. By generating huge files of data, these techniques were supposed to provide sufficient information to break the gap between genotype and phenotype (Karczewski and Snyder, 2018). Thus, it would be possible to get a holistic overview of cells global operating and even individual networks. For that purpose, statistics and bioinformatics have developed a number of novel methodologies to efficiently process, analyse, integrate and interpret "omics" data (Pesce et al., 2013; Fondi and Liò, 2015; Van Assche et al., 2015; Zapalska-Sozoniuk et al., 2019) that could drive the discovery of new important therapeutic targets that direct development of specific and efficient compounds addressing them (Iskar et al., 2012; Leung et al., 2013). Aside Pharma industry, they were supposed to be massively used in clinical laboratories to discover and then apply new useful biomarkers in either pathologies diagnosis or drug management as companion tests (Strunz et al., 2016). However, "omics" usefulness, either used alone or combined with system biology, remains controversial (Ein-Dor et al., 2006; Lay et al., 2006a; Lay et al., 2006b; Ghosh and Poisson, 2009; Ioannidis, 2010; Sung et al., 2012) and are still limited to research only and not yet used for patient's health management. A few "omics" protocols have been applied to ETC/OS explorations but are still unable to be used in clinical handling of mitochondrial diseases (Go et al., 2018; Rahman and Rahman, 2018). Indeed, to reach clinical biology criteria, two major points should be considered:

- The first one concerns quantification as an important issue. Whereas random biological errors could not be circumvented, introducing errors at the beginning of the process and accumulating such errors at each step of aggregating data will unavoidably distort mathematic model calculations (**Figure 1**). As both accuracy and precision of multi-step biological measurement procedures are always the sum of many different elementary errors resulting from either systematic or stochastic influences, integration of results suffering error accumulation would possibly generate false predictions. In a simple comparison system where large difference between two studied cases is expected, even if systematic and stochastic errors do occur, it will be still possible to draw up true concluding remarks. In a complex system in which differences can range within a few percent, no clear answer or no answer at all would be made. This adverse error propagation can be approximated by statistical calculations but unfortunately is rarely performed (**Figure 1**) (Moseley, 2013; Noack and Wiechert, 2014). If these results are sustained by economic considerations, like in the field of drug discovery, or by vital ones, in the medical field, availability of accurate quantitative methods looks like a mandatory challenge.
- The second one concerns measurement reproducibility (Perng and Aslibekyan, 2020). A survey published approximately two years ago reported that more than 70% of researchers have tried and failed to reproduce another scientist's measurement (Baker, 2016). This is quite impossible in clinical biology as reproducibility of results is mandatory and mainly the consequence of low CV % (either intra- or inter-assays CV%) of automated devices that display sufficient power to significantly detect tiny variations (**Figure 2**). In contrast, high CVs have low power to detect small-scale differences and, the only way to increase power with high CVs techniques relies in increasing the number of replicates. Using tests with the least inherent variability will induce the least replication and thus will be the most cost-effective (for example, in every clinical biochemistry laboratory in the world, blood

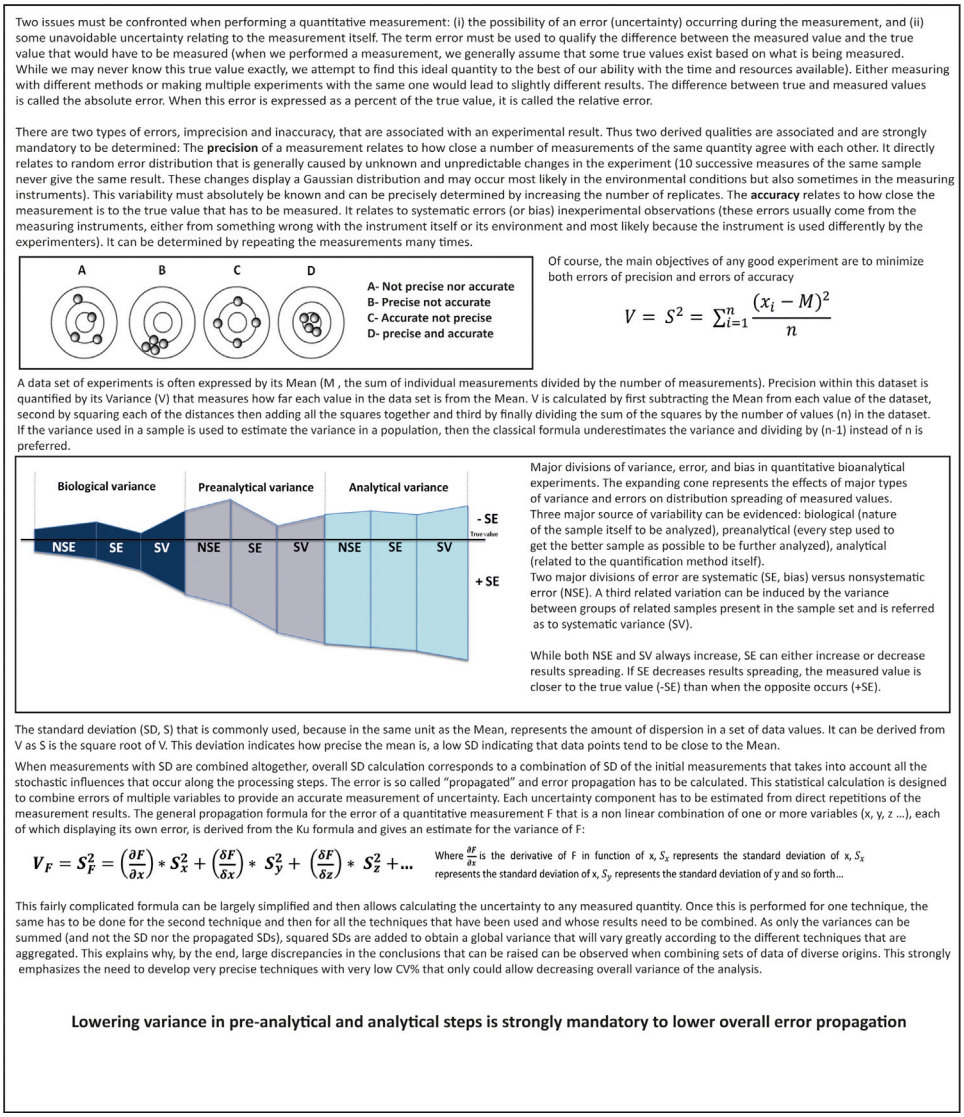

FIGURE 1 | Error definitions and propagation.

measurement of every parameter is only performed once as test CV% are very low). Developing the lowest CV% techniques in research or in drug development strategies will increase confidence in data. Study results will then be considered with the most attention and avoid criticisms.

We report here, as a significant example, the development of a robust and accurate system to evaluate in a combined fashion either mitochondrial function or subsequent OS in different situations (cell culture, tissues, blood samples). This system, based on the use of a multiparametric analyzer (normally devoted to clinical biochemistry laboratories) allows to measure a panel of several ETC (complexes I to V) and OS (SOD1,2, GPX, Catalase, Glutathione reductase) markers on a unique sample. Every test is performed in parallel with high precision, without any bias due to either inter-experiments variability or sample degradation. This measurement method not only allows to precisely evaluate in parallel on number of samples both mitochondrial function and OS involvement in an integrative way, but it also allows comparing drug effects in a dynamic fashion. Because of its versatility, it can be used not only in heath management of patients but also in research on cell culture and animal testing.

MATERIAL AND METHODS

Reagents

All chemicals purchased from Sigma. RPMI-1640 media were purchased from Gibco/ThermoFischer. Penicillin-Streptomycin solution (100x) and 1x PBS were purchased from Corning. Standard cell media was made by adding 10% serum (either

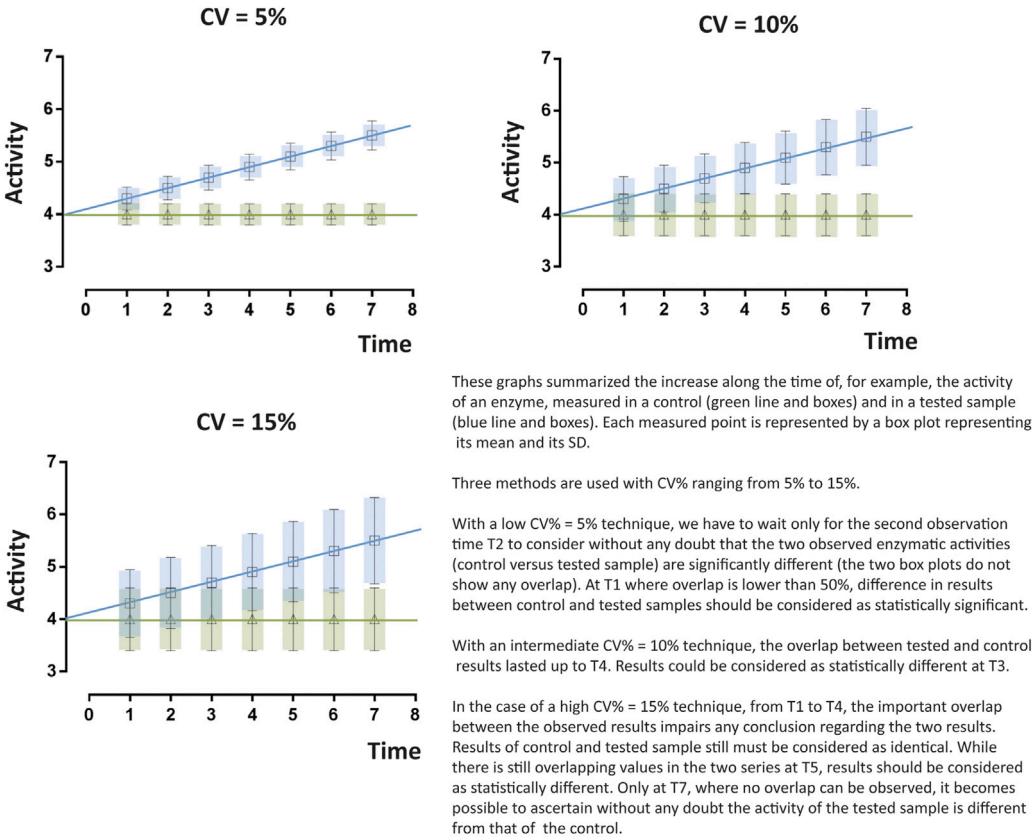

FIGURE 2 | Importance and influence of the coefficient of variation (CV%). The dimensionless and unitless coefficient of variation CV% is often preferred to standard deviation (S) as it is more convenient to describe data spreading regarding a measurement. Indeed, CV%s values are interesting ones as they can be used easily to compare variations of two or more quantitative datasets. Low CV%s, as compared to high ones, allows detecting significant tiny variations.

$$CV\% = \frac{S}{M} * 100$$

FCS or DC-FCS) to 500 ml RPMI-1640, together with pen/strep and L-glutamine (ThermoFischer).

Cell Culture and Harvest

VCaP and PC3 prostate cancer (CaP) cell lines were purchased from ATCC, PNT1A from Sigma and were routinely maintained in RPMI medium containing 1 g/L glucose and supplemented with 10% fetal calf serum. PNT1A is an SV40 immortalized cell line, derived from normal prostate, mimicking normal epithelial prostate cells. VCaP cell line has been derived from CaP patient lumbar vertebral metastases, and behaves as a classical androgen-sensitive prostate adenocarcinoma cell line. PC3 cell line derived from bone metastasis of grade IV CaP patient, and is no more responsive to androgens, glucocorticoids or fibroblast growth factors.

Cells were scraped using rubber policeman then washed two times with cold PBS (Trypsin was avoided as it generates cellular stress). Cell suspension was then centrifuged at 400 *g* for 5 min. After careful supernatant withdrawing, cell pellet was snap frozen in liquid nitrogen and kept at −80°C. Before treatment, the resulting pellet was resuspended in 20 mmol/L phosphate buffer (pH 7.4) then homogenized on ice in a Bandelin Sonoplus ultrasonic homogenizer for 20 s at 30% power. An aliquot was immediately collected on 2% metaphosphoric acid for further ATP and GSH measurements. This acidic suspension was centrifuged at 13,000 rpm for 15 min at 4°C and supernatants were collected, aliquoted, and stored at −80°C until analysis. The remaining preparation was centrifuged a final time at 600 *g* for 10 min, and the supernatants were kept at 4°C until analysis on the automated system.

For 5 g/L time dependent experiments, cells were initially grown in 1 g/L. Then after washing them two times in PBS, 5 g/L medium was added for 10 min (t10 m), t6 h and t12 h. At the end of the period, cells were washed again in cold PBS as described.

Tissue Sample Preparation

Tissue biopsies of gastrocnemius muscle of Sprague Dawley rats (Charles River) have been originally obtained for another study. Rat housing, surgical procedures, and assessment of analgesia have been performed in strict agreement with the regulations set out in EU laws and approved by local university ethical and animal care committees. Efforts were made to minimize animal suffering and to only use the number of animals necessary to produce reliable scientific data. Animals were housed in smooth-bottomed plastic cages at 22°C with a 12 h light/dark cycle. Food and water were available ad libitum. At the end of the study, rats were anesthetized with isoflurane anesthesia and sacrificed by exsanguination [complete blood draw (about 1 ml) from the vena cava, followed by perfusion with phosphate buffered saline]. Gastrocnemius samples were obtained as follows. A 2 cm incision was performed over the gastrocnemius muscle and 0.5 cm^3 segments of muscle tissue were obtained under sterile conditions. Muscle tissue samples were immediately snap frozen and stored at −70°C.

Tissue samples from *Caenorhabditis elegans* were obtained as follows. The *C. elegans* strain N2 was obtained from *Caenorhabditis* Genetics Center. Worms were cultured on bacterial lawns containing OP50 bacteria on NGM plates at 20°C according to standard methods (Nass and Hamza, 2007). For rotenone experiments, NGM plates were pre-coated with rotenone in DMSO to achieve a concentration of 2 or 4 µM, a concentration that did not cause any mortality throughout the course of experiments. After synchronization, a cohort of 1st larval stage (L1) was obtained. After washing, L1 worms were then loaded on rotenone-coated plates. After 24 h, worms were separated, snap frozen then stored at −70°C.

For our retrospective analysis, frozen tissue samples were weighed prior homogenization using an Ultra-turrax Ika T25 for 30 s in an adequate volume of 20 mM phosphate buffer saline pH7.4. An aliquot of the suspended material was immediately collected on 2% metaphosphoric acid for further ATP and GSH measurements. Samples were then centrifuged at 600 g for 10 min at 4°C, and the supernatants were kept on wet ice until analysis on the automated system.

White Blood Cell Preparation

Antecubital human blood draws were performed in one 5 ml EDTA vacutainer. A volume of blood was then diluted with the same volume of NaCl 0.9%, (V/V). One volume of this diluted blood was carefully poured onto one volume of Lymphoprep (Biovision). After centrifugation at 4,000 rpm 20 min at 20°C, plasma was discarded, and the lymphocyte layer was transferred for washing with NaCl 0.9%. After shaking and centrifugation at 600 g for 5 min at + 4°C, supernatant is removed, and cell pellet was frozen as quickly as possible at −80°C.

Before analysis, the resulting pellet was resuspended in 20 mmol/L phosphate buffer (pH 7.4) then homogenized on ice in a Bandelin Sonoplus ultrasonic homogenizer for 20 s at 30% power. An aliquot was immediately collected on 2% metaphosphoric acid for further ATP and GSH measurements. This acidic suspension was centrifuged at 13,000 rpm for 15 min at 4°C and supernatants were collected, aliquoted, and stored at −80°C until analysis. The remaining preparation was centrifuged a final time at 600 *g* for 10 min, and the supernatants were kept at 4°C until analysis on the automated apparatus.

Three blood samples were analyzed. The first one (referred as normal one) was obtained from a normal volunteer devoided of any known illnesses (male, aged 23). Two young patients were also analyzed: One is a 2.5 year-old male patient who was screened for potential Leigh syndrome (LS) at the age of 7 months presenting muscle hypotonia, ataxia and developmental delay (and already related to MTND5 mutation). The second one is a 14 year-old male suffering seizures and myasthenia who was screened for potential late-onset LS syndrome.

Automate Apparatus

The apparatus, a Roche/Hitachi-ModularP analyzer, normally dedicated to routine clinical biochemistry, consists in an automated spectrophotometer that can analyze up to 300 samples, with continuous loading of five-position sample racks and a maximum capacity of 800 tests/h. It can perform in parallel a maximum of 86 different photometric tests. Each programmed assay can use up to 4 different reagents. First reagents are dispensed into the cuvette as few as 10 s after the sample. Other reagents can be dispensed between 1 and 10 min, after the first one. Absorbance readings are taken every 20 s at a specified wavelength in a kinetic fashion, allowing very precise reaction follow-up. Automation allows launching one assay every 6 s. Thus, a multi-panel of analysis consisting of more than 20 assays may be launched in 2 min time limiting the biological sample evolution. All measurements are performed on a same sample at the same time and as all results are given in less than 30 min, it becomes possible with the same apparatus and reagents to rerun samples, whose results seem unfitting, without time degradation between series.

These technical specificities give these biochemical assays a very high reliability and generate small CV% variation coefficients. The lower the CV, the less variation there is and the higher the confidence in the results given by this precise test, so there is no need to iterate the test to ascertain the validity of results, saving sample volumes and being able to carry out all the experiments on the same aliquot. With such accuracy, tests panel can be performed on as few as 3 10^6 cells or 5–10 mg biopsies. Last, it is possible to measure in the same series either cell homogenates, biopsies or tissues homogenates from different species, nucleated cells and red blood cells hemolysates (one 5 ml vacutainer is sufficient).

Methods

Originally, all the techniques reported below have been developed and used manually for years, either for patients in the context of metabolic exploration of innate pathologies in our hospital clinical biology laboratory, or for our research work. As manual techniques are largely time consuming, need large samples volumes and do not preserve material stability, we were prompted to transfer these techniques to automated analyzers. To adapt them, we had to modify volume ratios of

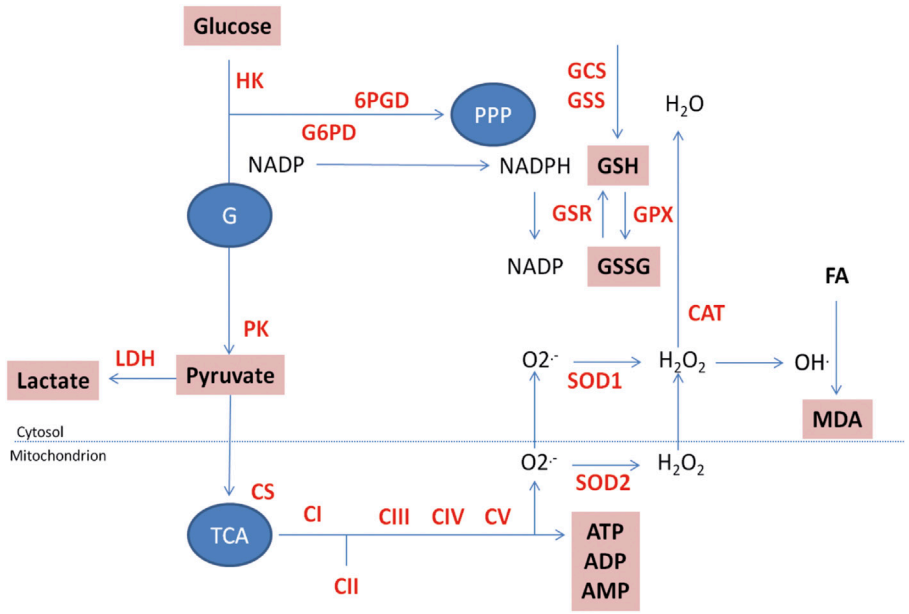

FIGURE 3 | Functional energy metabolism: visualization of metabolic cascades. To date, 22 analytes and enzymes, presented on the schema above, are measured in parallel on the platform. GSH and GSSG, ATP, ADP, and AMP are measured by capillary electrophoresis. All CV% of the different techniques are below 5%. HK: hexokinase; G6PD: glucose 6-P dehydrogenase; 6PGD: 6P-gluconolactone dehydrogenase; PK: pyruvate kinase; LDH: lactate dehydrogenase; CS: citrate synthase; CI to CV: ETC complexes I to V; SOD1: CuZn superoxide dismutase; SOD2: MnSOD; CAT: catalase; GSR: glutathione disulfide reductase; GPX: glutathione peroxidase; GCS: γ-glutamyl cysteinyl synthase; GSS: glutathione synthetase; PK: Pyruvate kinase; LDH: lactate dehydrogenase. GSH: reduced glutathione; GSSG: oxidized glutathione; MDA: malonedialdehyde. G: glycolysis; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle; FA: Fatty acids. Measured enzymes in red; measured molecules in bold in boxes.

samples and reagent concentrations because of the stringent mechanical constraints of the Roche apparatus. Total reaction volume is strictly limited to 250 µL. Sample volume cannot exceed 20 µL. Only two reagents can be used to perform the analysis. The volume of the first reagent, generally the one that starts the reaction and measures non-specific reactions, cannot exceed 190 µL. The second one, that brings specificity, is limited to 50 µL. Therefore, to use 20 µL of test sample, it was therefore necessary to reduce the number of reagents to only two by making premixes whose stability need to be tested prior any experiment. Then, the concentrations of the different reagent components have to be tightly adapted so that the final concentrations in the reaction medium remained identical to those of the reagents used manually. Of course, each step of this process was carefully compared to reference manual technique results then validated. This process took place during our 30 years of experiences in the field. Quality controls (QC) (both frozen mix of crude enzymes and tissue homogenates) were performed during each run of analysis and Levey-Jennings graphs were used to monitor QC values.

Oxidative Phosphorylation Complexes Measurements

ETC complexes, coenzyme Q, and cytochrome C transform energy transduction from respiratory substrates to a proton motive gradient needed for ATP synthesis (**Figure 3**). This pathway is made up of five multi-enzymatic complexes (complexes I–V) whose function is essential for ATP production (complex V). All respiratory chain complex assays were based on methods described by Kramer (Kramer and Nowak, 1988) and Krakenbuhl (Krähenbühl et al., 1994; Krähenbühl et al., 1996), both modified to match our apparatus requirements. The activities of all the complexes in each sample were normalized by the amount of protein or referred to citrate synthase activity to allow sample comparison.

- Complex I activity measurement (NADH dehydrogenase)

Briefly, reduced nicotinamide adenine dinucleotide phosphate (NADH)-ubiquinone reductase activity (complex I) was measured by following the disappearance of NADH using rotenone as a specific inhibitor to ensure the specificity of the assay.

- Complex II activity measurement (succinate dehydrogenase)

Complex II activity, succinate-ubiquinone reductase, was assayed through the reduction of 2,6-dichlorophenolindophenol, a final electron acceptor, after the addition of succinate.

- Complex III activity measurement (ubiquinone bc1 complex)

The activity of complex III, ubiquinone-cytochrome c reductase, was determined by assaying the rate of reduction of cytochrome c.

- Complex IV activity measurement (Cytochrome C oxidase)

The cytochrome c oxidase (complex IV) activity was based on the same assay as for complex III using potassium cyanide to inhibit the activity of this enzyme.

- Complex V activity measurement (ATP synthase)

Complex V activity was measured according to a method coupling ADP production to NADH disappearance through the conversion of phosphoenol-pyruvate into pyruvate then into lactate (Rustin et al., 1993).

- Citrate synthase (CS) activity measurement

The activity of CS was assayed as described previously (Itoh and Srere, 1970) with the reduction of DTNB caused by the de-acetylation of acetyl-CoA.

- Evaluation of the energetic capability

ATP/ADP/AMP cellular levels were assessed by capillary electrophoresis after protein precipitation with 2% PCA (Uhrová et al., 1996; Markuszewski et al., 2003). Two calculations can then be derived:

- Adenylate energy charge (AEC) is a measure of disposable energy at a given moment in the cell. AEC = [(ATP)+1/2(ADP)]/[(ATP)+(ADP)+(AMP)].
- Total adenine nucleotides (TAN) evaluation gives an idea of the pool of adenine nucleotide available in the cell. TAN = AMP + ADP + ATP

- Assessment of anaerobic glycolysis

When mitochondria cannot supply sufficient ATP for cell metabolism, anaerobic glycolysis is activated. We therefore see in the activation of anaerobic glycolysis an indirect proof of mitochondrial dysfunction. G6PD catalyzes the production of ATP in anaerobic conditions using glucose as substrate and generating lactate. It was assayed as described by Beutler (Beutler and Mitchell, 1968).

OS Pathway Measurements

To study the reduction of O_2 into water, we measured the activities of each of the main antioxidant enzymes and antioxidant compounds in this pathway (**Figure 3**). When the superoxide anion is produced, this highly toxic entity must be quickly detoxified. Superoxide dismutases (SOD) will reduce it into hydrogen peroxide (H_2O_2), an even more oxidizing entity than superoxide anion itself that will be even more toxic. Thus, it is mandatory to combine SOD measurement with the quantification of the activities of the two enzymes [glutathione peroxidase (GPX) and catalase (CAT)] able to reduce H_2O_2 into a non-toxic molecule, namely water. As GPX need glutathione, oxidized (GSSG) and reduced glutathione (GSH) must be quantified to evaluate if GSH is in sufficient amount to allow H_2O_2 reduction. As GSH is either produced through glutamate-cysteine ligase (GCS) and glutathione synthetase (GSS) synthesis pathway or reduced from GSSG by NADPH-dependent glutathione reductase (GSR), these three enzymes must be quantified. In addition, as NADPH is mandatory for glutathione reduction, Glucose-6-Phosphate Dehydrogenase (G6PD) activity is required. Last, to evaluate if antioxidant defense is efficient, and because lipid peroxidation is the OS primary target, the measurement of a specific marker like malonedialdehyde (MDA) is required.

- Assessment of superoxide radical reduction

To study O2 reduction to H_2O_2, cytosolic SOD (Cu/Zn-SOD, SOD1) and mitochondrial SOD (Mn-SOD, SOD2) activities were both measured. Using a slightly modified protocol based on the method developed by McCord and Fridovich (McCord and Fridovich, 1969; Huet et al., 2011), total SOD was measured at pH7.8 and SOD1 was measured at pH10.2. SOD2 activity was calculated as the difference between total SOD and SOD1 activities.

- Assessment of H_2O_2 reduction

H_2O_2 is reduced to water mainly by the glutathione antioxidant pathway. GPX activity was measured using a method based on the one developed by Paglia and Valentine (Paglia and Valentine, 1967). CAT activation being another mode of H_2O_2 removal, CAT activity was measured using derived method from the one developed by Johansson (Johansson and Håkan Borg, 1988).

- Assessment of the Glutathione antioxidant pathway

This pathway includes the antioxidant enzymes GPX, GSR, G6PD, and non-enzymatic antioxidant compounds such as reduced and oxidized glutathione (GSH and GSSG).

GSR and G6PD activities were estimated as already described by Beutler (Beutler and Mitchell, 1968). Glutathione synthesis was estimated by measuring the activity of GCS and GSS as described previously (Huang et al., 1993).

GSH and GSSG concentrations were assayed by capillary zone electrophoresis after protein precipitation with 2% PCA (Muscari et al., 1998; Serru et al., 2001). Total glutathione is calculated as the sum of GSH plus GSSG.

- Lipid peroxidation assay

MDA concentration was measured by spectrofluorometry as previously described (Conti et al., 1991). Briefly, the sample was treated with diethylthiobarbituric acid (DETBA) and the fluorescent compound was then extracted with butanol and determined by synchronous fluorescence spectroscopy. MDA in each sample was also normalized with total proteins assayed by the method of Bradford (Bradford, 1976).

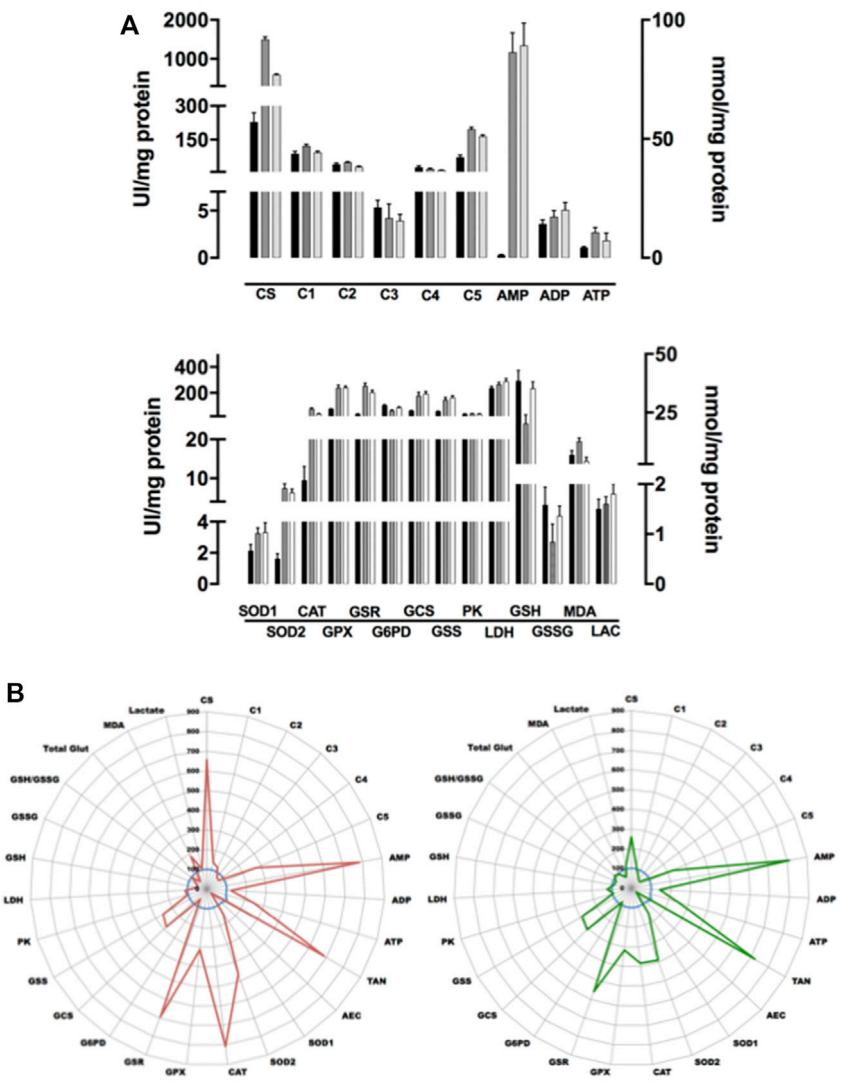

FIGURE 4 | Comparative analysis of three prostate cell lines. **(A)**: Figure 4A depicts results of the three cell lines (mean of five different experiments) in conventional box plots showing mean and standard deviation. PNT1A cells are in black bars, VCaP in grey and PC3 in white ones. Upper figure: AMP, ADP, and ATP are plot to right axis while all other parameters refer to left one. Lower figure: GSH, GSSG, MDA, and LAC are plot to right axis while all other parameters refer to left one. **(B)**: Radar graph depicting the metabolic results of the three cell lines (prostate PNT1A, blue line, normal control as 100%; VCaP cells, red line and PC3 cells, green line). Each point represents the mean of 5 independent experiments. All enzyme results are expressed in UI/g protein, lactate, glutathione and MDA respectively in mmol, µmol, nmol/g protein. The scale on the radar graph represents % of variations versus control. TAN: total adenylate (ATP + ADP + AMP), AEC: adenylate energy charge (ATP+1/2ADP)/TAN.

Other Measurements

- Lactate dehydrogenase (LDH) activity measurement

The activity of LDH was determined using the Cobas commercial LDHI2 kit (Roche). This spectrophotometric method, based on the rate of the NADH formation that is directly proportional to the catalytic LDH activity, is derived from the formulation recommended by the IFCC (Schumann et al., 2002) and was optimized for performance and stability on Roche apparatus.

- Pyruvate kinase (PK) activity measurement

Pyruvate kinase activity was assayed spectrophotometrically using a derived method measuring the decrease in absorbance at 340 nm using a coupled system with LDH and NADH as described previously (Carbonell et al., 1973). After stabilization between homogenates and reagents, phosphoenolpyruvate is used as the starting reagent.

- Lactate measurement

Lactate concentration was measured using the Cobas commercial LACT2 kit. Briefly, L-lactate is oxidized to pyruvate by the specific enzyme lactate oxidase (LOD).

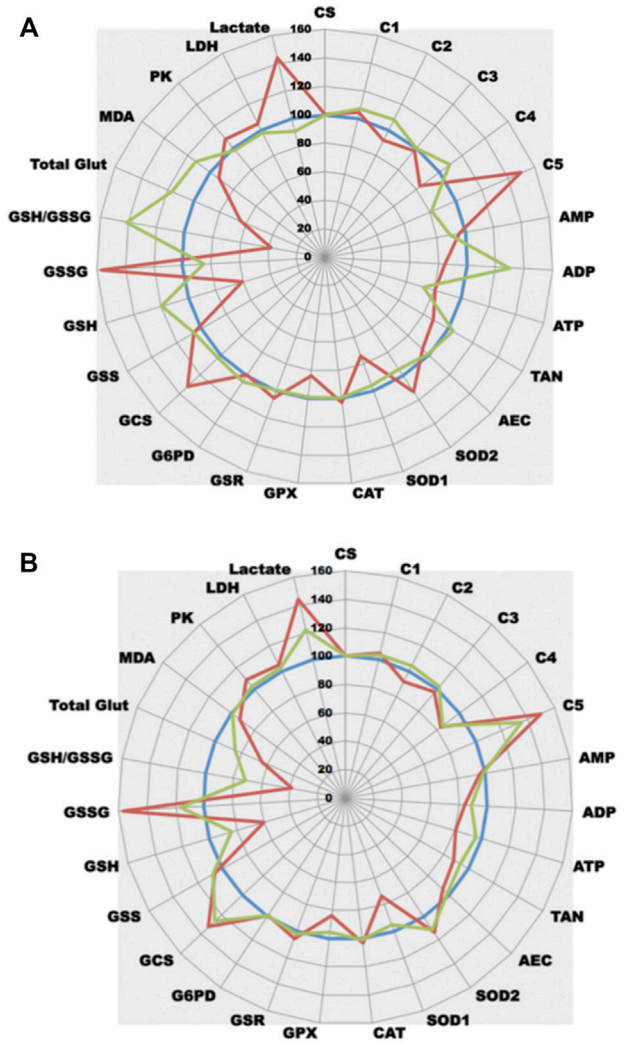

FIGURE 5 | Comparative analysis of the effect of glucose on cell metabolism. **(A)** Three different glucose concentrations were tested at t12h (1 g/L blue line, normal control as 100%; 2.5 g/L green line; 5 g/L red line) in a cell line model (prostate PNT1A cells). Each point represents the mean of 5 independent experiments. **(B)** A unique dose of 5 g/L glucose was tested at 3 different times t10m (blue line, normal control as 100% obtained with 10 min contact of cells with 5 g/L medium), t6h (green line) and t12h (red line) in a cell line model (prostate PNT1A cells). Each point represents the mean of 5 independent experiments.

Peroxidase (POD) is used to generate a colored dye using the hydrogen peroxide generated in the first reaction (Trinder, 1969).

As mentioned in **Figure 2**, low CV% are mandatory to compare enzymatic activities. Analysis of the intra-assay variability of the ETC/OS panel tests was performed (the same cell extract was measured 20 times in the same experiment). For the 22 parameters, intra-assay CV% vary from 2,7 to 5,5% (data not shown). Inter-assay variability (the same cell extract was frozen, kept at −80°C and was thawed then analyzed 10 more times in 10 different experiments) of all the tests of the ETC/OS panel varies from 3,5 to 7,7%, which is quite acceptable for inter-assay measurements (data not shown). Once validated, tests can be used to characterize cell lines.

Data Analysis

All experiment data were processed by GraphPad Prism 8.3 software. The results were shown as median ± SD or SEM. Non-parametric tests were used in all different groups, and $p < 0.05$ was considered as statistically significant (Snedecor and Cochran, 1967).

RESULTS

Combined analysis of ETC and OS supposed to quantify the different elements represented in **Figure 3**. Additionally, to ETC and OS pathways, a glance at glycolysis is made through PK, LDH, and lactate measurements. Except GSH/GSSG, ATP, and MDA, every analysis has been performed on the same sample and in parallel on the Roche automated apparatus.

Cell (or Tissue) Metabolism Comparison

As an example, and because our research mainly focuses on prostate cancer evolution, ETC/OS panel was firstly performed on three well-known prostate cell lines, namely PNT1A, VCaP and PC3 cells. **Figure 4A** depicts results of the three cell lines in conventional bar graphs showing standard deviation. While less informative in term of true quantitative results, radar panel is more convenient than bar graphs as it allows immediate comparison of different cultured cells at their metabolism level. Therefore, using that graphic representation, it becomes easy to compare a control cell line (here, the normal prostate PNT1A cells) to its cancer neighbors (VCaP and PC3 cells) (**Figure 4B**). When normalized with sample protein content, and compared to PNT1A, VCaP and PC3 cells undergo a very significant increase in ETC complex V activity ($p < 0.001$) while other I to IV complex activities remain stable or slightly decreased (CIII and CIV activities in PC3 cells). This increase in ATP synthase activity leads to significant ATP increase in VCaP cells ($p < 0.001$) while ATP is only very slightly increased in PC3 cells (not significant). A very interesting finding is the dramatic AMP increase both VCaP and PC3 cells experienced ($p < 0.0001$) indicating that adenosine metabolism is likely to be crucial in CaP progression. Adenylate kinase that directs adenosine phosphorylation into AMP (the substrate of the central metabolic regulator AMP kinase) has been shown to be important in cancer cells capability to rewire bioenergetics and metabolic signaling circuits to fuel their uncontrolled proliferation and metastasis (Klepinin et al., 2020). As adenylate energy charge (AEC) is decreased in both cells, it is tempting to speculate AMPK is certainly activated in these cells to restore AEC when intracellular levels of ATP drop down (Iommarini et al., 2017). When activated, AMPK promotes catabolic processes and mitochondrial biogenesis. In both VCaP and PC3 cells, it seems that mitogenesis is likely to be increased (more importantly in VCaP than in PC3 cells) as citrate synthase activity, a constitutive enzyme of the mitochondrial matrix and a validated marker of the mitochondrial content (Vigelsø et al., 2014), appears to be increased. Interestingly, these cells do not enter a glycolytic process to restore ATP production as PK and LDH activities remain stable without production of increasing amounts of lactate.

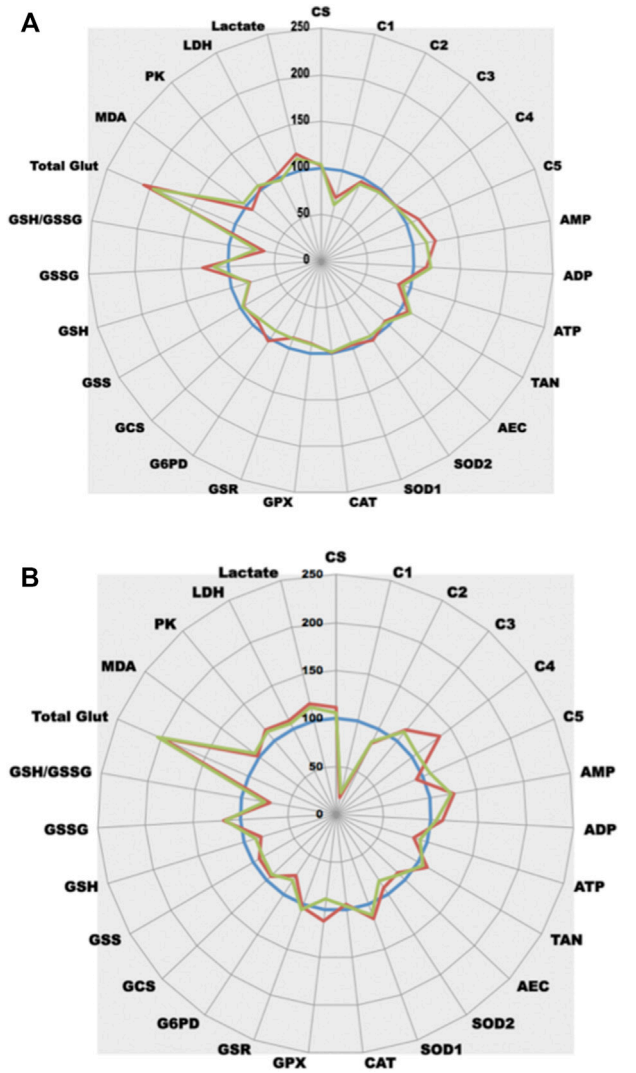

FIGURE 6 | Comparative analysis of the effect of rotenone on cell metabolism. **(A)**-Rotenone-induced complex I dysfunction in PNT1A cells (blue line, control = 100%; green line, + rotenone 5 nM; red line, + rotenone 50 nM). **(B)**-Rotenone effect in an animal model (Caenorhabditis elegans) (blue line, control = 100%; green line, + rotenone 50 nM; red line, + rotenone 100 nM).

Concerning OS, it is interesting to notice that both VCaP and PC3 undergo an important oxidative stress as all antioxidant enzymes activities are increased (SOD2 and CAT, $p < 0.0001$; SOD1 and GPX, $p < 0{,}001$ when compared to PNT1A) leading to lipid peroxidation in VCaP cells (MDA concentration increases). Stress seems to be largely mitochondrial as higher SOD2 activity than SOD1 can be found in both cells. In the meantime, GSR reductase activity increases to refill GSH and GSH synthesis is also induced (GCS activity increases).

Drug Effect on Energy Metabolism

With such test panel, it is also possible to easily compare drug concentrations respective effects. Here, we use glucose as a well-known toxicant for cells at high concentrations. It is recognized that high glucose concentration can generate OS and induce mitochondrial dysfunction (Blake and Trounce, 2014; Liemburg-Apers et al., 2015) and is responsible of well-described oxidation particularly in many organs (kidney, vessels, eyes, heart, brain) (Forbes and Cooper, 2013). Glucose effect on cells remains an interesting issue as a major nutriment, its role in the context of tumor cells is still a matter of debate (Kamarajugadda et al., 2012; Yin et al., 2012).

As shown in **Figure 5A**, three glucose concentrations varying from 1 g/L (the normal one) to 5 g/L (the classical glucose concentration of the universally used DMEM medium for cell culture) were tested for 12 h on PNT1A cells. The highest glucose concentration decreases ETC (complex II, III, and IV activities) with a drop in ATP synthesis (about 30%) that try to be compensated by a large increase in complex V activity (+ 150%). It is interesting to notice that in cells under 2,5 g/L glucose, ETC remains unchanged when compared to control while ATP is already decreased. TAN in these cells is conserved as ADP concentration largely increases. High glucose generates also an important OS, mainly of mitochondrial origin (while SOD1 activity remained unchanged, an increased in SOD2 was observed). This OS induces an important GSH depletion and a dramatic increase in GSSG at 5 g/L. To efficiently reduce GSSG, GSR reducing activity increases slightly. In parallel, to fill in GSH, GSH production also increases (GCS activity largely increases). It is interesting to notice that up to 2,5 g/L, OS can be controlled by antioxidant defenses (while GSH refilling is already in process, GSH is still in sufficient amount). Additionally, as one would expect, large glucose moiety generates through glycolysis important amounts of pyruvate. This neosynthesized pyruvate will be transformed into lactate (lactate concentrations are increased) rather than being used in the TCA cycle.

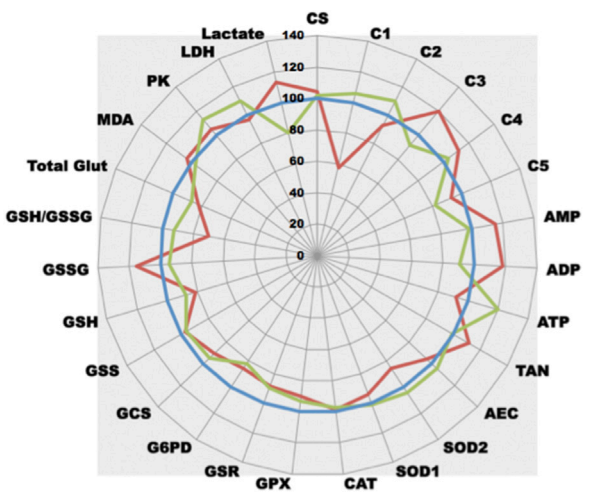

FIGURE 7 | Comparative analysis of the effect of complex I deficiency. After blood draw and further white blood cells careful separation, white blood cells of patients were compared (normal patient as control, blue line, control = 100%; green line, patient 1; red line, patient 2).

	Nom	mg Prot /mg biop	CS U/g prot	CP1 U/g prot	CP2 U/g prot	CP3 U/g prot	CP4 U/g prot	CP5 U/g prot	SOD1 U/g prot	SOD2 U/g prot	Gpx U/g prot	CAT kU/g prot	GSR U/g prot	GSH µmol/g prot	GSSG µmol/g prot	Total GSH µmol/g prot	GSSG/GSH ratio	MDA µmole/g prot
1	old 1	0,112	607	23,85	40,6	0,387	77,1	1108	5,82	6,16	2959	207,0	757	20,80	0,93	21,73	0,044	0,015
2	old 2	0,058	524	9,28	28,0	0,352	52,1	666	7,58	8,25	3576	278,9	522	10,63	0,93	11,56	0,088	0,035
3	old 3	0,077	339	6,68	19,5	0,308	25,4	579	7,20	7,61	4307	191,2	606	12,90	0,93	13,83	0,072	0,029
4	old 4	0,070	480	16,71	28,0	0,290	41,4	496	7,86	9,01	4219	133,9	601	9,01	0,91	9,92	0,101	0,029
5	old 5	0,059	545	12,42	32,6	0,218	37,0	423	7,10	8,89	3619	193,6	771	20,69	0,96	21,65	0,046	0,016
6	old 6	0,061	348	5,67	18,8	0,325	16,4	509	5,95	7,77	3643	205,6	471	10,75	0,92	11,67	0,086	0,020
7	old 7	0,083	489	6,98	31,4	0,328	34,3	660	6,61	8,42	4367	210,7	539	10,51	0,92	11,43	0,087	0,029
8	old 8	0,088	320	16,04	17,5	0,361	25,6	539	6,31	6,45	4015	189,3	524	11,53	0,94	12,47	0,082	0,021
9	old 9	0,082	562	42,06	32,9	0,282	47,0	754	7,87	6,47	4853	190,6	517	12,24	0,93	13,17	0,076	0,028
10	old 10	0,077	467	26,15	31,0	0,287	43,5	658	7,39	9,65	2983	231,0	222	9,66	0,98	10,64	0,101	0,025
11	old 11	0,063	418	20,00	16,9	0,338	42,7	770	5,35	7,43	3446	154,4	472	10,64	0,92	11,56	0,087	0,035
12	old 12	0,092	557	13,20	26,4	0,335	58,9	617	7,71	9,78	3649	212,9	641	8,32	0,96	9,28	0,115	0,039
13	old 13	0,085	503	13,61	29,4	0,289	52,9	796	6,59	8,49	4735	238,7	534	7,64	0,90	8,53	0,117	0,036
14	old 14	0,077	523	9,87	31,7	0,348	36,6	703	6,61	12,13	4347	284,2	462	6,99	0,99	7,98	0,142	0,043
15	old 15	0,069	546	26,71	31,7	0,351	40,1	616	5,97	6,70	4175	202,8	494	11,68	0,93	12,60	0,079	0,048
16	old 16	0,056	350	12,18	21,1	0,384	22,2	438	7,36	9,61	4001	237,3	531	8,78	0,95	9,72	0,108	0,042
	m	0,07556	473,6	16,34	27,34	0,3239	40,83	645,8	6,83	8,301	3931	210,1	541,5	11,42	0,9375	12,36	0,08944	0,03063
	SD	0,01495	91,26	9,563	6,77	0,04349	15,17	167,1	0,793	1,565	555	38,87	126,1	3,99	0,02463	3,99	0,02505	0,00977
	SEM	0,003738	22,81	2,391	1,692	0,01087	3,793	41,78	0,1983	0,3912	138,7	9,717	31,53	0,9974	0,006158	0,9976	0,006263	0,002442
17	young 1	0,051	425	38,36	36,0	0,316	42,8	796	4,89	5,72	3501	154,9	572	19,91	0,94	20,84		0,021
18	young 2	0,057	377	62,53	41,0	0,378	62,8	1002	3,71	7,17	3984	166,3	514	15,55	0,91	16,46	0,059	0,014
19	young 3	0,064	485	36,98	36,7	0,348	51,0	733	5,98	6,63	2678	170,2	448	14,62	0,94	15,56	0,064	0,025
20	young 4	0,054	523	59,07	42,4	0,405	72,5	862	4,97	6,14	3586	190,2	596	12,39	0,93	13,32	0,075	0,024
21	young 5	0,053	426	45,50	42,4	0,315	76,0	737	7,02	7,12	3860	213,6	630	13,25	0,91	14,17	0,069	0,008
22	young 6	0,051	309	35,78	27,6	0,372	38,8	561	6,12	7,76	2504	146,7	450	12,85	0,93	13,78	0,073	0,015
23	young 8	0,046	422	25,26	35,3	0,353	65,2	1015	7,33	8,39	2101	172,4	503	18,19	0,90	19,09	0,050	0,020
24	young 9	0,039	462	15,45	42,6	0,431	53,6	929	5,42	6,15	4104	169,4	500	12,27	0,93	13,19	0,076	0,028
25	young 10	0,057	309	21,69	30,6	0,416	53,1	721	6,19	6,33	3550	180,2	433	16,64	0,95	17,59	0,057	0,015
26	young 11	0,071	460	48,43	38,2	0,431	66,6	1043	6,13	7,09	3778	161,4	397	12,90	0,93	13,83	0,072	0,032
27	young 12	0,075	398	64,80	36,2	0,356	71,2	1031	4,58	6,32	3899	171,3	397	15,70	0,94	16,64	0,060	0,018
28	young 13	0,064	151	7,10	16,4	0,217	36,6	311	6,44	9,50	4723	247,8	548	7,55	0,94	8,48	0,124	0,047
29	young 14	0,065	373	70,26	35,3	0,385	48,9	1006	6,18	7,25	2250	167,0	465	11,01	0,91	11,92	0,082	0,038
30	young 15	0,049	398	74,65	36,3	0,448	68,0	1123	4,63	8,26	3006	188,2	570	17,45	0,89	18,34	0,051	0,009
	m	0,05686	394,1	43,28	35,5	0,3694	57,65	847,9	5,685	7,131	3395	178,5	501,6	14,31	0,925	15,23	0,0685	0,02243
	SD	0,009953	92,38	21,16	7,002	0,06043	12,97	222,8	1,018	1,051	771,9	25,8	73,8	3,222	0,01787	3,219	0,01925	0,01101
	SEM	0,00266	24,69	5,655	1,871	0,01615	3,468	59,56	0,2722	0,281	206,3	6,894	19,72	0,8611	0,004775	0,8604	0,005146	0,002942
	p value	0,0004	0,0225	0,0003	0,002	0,0122	0,004	0,0062	0,0037	0,0172	0,0425	0,0079	0,1761	0,0071	0,331	0,0071	0,0071	0,0265

FIGURE 8 | Comparative analysis of functional metabolic activities of rat muscles. Two series of old (n = 16) and young (n = 14) were metabolically analyzed. A red white green 3-color diverging scale heat map (with high values getting the red color and low values getting the green ones) was used to represent the results.

Glucose behaves as a global toxicant as it unbalances normal metabolism at different levels (it modulates energy production, but it also generates an important OS). We have also tested rotenone, a well-known specific inhibitor of ETC complex I. As already shown for glucose, the use of a specific drug, like rotenone, not only generates dysfunction of the rotenone target enzymes but also modifies cell metabolism (**Figure 6A**). As a clear resultant of rotenone action, complex I activity largely drops down. Consequently, ATP production decreases and ATP precursors (ADP, AMP) accumulate leading to an unchanged TAN. It is interesting to observe that one of the side effects of rotenone is an important generation of GSSG, a drop in GSH content and further MDA increase suggesting an associated OS could occur. This OS is likely to be a consequence of the mitochondrial electron leaking as a slight SOD2 increase is also present. A partial GPX inhibition is also observed, contributing to OS settlement. An increase in NADPH synthesis occurs (G6PD activity increases) to unlimit GSR reductive capabilities to replenish the GSH pool that is consumed to counterfight OS. Interestingly, as ETC complex I dysfunctions, pyruvate do not enter mitochondria for TCA then OXPHOS but is transformed by LDH into lactate.

This puzzling effect of rotenone can be evidenced not only on cultured cells but also can be confirmed in animal models as all animals share the same enzymes (with slight modifications of Km and Vmax values as shown in the Brenda database (https://www.brenda-enzymes.org/). The same drop in *Caenorhabditis* Elegans ETC complex I activity can be observed (**Figure 6B**), contributing to lower ATP synthesis. As for cultured cells, ETC dysfunction also generates OS in animals that seems to be more of cytosolic origin (SOD1 increase rather than SOD2). A same drop in GSH related either to GSH expenditure (GSH synthesis is decreased) or decreased NADPH synthesis (a significant drop in G6PD activity is observed) can be evidenced. *In vivo*, GPX inhibition does not appear. Likewise, an increase in glycolysis (increased PK activity) with endpoint lactate production can be evidenced.

After white blood cell isolation, the test panel can be also used to evidence complex I dysfunction in human patients (**Figure 7**). As compared to a normal control patient, patient 2 displays an important drop in complex I activity leading to a defect in ATP synthesis. Interestingly, he also shows a reactive increase in complex III and IV activities suggesting that ETC electron flux is activated through complex II. As for the rotenone-induced effect, GSH content is also decreased with related GSSG increase, certainly due to ETC dysfunction associated OS. Patient 1 who was addressed for potential complex I dysfunction displays a rather normal pattern. These results on human samples confirm those observed when complex I inhibition occurs either *in vitro* or *in vivo*. Interestingly, Patient 1 undergoes a global increase of ETC activity leading to increasing ATP production. This increased in OXPHOS is associated with an increased glycolysis (PK activity rises). More pyruvate is produced but most of this pyruvate enters mitochondria (lactate concentration is largely decreased).

Time-Dependent Changes of Energy Metabolism

Static data aside, it is also possible to iterate quantifications and observe metabolic dynamic changes, low CV% allowing to unravel slight modifications. When compared at three different times the treated cell line (**Figure 5B**), it becomes

possible to observe the progressive effect of high glucose concentrations on cell metabolism. **Figure 5B** depicts time effect of 5 g/L glucose concentrations on ETC/OS metabolisms. ETC complex I and III activities remain stable at t6 h while complexes II and IV are largely decreased at t12 h. Important complex V activation already appears at t6 h (mitochondrial OS seems an early event as at t6 h SOD2 activity is also increased). Interestingly, the observed but unexplained t12 h SOD1 and GPX decrease are already present at t6 h. Glutathione reductase and synthase activities are prematurely induced at t6 h. GSR was slightly increased (about 10%). Interestingly, a 40% increase in GCS occurs to efficiently counterbalance the beginning GSH depletion. Finally, redistribution of pyruvate through LDH pathway seems to be an early event since lactate begins to be largely produced at t6 h.

Large Scale Analysis of Energy Metabolism

Automation allows the combined analysis of a series of cell metabolism markers. As we used analyzers normally devoted to human sample analysis, their high throughput performance allows us to analyze such large panel in parallel with number of different samples. **Figure 8** depicts the results that can be obtained on rat tissues (here, gastrocnemius muscle tissue samples). As all the different muscle extracts are launch altogether on the machine, no batch effect can be observed as all the reagents are identical during the time course of the different tests. As they are kept on the machine at low temperature, neither reagent modification nor degradation can be observed. Two adult rat groups were analyzed: the first one comprised 16 elder rats (mean age 1,76 years) and the second 14 younger adult ones (mean age 0,92 years). Careful analysis of results shows that muscles from old and young rats largely differ from young ones in term of metabolism (except for GSR activity and GSSG content where no significant difference can be evidenced, all other metabolic markers statistically differ between the two groups with p values ranging from $p < 0.0001$ (CI) to $p < 0{,}05$ (CS, SOD2, GPX, MDA). Heat map show that globally elder rat's muscles display lower ETC and higher OS than younger ones where we observed the exact opposite with high ETC and low corresponding OS. In elder rats, OS induces increasing antioxidant enzymes. SOD1 and SOD2 are both elevated indicating the existence of a mixed cytosolic/mitochondrial OS. GPX and CAT are also increased generating an important GSH depletion. As GSH is not sufficient to counterbalance OS, GSSG increases and peroxidation occurs (except for young rat 13, the highest MDA concentrations are all found in elder rats). As no batch effect can be evidenced in the different series, the heat map allows at a glance to discriminate outliers from the general population: elder rat 1 can be seen immediately as an outlier in his own group as well as younger rat 13 in the youngest one. Unfortunately, no clear explanations could account for these discrepancies. These results are in accordance with what has been already published. Altered mitochondrial function has been linked to aging-related declines in performance (Short et al., 2005) and was more recently related to mitochondrial proton and electron leaking that will have a very detrimental effect on coupling efficiency (Treberg et al., 2018).

DISCUSSION

The human body comprises about 40 trillion cells, each one containing up to 6 billion proteins—the entire system being in continual change to adapt to either internal or external changing conditions (Moseley, 2013; Noack and Wiechert, 2014). It is well acknowledged that slight cell modifications could easily impair protein expression or activity levels thus altering global system metabolic performances leading into diseases. Moreover, some drugs affect cell function in an acute way while others may lead to cell dysfunction in a continuous manner leading with time to chronic diseases. This is particularly true for mitochondrial diseases as slight mitochondrial alteration could lead to global cellular dysfunction. To overcome limitations of conventional explorations, combination of multiple technologies is mandatory. Data aggregation of combined omics technologies may represent an elegant way to solve the problem. However, in most studies, static quantities of one biomolecules class (DNA, RNA, proteins, metabolites) are measured but do not preclude on system functionality. Transcriptomics allows semi-quantitative determination of transcripts but does not give any information on their translatability. As post-transcriptional and post-translational modifications are highly regulated, no information regarding protein synthesis and activity could then be derived. It holds true for proteomics that allows quantification of large number of proteins but does not preclude on their functionality. Indeed, proteins displaying activity (such as enzymes) can be present in a relative amount, but no one knows whether it has been activated and able to work. Measuring metabolites using metabolomics allows visualizing metabolic chains with intermediate and/or end-products but does not give any information on functionality of cascade enzymes. To bypass such limitations, monitoring temporal changes of any molecules in the body is interesting. However, either newly synthetized products or clearance level of already existing ones need to be observed. For that purpose, fluxomics have been developed using stable isotopes in kinetics studies (Kohlstedt et al., 2010) allowing determination of metabolites intracellular flux (Claydon and Beynon, 2012; Previs and Kelley, 2015). Nevertheless, as it is very expensive and heavy to process, isotope use has been limited.

One alternative way to address a dynamic issue should be sampling at different times. However, even if measurement iterations are performed, it presumes that quantitative measurement techniques are sufficiently precise and reproducible to be able to compare results in a dynamic fashion. For quantification, overall variance of biological measurement can be deconvoluted into specific variances due to either nature of sample that would be studied or methods that would be used to perform quantification (**Figure 1**). Living material is notoriously variable and most variations are unpredictable. Thus, the experimenter has only little to no control on them. For example, it remains tricky to separate

viable from non-viable cells in cell culture dish as it is also largely problematic to evidence exact percentage of tumor versus normal cells in a tissue, but efforts can be made to lower these errors which may impair further interpretation. Pre-analytical and analytical phases are also very significant. As an important source of variability that can be controlled (Nunnally et al., 2008), both must be correctly mastered. It is commonly admitted that processing any measurement with bad starting sample will undoubtedly generate awful results. Many studies have evidenced, more notably in the field of clinical biology as biological results have direct impact on patient's handling, that most of errors are found outside the analytical phase. Moreover, pre-analytical steps have been found to be the most vulnerable to risk of error. To limit their impact, recent technical recommendations regarding sampling, storage, transport and identification have been developed by consensus organizations (Lippi et al., 2015).

In industrial health research, whereas relative quantification may lead to meaningful results during early stages of drug development, transfer to clinical biology must be considered as a major goal as it represents a validation of its usefulness. Clinical biology criteria in terms of accuracy and precision, are largely more stringent than research criteria. As high CV%s tests display low power to detect small-scale differences (**Figure 2**), low CV% tests are generally preferred as they can significantly detect very small variations. Generally, the only way to compensate and increase high CV% techniques relies in increasing replicates number thus increasing starting material. Using tests with the least inherent variability will induce the least replication and thus will be the less material consumer thus the most cost-effective. Indeed, due to high precision level, experiments can be performed only once for each sample like what is routinely done for biomarkers in patient's blood samples. Such procedure saves sample volumes and allows carrying out a great number of experiments on the same plasma aliquot.

Using our new platform, we have primarily focused on mitochondrial function and oxidative stress that are commonly linked together. Indeed, catalytic dysfunctions in mitochondrial ETC and/or OS pathways that lead to bioenergetics defects have been reported in large number of species (from unicellular to multicellular eukaryotes, from vegetal to animal) (Schwarzländer and Finkemeier, 2013; Song et al., 2016). In mammals, whereas OS seems ubiquitously distributed, ETC dysfunction is frequently observed in specialized tissues. As compound evaluation can be performed either in preclinical or clinical stages, development of versatile diagnostic assays usable either on a large subset of cells and/or tissues or on a variety of living species (from yeast to humans) is strongly mandatory. Traditional quantification methods either for ETC or OS are usually labor-intensive and time-consuming because it is still largely manual. Thus, they are only available in a small number of very specialized laboratories able to extract with appropriate means mitochondria from whole cells. Most of conventional techniques lack analytical performances, especially precision, with intra- and inter-CV% largely above 10%, impairing detection of tiny variations that are very common in chronic illnesses. Traditionally, measurement of a large panel of markers requires the use of a large range of different techniques. It also requires measuring parameters successively and never in parallel, generating an unavoidable degradation of starting material.

The use of a random-access analyzer for automated analysis of mitochondrial enzymes is not new as it was originally described in cultured skin fibroblasts by Williams et al. (1998) and later extended to the four complexes of the respiratory chain (Kramer et al., 2005). Such automated method was faster to perform, less expensive, and required less than one half of the sample material needed for traditional manual methods. Moreover, as multiparametric analyzers are now able to quantify in parallel up to 40 different biomarkers, it becomes possible on a unique sample to uncover different metabolic parts of the cellular machinery.

When applied to cell lines, as cellular enzymes work together, it becomes possible to evaluate the relative importance of each metabolic cascade. At the light of our results, it is tempting to postulate that, when compared to PNT1A normal prostate cells, both VCaP and PC3 prostate cancer cells undergo OS but this OS is still well controlled (limited lipid peroxidation) by increased global antioxidant defense. This strong capability of CaP cells to efficiently fight OS is certainly a cardinal feature that allows them to resist to conventional prooxidative chemotherapy. It is also possible to evaluate the influence of a metabolite (like glucose in **Figures 5A,B**) or a drug on cell metabolism (like rotenone in **Figures 6A,B**). The use of reliable techniques allows comparison between cell lines and evidence that cells behave differently under treatment (**Figures 5**, **6**). It is also interesting to notice that a well-known ETC complex I inhibitor can have effects not only on complex I but also on other parts of metabolism (it decreases ATP production but, for yet unknown reasons, it lowers also GPX activity) (**Figure 6A**). When applied *in vivo* to specific tissues or whole animal, (**Figure 6B**), it allows to understand the impact a compound may have on cell metabolism and how the cell will react to its presence. Such results are largely complementary to omics data. Identifying transcripts, metabolites or protein do not preclude whether the system is functional or not. The ability to measure enzyme activities gives invaluable information not only on protein amount but also on protein functionality. Looking at the results, the enzyme is present in a given amount but also is more or less active. Combining this enzyme activity with others in the same cellular metabolic pathway allows getting an insight on the overall functionality of this pathway. In addition, measuring, in parallel on the same starting material, different parameters allow the establishment of ratios (as enzyme activity ratios). Because CV% of each enzymatic technique is below 5%, it becomes possible to compare these ratios. As it is also possible to very precisely measure protein sample concentration in the same set of measurements, all the quantitative tests can be reported to this content and can be compared together. Using such ratios may have an importance to unravel complex metabolism features. As an example, looking at OS cellular effects creates the thought that an observed SOD increase may certainly have a cellular benefit as it reflects a positive cell response. Nevertheless, as superoxide anion detoxification leads to detrimental H_2O_2 production, a parallel increase in

GPX activity is mandatory to reduce H_2O_2. Thus, a stable GPX/SOD ratio may reflect an efficient cellular response while a decreased one may indicate a decrease in H_2O_2 reducing power that will certainly have deleterious consequences such as cellular peroxidation of multiple cellular elements. This peroxidation can lead to read-outs modification such as increased GSH consumption and/or potential MDA increase. Indeed, the use of tests panel rather than a single test in such complex metabolic cascades greatly increases both analysis quality and interpretation fineness. Thus, performing in parallel several panels of tests uncovering multiple metabolic pathways will definitely pave the way to metabolic integration.

At the scale of a multi-person study on the effect of a compound, it becomes possible to perform enzyme measurements on tissues from a large set of individuals and easily detect outliers that behave differently (**Figure 8**). Because of the very low CV% techniques, aside comparison of drug effect on cell or organ tissue behavior, it can also be used to evaluate dose effects on whole animal (**Figure 6B**), or time-dependent drug effects on human cells (**Figure 5B**), measuring effects not only in a static but also in a dynamic fashion. In the latter case, if a time-dependent experiment can be designed, all samples are analyzed in parallel on the same apparatus at the same time with the same reagents and technical constraints, hereby limiting possible drawbacks (batch effect).

To increase results reliability and comparability between cell culture, animal testing or human follow-up, from preclinical studies to clinical ones, it seems essential to perform tests using the same technologies. Metabolites and enzyme activities measurements are strong biomarker candidates as they encompass species differences. Indeed, as shown in our example studies, enzyme activities behave globally similarly either in *Caenorhabditis* Elegans and rat tissues or in human white blood cells.

In the chemical toxicity testing field, new efficient and reliable methodologies are strongly required by regulatory agencies. By its versatility (from cultured cells and small animals to humans with the same analytical performances), its high precision and robustness, its high throughput capabilities (some analyzers may launch from 2,000 to 4,000 tests/h), our platform fulfill some of the NAMs requested features. As it may be expanded to different central cellular metabolisms (parallel implementation on the platform of other metabolisms, i.e., glycolysis, TCA cycle, fatty acids degradation, ketogenesis), it can give very precise information regarding the effect a compound may have on global cellular metabolism and bioenergetics. As an example, in humans, compound dose- or time-dependent effects can be observed either on circulating cells (white or red blood cells depending on the need of an OXPHOS activity evaluation) or specific tissue biopsies with the same accuracy.

CONCLUSION

Assessing global functional metabolic behavior earlier and more comprehensively in drug development process will help avoid costly late-stage attrition, and more importantly, will improve drug safety. As more novel drugs, heading for restoring congruous cell, tissue or organ equilibrium in every disease that is metabolic in nature, are supposed to be developed in the future, the probability of concomitant mitochondrial and cytoplasmic metabolic pathways dysfunctions will largely increase. Because most of the defects are likely to be insidious, they will necessitate more sensitive, frequent and dynamic evaluation of complexes functionalities.

As complementary to classical "omics" studies, the use of multi-parametric equipment able to measure several markers in parallel with high precision, without any bias due to either inter-experiments variability or sample degradation, is certainly an interesting opportunity to evaluate compound impact along the different phases of its development. By its unique characteristics, its versatility (working from early pre-clinical to late clinical phases), its development capabilities, such platform will certainly help strategic decisions regarding subsequent drug testing or development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes to Predict the Cardiotoxicity Potential of Next Generation Nicotine Products

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Combustible cigarette smoking is an established risk factor for cardiovascular disease. By contrast, the cardiotoxicity potential of non-combustible next generation nicotine products (NGPs), which includes heated tobacco products (HTPs) and electronic vaping products (EVPs), and how this compares relative to combustible cigarettes is currently an area of scientific exploration. As such, there is a need for a rapid screening assay to assess this endpoint. The Cardio *quick*Predict is a metabolomics biomarker-based assay that uses human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) to screen for potential structural and functional cardiac toxicants based on the changes of four metabolites, lactic acid, arachidonic acid, thymidine, and 2′-deoxycytidine. The study aims were to investigate the cardiotoxicity potential of NGPs compared to cigarettes, in addition to nicotine. To accomplish this, hiPSC-CM were exposed to smoke or aerosol bubbled PBS samples: reference cigarette (1R6F); three variants of HTP; and three EVP variants. The 1R6F bPBS was the most active, having cardiotoxic potential at 0.3–0.6% bPBS (0.4–0.9 µg/mL nicotine), followed by HTP, which displayed cardiotoxic potential at a 10 times higher concentration, 3.3% bPBS (4.1 µg/mL nicotine). Both 1R6F and HTP bPBS (at 10-fold higher concentration than 1R6F) affected all four predictive metabolites, whereas none of the EVP bPBS samples were active in the assay up to the maximal concentration tested (10% bPBS). Nicotine tested on its own was predicted to have cardiotoxic potential at concentrations greater than 80 µg/mL, which is higher than expected physiological levels associated with combustible cigarette smoking. The application of this rapid screening assay to NGP research and the associated findings adds to the weight-of-evidence indicating that NGPs have a tobacco harm reduction potential when compared to combustible cigarettes. Additionally, this technique was shown to be sensitive and robust for the assessment of different NGPs and may be considered as part of a larger overall scientific framework for NGP assessments.

Keywords: cigarette, NGP, nicotine, cardiotoxicity, hiPSC-CM, *in vitro*, NAMs

1 INTRODUCTION

Cigarette smoking is a cause of serious diseases in smokers and is an established risk factor for cardiovascular disease (Messner and Bernhard, 2014). Cigarette smoke is a complex mixture comprised of both gas and particle phases containing around 7,000 chemical constituents, (Smith et al., 2001; Rodgman and Perfetti, 2008; Rodgman, 2014). Around one hundred of these chemicals have been identified as harmful and potentially harmful constituents (HPHCs) by public health bodies (Burns et al., 2008; FDA, 2012). Certain HPHCs have been classified as having cardiotoxic activities, including: carbon monoxide, acrolein, arsenic, benz [a]anthracene, benzene, benzo [b] fluoranthene, benzo [k]fluoranthene, chrysene, cobalt, hydrogen cyanide, lead, phenol, and propionaldehyde (FDA, 2012). Non-combustible nicotine products, which deliver nicotine but with fewer and lower levels of toxicants compared to cigarette smoke, represent a promising tobacco harm reduction (THR) tool for adult smokers who are unwilling or uninterested to quit smoking and who would otherwise continue to smoke. Collectively these are known as Next Generation Products (NGPs) and encompass many non-combustible tobacco as well as tobacco-free products, including heated tobacco products (HTPs) and electronic vapour products (EVPs). Whereas EVPs aerosolise a nicotine-containing liquid (typically with propylene glycol and vegetable glycerine carriers) to create an inhalable aerosol, HTPs electronically heat a portion of tobacco at temperatures well below the level necessary to burn tobacco to generate the inhalable aerosol. By eliminating tobacco combustion, NGP aerosols have been shown to have a relatively simple chemical composition with fewer and substantially reduced levels of HPHCs and other chemicals when compared to tobacco smoke (Schaller et al., 2016b; Jaccard et al., 2017; Eaton et al., 2018; Forster et al., 2018; Bentley et al., 2020; Rudd et al., 2020).

Cigarette smoking, and associated HPHCs, are major causes of cardiovascular disorders including coronary heart disease, stroke, aortic aneurysm, and peripheral artery disease (Benowitz and Burbank, 2016). The risk is seen both as an increased risk of acute thrombosis of narrowed vessels and as an increased degree of atherosclerosis in the blood vessels involved and damage to the myocardium. Cigarette smoke -induced cardiac damage is divided into two major and interchangeable mechanisms consisting of 1) direct adverse effects on the myocardium causing smoking cardiomyopathy due to oxidative stress, inflammation metabolic impairment and cell death or 2) *via* indirect effects on the myocardium by causing comorbidities such as the development of atherosclerosis and hypertension that eventually leads to damage and remodelling of the heart (Kaplan et al., 2017). The cardiovascular risks attributable to cigarette smoking increase with the number of cigarettes smoked and with the duration of smoking (US Surgeon General, 2010) and there are a number of mechanisms involved in the development of associated cardiovascular disease pathologies. These include, inflammation, endothelial dysfunction, lipid abnormalities, such as oxidation of low-density lipoprotein (LDL), and platelet activation (Burke and FitzGerald, 2003); all of these are associated with atherogenic and atherosclerotic progression, in addition to the key role of oxidative stress (Marchio et al., 2019).

Cigarette smoke is known to induce cellular oxidative stress, which is in fact reported to be a driving factor in many smoking related diseases (Laniado-Laborin 2009; Kamceva et al., 2016). Extensive research conducted over the last 30 years has revealed the mechanism by which continued oxidative stress can lead to chronic inflammation, ultimately leading to numerous chronic diseases including diabetes, cancer, cardiovascular, neurological, and pulmonary diseases (Reuter et al., 2010). Oxidative stress has been shown to activate various transcription factors including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt and Nrf2, which leads to changes in the expression of multiple genes, including growth factors, inflammatory cytokines, chemokines, molecules regulating cell cycle, and anti-inflammatory molecules (Reuter et al., 2010). Delivery of free radicals and oxidants present in both the gas and particulate phases of cigarette smoke, as well as endogenously produced oxidants and radicals (resulting from the smoke chemical-induced alteration in the cellular redox system), cause a pro-oxidative environment. This general shift is likely to contribute to lipid oxidation and to a general increase in oxidative modification (and inactivation) of biomolecules (Messner and Bernhard, 2014). Morrow et al. (1995) reported the increased presence of lipid peroxidation products in the serum of smokers due to the stable aldehydes in cigarette smoke increasing reactive oxygen species production by the activation of NADPH oxidases (Messner and Bernhard, 2014).

Mitochondrial dysfunction caused by cigarette smoke is involved in the pathology of respiratory diseases caused by oxidative stress and reducing the levels of harmful and potentially harmful components, by heating instead of burning tobacco, can further reduce mitochondrial changes that contribute to oxidative stress and cell damage (Malinski et al., 2018). However, there is some evidence that NGPs may demonstrate some association with oxidative stress and also cardiovascular disease. NASEM (2018), in their review of e-cigarettes concluded that "There is substantial evidence that components of e-cigarette aerosols can promote formation of reactive oxygen species/oxidative stress. Although this supports the biological plausibility of tissue injury and disease from long-term exposure to e-cigarette aerosols, generation of reactive oxygen species and oxidative stress induction is generally lower from e-cigarettes than from combustible tobacco cigarette smoke." Buchanan et al. (2020) undertook a review of the clinical and pre-clinical studies conducted on e-cigarettes and concluded that the studies presented in their review "have shown that e-cigarettes can induce negative cardiovascular effects through various mechanisms such as oxidative stress, inflammation, DNA damage, arterial stiffness, and altered haemodynamics, and platelet activation." Furthermore, recent research suggests that HTP and EVP aerosols can affect the cardiovascular system, albeit it at exposures many magnitudes higher than combustible cigarette cardiotoxic exposures (Abouassali et al., 2021; Qasim et al., 2017; Znyk et al., 2021). The role of NGPs in relation to cardiovascular diseases is however, still a source of debate and controversy (NASEM 2018; Fried and Gardner, 2020; Tsai et al.,

2020). Given the reported association between exposure to cigarette smoke and cardiovascular disease risk, and emerging evidence of potential effects of NGPs, there is a need for models to screen for such effects and to determine the potential of NGPs to induce cardiotoxicity. These models would ideally be high throughput and possess human relevance. Gathering mechanistic information on NGP samples relative to combustible cigarette would also help to determine their THR potential and to determine if the novel aerosol mixtures have the potential to induce cardiotoxicity, preferably using a high throughput human cell-based system, for increased human relevance. The implementation of rapid screening assays that can identify functional and structural cardiotoxicity earlier in the drug development pipeline has been shown to have the potential to improve safety and the cost and time required to bring new drugs to market (Palmer et al., 2020). With respect to NGPs, such assays allow for a broad screening assessment of potential toxicity when used as part of a wider overall scientific assessment framework for substantiating the THR potential of products as part of a weight-of-evidence approach. Current *in vitro* testing strategies for cardiotoxicity predominantly focus on the compound impact on individual ion channels. In addition, pre-clinical animal models used to assess cardiotoxicity are limited due to being low throughput, costly and may lack human relevance (Burnett et al., 2021). The Stemina Cardio *quick*Predict™ (Cardio^{qP}) is a human induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM)-based assay that predicts a test sample's cardiotoxicity potential using changes in hiPSC-CM metabolism and cell viability. The assay was developed and commercialized by Stemina Discovery Inc. The assay's metabolic endpoints are generated using known structural and functional reference toxicants to identify predictive metabolites released in the secretome of the hiPSC-CM. The assay's prediction model uses four metabolites (lactic acid, 2′-deoxycytidine, thymidine, and arachidonic acid) and cell viability to predict the concentration at which a test sample shows cardiotoxicity potential (cTP). These metabolites were found to be the most responsive when hiPSC-CM were exposed to known cardiotoxic drugs (Palmer et al., 2020). The reported accuracy, sensitivity, and specificity of the Cardio^{qP} assay are 86%, and 90%, respectively (Palmer et al., 2020). hiPSC-CM expresses most of the human cardiac ion channels and sarcomeric proteins, some of which contribute to action potential generation and are regulated by adrenergic stimulation (Huo et al., 2017; Zhao et al., 2018; Burnett et al., 2021). This assay adds significantly to other cardiac assays available when used in isolation, or as part of a combined approach, and can be used to screen test articles for potential cardiotoxicity.

To allow for the *in vitro* exposure of cigarette smoke and NGP aerosols, a number of trapping methodologies have been employed (Smart and Phillips, 2021). The bubbling of whole smoke or aerosol in phosphate buffered saline (bPBS) is a commonly used method and allows for the direct addition of bPBS into an *in vitro* cell system (Johnson et al., 2009). The bubbling procedure largely captures the water-soluble gaseous fraction of the smoke or aerosol, making this an alternative delivery mechanism to the historically used filter trapped total particulate matter or aerosol collected mass only. Work by Buratto et al., has shown that a number of carbonyls can be successfully trapped and quantified within bPBS from both combustible cigarettes and HTPs (Buratto et al., 2018).

This study aimed to screen the cardiotoxic potential of six NGP aerosols (from three HTP variants and three EVP variants) and reference cigarette smoke (1R6F), trapped directly in PBS, and then added to the cell culture medium of hiPSC-CMs. For each test sample, the concentration predicted to have cardiotoxic potential was determined using the Cardio*qP* prediction model. Nicotine was also tested in isolation to assess its effects in this system. To our knowledge, this study provides the first published assessment of the cardiotoxicity potential for a variety of NGP aerosols and cigarette smoke using the Cardio^{qP} assay.

2 MATERIALS AND METHODS

2.1 Test Samples

The test samples evaluated in this study with the Cardio^{qP} assay are described below in **Table 1**. The test sample were evaluated in three separate batches in 2019 (study 1: 1R6F S1 and cHTP, EVP-FB, EVP-0 and EVP-NS; study 2: 1R6F S2, pHTP1, pHTP2; study 3: nicotine). The bPBS samples for the studies were analysed within 3–4 months of generation.

Prior to smoke/aerosol generation, the 1R6F reference cigarette (batch #V062X53D1), and the respective HTP tobacco consumable sticks were stored in an airtight container at 4°C until use. The reference cigarettes and HTP sticks were allowed to come to room temperature for 15 min before opening and conditioned for at least 48 h in a standard humidified chamber following the International Organization for Standardisation (ISO) 3402 Guideline (International Organization for Standardization, 1999). Additional information of the device used in this study is proved in the **Supplementary Material**.

2.2 Smoke and Aerosol Extract Generation

Fresh whole aerosol and smoke was trapped in phosphate-buffered saline (bPBS) using the VITROCELL VC 10 S-TYPE smoke robot (VITROCELL Systems GmbH, Waldkirch, Germany), connected to glass impingers containing 30 mL PBS in total. The smoke from the 1R6F reference cigarette and aerosols from HTPs and EVPs were generated as detailed in **Table 2**. For the 1R6F reference cigarette a total of 56 puffs (8 puffs per stick, 7 cigarette sticks) per 30 mL (1.87 puffs per mL) PBS was generated. For all the NGPs, a total of 120 puffs (for pHTPs: 8 puffs/stick, 15 sticks; for cHTP: 10 puffs/stick, 12 sticks; for EVPs: 60 puffs per device, 2 devices) per 30 mL (4 puffs per mL) PBS were generated. Smoke or aerosol extracts were prepared by bubbling the sample generated smoke or aerosol into 3 in-line impingers, each containing 10 ml PBS. The three 10 ml samples were then combined to make a total stock solution of 30 mL prior to being aliquoted, rapidly frozen, and stored at −70°C, Samples were shipped on dry ice to Stemina, where the biological assay was carried out. The 30 mL PBS stocks contained 56 puffs for 1R6F and 120 Puffs for the NGPs to reduce the length of time the samples were collected in. All samples were collected over half an hour to try to minimise losses of any volatile constituents, that may be seen with an extended

TABLE 1 | Test samples evaluated in the Cardio^{qP} assay.

Study	Sample	Product category	Coding	Manufacturer
1, 2	1R6F reference cigarettes	Cigarette	1R6F S1[a] 1R6F S2[b]	University of Kentucky
1	IQOS with Amber HEETS	HTP	cHTP	Philip Morris International
2	Prototype HTP	HTP	pHTP1[c]	Imperial Brands
2	Prototype HTP	HTP	pHTP2[d]	Imperial Brands
1	*my*blu with tobacco flavour, 0% nicotine[e]	EVP	EVP-0	Imperial Brands
1	*my*blu with tobacco flavour, 1.6% nicotine (freebase)[e]	EVP	EVP-FB	Imperial Brands
1	*my*blu with tobacco flavour, 1.6% nicotine salt (nicotine lactate)[e]	EVP	EVP-NS	Imperial Brands
3	Nicotine	—	—	Sigma-Aldrich

[a]*1R6F S1 1R6F reference cigarette used in Study 1*
[b]*1R6F S2 1R6F reference cigarette used in Study 2*
[c]*pHTP1 Imperial Brands prototype HTP with tobacco variant consumables.*
[d]*pHTP2 Imperial Brands prototype HTP with menthol variant consumables.*
[e]*All* myblu *e-liquids were custom made by Nerudia Ltd., a subsidiary of Imperial Brands, for research purposes only. The nicotine was derived from tobacco.*

TABLE 2 | Smoke/aerosol generation standardized parameters.

Product	Smoking regime	Total puff number	Puffs per mL PBS
1R6F Reference cigarette	ISO 20779:2018 intense smoking regime (International Organization for Standardization, 2018b) *(bell-shaped puff profile, 55 mL puff volume, 2 sec duration, 30 sec interval with 100% ventilation blocking)*	56	1.87
HTPs	Modified ISO 20779:2018 (International Organization for Standardization, 2018b) *(bell-shaped puff profile, 55 mL puff volume, 2 sec duration, 30 sec interval, no ventilation blocking)*	120	4
EVPs	ISO 20768:2018 (International Organization for Standardization, 2018a) *(square-wave puff profile, 55 mL puff volume, 3 sec duration and a 30 sec interval, no ventilation blocking)*	120	4

The smoke/aerosol generation was performed using the standardized methods adopted by ISO to ensure repeatability of sample generation. All samples used a 55 mL puff volume collected over 2 or 3 sec. there was a 30 sec interval between puffing. The bell and square wave puff profile relates to the the smolder phase between puffing from cigarettes (bell shaped), square wave due to the either on or off nature of the heating elements for the NGPs. sec = second

collection time (hours) for higher puff numbers per mL of PBS. The 56 Puffs for 1R6F was based on the cytotoxicity seen with higher puff numbers. Nicotine for the nicotine sample was simply diluted in the culture medium (containing 0.1% DMSO) at a concentration of 1 mg/ml).

2.3 Characterisation of bPBS

Nicotine and eight selected carbonyls, based on the list described by Buratto et al. (2018), were quantified within the aerosol and smoke bPBS samples using the previously described methodology (Simms et al., 2020; Czekala et al., 2021). In brief, nicotine was quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX, Framingham, MA, United States). Carbonyl detection methodology in bPBS was adapted from ISO 21160:2018 (ISO, 2018) methodology for carbonyl detection. Carbonyl-DNPH derivates, were quantified using high-performance liquid chromatography with a diode-array detector (HPLC-DAD, Agilent Technologies 1100 Series, Agilent Technologies, Santa Clara, CA, United States).

2.4 hiPSC-CM Culture and Treatment

Cryopreserved hiPSC-CM (iCell® Cardiomyocytes) were obtained from FUJIFILM Cellular Dynamics, Inc. (FCDI, Madison, WI, United States) and cultured as previously described (Palmer et al., 2020). Cardio^{qP} has not undergone a formal validation study but has been extensively tested in over 80 compounds known to cause a wide range of cardiotoxic effects. Additionally the reproducibility of the Cardio^{qP} biomarkers and prediction model was evaluated during assay development (Palmer et al., 2020). Sixty-six drugs were evaluated a minimum of three times by multiple scientists with multiple lots of hiPSC-CM and reagents over the course of 3 years. These studies demonstrated that the drugs altered the metabolism of the same biomarkers at similar concentrations, indicating that both the biomarker response and the concentration where cardiotoxicity potential was observed were reproducible over multiple replicates (Palmer et al., 2020).

HiPSC-CM were exposed to 16 concentrations of nicotine (Sigma-Aldrich, St. Louis, MO, United States) ranging from 0.01 to 1,000 μg/ml. For smoke and aerosol bPBS testing, exposures were based on the bPBS concentration, which was used to calculate nicotine exposures during data analysis. Cells were exposed to eight concentrations of each test article, ranging from 0.053–3% with quarter-log dilutions (1R6F S1 or S2 bPBS) or 0.18–10% with quarter-log dilutions (all NGP bPBS). Test articles were diluted in iCell Cardiomyocytes Maintenance Medium containing 0.1% DMSO such that the final concentration of DMSO was 0.1% in all treatments.

Test article exposure began 4 days after plating. HiPSC-CM were exposed to test article for 72 h, with media ± test article replacement every 24 h. The spent media from the last 24-h treatment period was collected for analysis of metabolites. Cell viability was assessed after sample collection using the CellTiter-Fluor Cell Viability Assay (Promega, Madison, WI, United States). All experimental treatments were carried out in a 96-well plate format. Each test plate included cells exposed to 30 μM verapamil (Toronto Research Chemicals, North York, ON, Canada) as the positive control (n = 3 wells), 0.005 μM verapamil as the negative control (n = 3 wells), 0.1% DMSO as the reference (vehicle) control (n = 3 wells), eight concentrations of two test samples (n = 3 wells per concentration), and media controls for each treatment (lacking cells, ± test article). A single biological replicate (or repeat) was performed for each test article.

2.5 Sample Preparation

Proteins were precipitated in spent media samples using 60% methanol/40% acetonitrile solution cooled to −20°C containing thymidine-$^{13}C_{10}$, $^{15}N_2$, 2′-deoxycytidine-$^{15}N_3$, sodium L-lactate-$^{13}C_3$ (Cambridge Isotope laboratories, Tewksbury, MA, United States), and arachidonic acid-D_8 (Sigma-Aldrich). Samples were centrifuged at 2000 × g at 4°C for 10 min to pellet the precipitated proteins and the supernatant was transferred to a new 96-well plate for analysis.

2.6 Mass Spectrometry

Ultra-Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS) data were acquired as described in Palmer et al. (2020) using an Agilent 1290 Infinity LC system interfaced with an Agilent G6530 QTOF high-resolution mass spectrometer (Agilent Technologies). Briefly, an ACQUITY UPLC BEH Amide column (Waters, Milford, MA, United States) maintained at 45°C was used for metabolite separation. A 2 μL volume of sample was injected and data were collected over 7 min using a 6.1-min solvent gradient containing 5 mM ammonium acetate (pH 5.7) in water and 5 mM ammonium acetate (pH 5.7) in 95% acetonitrile.

2.7 Quality Control

Two quality control procedures were included to ensure correct plate readout. Firstly, the vehicle control (0.1% DMSO) sample coefficient of variation for the viability relative fluorescent units (RFU) could not exceed 10%. Second, the positive and negative control treatments had to be correctly predicted to ensure that the hiPSC-CM metabolism was within the assay specifications. Quality control data is provided in Supplementary materials (**Supplementary Figure S4**).

2.8 Data Analysis

2.8.1 UPLC-HRMS Data Analysis

The extracted ion chromatogram height for lactic acid, thymidine, arachidonic acid, and 2′-deoxycytidine, and the internal standards (ISTDs) were determined using the Agilent Mass Hunter Quantitative Analysis software, version B.05.00 or newer (Agilent Technologies). The height of each endogenous metabolite was normalized to the corresponding ISTD by dividing the endogenous metabolite height by the corresponding isotopically labelled ISTD height. Relative fold changes were then calculated for each ISTD-normalized metabolite in each sample by dividing the sample response by the median ISTD-normalized response of the reference treatment (0.1% DMSO) samples, producing a reference-normalized value for each metabolite in each sample within a plate of cell culture samples. A Grubbs' test was then used to identify outlier samples within each treatment and exposure level, and outlier samples were then removed from further analyses.

TABLE 3 | Metabolite specific prediction thresholds based on upper lower band beyond which ratios or values are considered to be cardiotoxic.

Metabolite	Lower threshold	Upper threshold
Viability/Lactic Acid	0.325	1.44
Viability/Arachidonic Acid	0.675	1.72
Thymidine	0.895	2.27
2′-Deoxycytidine	0.865	1.26
Cardiotoxicity Threshold	1.025	

2.8.2 Viability Data Analysis

To determine the relative fold changes for cell viability, the RFU value for each sample was first background corrected by subtracting the RFU value of the treatment specific media blank from the cell sample RFU. Next, the values were reference-normalized by dividing the background-corrected RFU value of each sample by the average RFU value (background corrected) of the reference treatment.

2.8.3 Prediction Model Calculation

The prediction model uses the reference-normalized response for thymidine and 2′-deoxycytidine and the viability-normalized responses for lactic acid and arachidonic acid. The viability-normalized responses for lactic acid and arachidonic acid were calculated for each sample by dividing the reference-normalized viability value by the reference-normalized lactic acid or arachidonic acid value. The prediction distance for each metabolite was determined by dividing the median metabolite value at a given concentration by its nearest prediction threshold (**Table 3**). Values outside the prediction thresholds were predicted to be cardiotoxic. The prediction model value (combined prediction distance) at each concentration was the maximum prediction distance of the metabolites used in the model.

2.8.4 Dose-Response Analysis

For the smoke and aerosol bPBS treatments, the bPBS concentration ranges were converted to nicotine concentrations based on the measured nicotine concentration to create a normalized concentration range across samples based on the component analysis. Dose-response analysis was performed using GraphPad Prism (version 9.1, GraphPad Software, San Diego, CA, United States). Each data set was fitted with a nonlinear model. The Akaike information criterion was used to determine if an asymmetric (five-parameter) or multiphasic nonlinear model was a better fit for the data than a four-parameter model. The cardiotoxicity toxicity potential concentration (cTP) was then determined from the

TABLE 4 | Nicotine and carbonyl content (μg/mL) of test article smoke or aerosols used in this study were measured using LC-MS/MS and HPLC-DAD, respectively.

Concentration (μg/mL)	1R6F S1	1R6F S2	cHTP	pHTP1	pHTP2	EVP-FB	EVP-NS	EVP-0	LOQ (μg/mL)
Nicotine	127.1	152.0	123.0	101.0	140.0	165.1	187.0	0.5	0.01
Formaldehyde	12.6	13.1	0.9	0.6	0.8	5.0	9.0	4.4	0.25
Acetaldehyde	190.3	171.4	52.9	53.3	50.5	<LOQ	3.3	<LOQ	1.5
Acetone	55.8	21.76	5.4	5.1	5.5	<LOQ	<LOQ	<LOQ	1.0
Acrolein	4.0	5.0	1.3	<LOQ	0.4	<LOQ	<LOQ	<LOQ	0.5
Propionaldehyde	10.5	9.1	3.5	2.6	2.6	<LOQ	<LOQ	<LOQ	0.5
Crotonaldehyde	8.3	4.9	0.6	0.9	0.8	<LOQ	<LOQ	<LOQ	0.5
2-Butanone	11.9	4.8	1.3	1.1	1.1	<LOQ	<LOQ	<LOQ	0.5
n-Butyraldehyde	4.0	3.28	2.8	2.8	2.7	<LOQ	<LOQ	<LOQ	0.5

prediction model dose-response curve using the cardiotoxicity threshold value (**Table 3**). The cTP concentration for each sample is provided in both μg/mL nicotine and % bPBS (which could be used to determine the concentration of other constituents).

An extra sum-of-squares F test was used to determine if the dose-response curve of each endpoint (cell viability, lactic acid, thymidine, arachidonic acid, and 2′-deoxycytidine) for the cigarette reference sample (1R6F bPBS) were significantly different from each other, under the null hypothesis that one curve adequately fits all data sets (i.e., the data sets have the same top, bottom, IC_{50}, and hill slope best-fit values), and the alternative hypothesis that there is a different dose-response curve for each data set (i.e., the data sets have different top, bottom, IC_{50}, and Hillslope best-fit values).

If it was determined that the data sets had independent dose-response curves, an unpaired t test was conducted for each endpoint at each closest or overlapping concentration to determine the concentration where the curves differed from one another. The resulting p values were adjusted for multiple comparisons (q value) using Benjamini and Hochberg's method to control the false discovery rate. These analyses were also used to determine if the dose-response curves for the 1R6F bPBS was significantly different from the NGP bPBS and samples tested in the same experiment (e.g., 1R6F S1 was compared to cHTP, EVP-FB, EVP-0, and EVP-NS; 1R6F S2 was compared to pHTP1, pHTP2). Additionally, the response following cHTP bPBS exposures were compared to EVP-0, EVP-FB, and EVP-NS bPBS. The significance threshold was set at $p < 0.05$ for all statistical tests. All values in figures are given as mean ± standard error. If the standard error is not shown, the error bars are smaller than the size of the symbol.

3 RESULTS

3.1 Characterisation of bPBS for Reference Cigarette and NGPs

Nicotine and eight carbonyls were quantified in the bPBS matrix following the capture of the 1R6F smoke and the individual NGP aerosols. Quantification results are displayed as μg/mL of bPBS in **Table 4**. The 1R6F S1 and S2 bPBS samples contained the highest level of carbonyls. By contrast, the total quantified carbonyls were greatly reduced or not quantifiable in the NGP samples. Lower levels were detected for the HTP samples compared to 1R6F for all eight quantified carbonyls, whereas the majority of these were below the LOQ for the EVP bPBS samples. Formaldehyde levels in the EVP-NS bPBS samples were higher compared to the seven other carbonyls but did not exceed those measured in both 1R6F bPBS samples. The EVP-NS delivered the highest level of nicotine to the PBS, with both EVP-NS and EVP-FB bPBS containing more nicotine than all three HTPs and 1R6F bPBS samples. Low levels of nicotine (0.5 μg/mL) were detected in the EVP-0 bPBS, which was not recorded in the neat e-liquid sample, as per the Certificate of Analysis (determined using gas chromatography).

3.2 Nicotine Cardio^{qP} Results

The CardioqP prediction model was based on the perturbation of four key metabolites (lactic acid, arachidonic acid, thymidine and 2′-deoxycytidine) and cell viability in hiPSC-CM. The predicted cTP concentration for nicotine was 80 μg/mL and viability IC50 was 586 μg/mL (**Table 5**). Nicotine caused metabolic perturbations in all four predictive metabolites (**Figure 1**), with viability/lactic acid responding at the lowest concentrations, but viability/arachidonic acid and 2′-deoxycytidine crossing their respective prediction thresholds first. Viability/lactic acid ratio showed a downward trend after 10 μg/mL of nicotine, and the fold change continued to decrease as the concentration of nicotine increased. At 495 μg/mL, the fold change dropped below the lower metabolite-specific prediction thresholds. Viability/arachidonic acid ratio displayed a different trend to viability/lactic acid. Initially, an upward trend was observed after 50 μg/mL of nicotine, and the fold change continued to increase, exceeding the upper metabolite-specific prediction threshold at 120 μg/mL of nicotine. After 200 μg/mL of nicotine, the fold change decreased as the concentration increased. Thymidine (which dropped below threshold at 398 μg/mL) fold change followed a similar trend as viability/lactic acid (**Figures 1B,D**), whereas 2′deoxycytidine (which exceeded threshold at 56 μg/ml) was similar to viability/arachidonic acid (**Figures 1C,E**). Individual cell viability, lactic acid and arachidonic acid dose-response curves can be found in the Supplementary Material (**Supplementary Figure S1**).

3.3 Cardiotoxicity Potential of bPBS for Reference Cigarette and NGPs

The predicted cTP concentration and viability IC_{50} for all the test samples are summarized in **Table 5** and the dose-response curves for the prediction model are shown in **Figure 2**. The 1R6F bPBS samples from both studies were predicted to be cardiotoxic: 1R6F S1 at 0.3%

TABLE 5 | Cardiotoxicity potential summary for smoke or aerosol trapped bPBS from reference cigarettes and NGPs.

Sample bPBS	Concentration range tested (% bPBS)	cTP[a] (% bPBS)	Viability IC_{50} (% bPBS)	Exposure range (μg/mL nicotine)	cTP[a] (μg/mL nicotine)	Viability IC_{50} (μg/mL nicotine)
1R6F S1	0.053–3	0.3	1.0	0.07–3.81	0.4	1.3
1R6F S2	0.053–3	0.6	2.3	0.08–4.6	0.9	3.5
cHTP	0.18–10	3.3	10.4	0.22–12.3	4.1	12.8
pHTP1	0.18–10	6.2	ND	0.18–10.1	6.3	ND
pHTP2	0.18–10	7.0	ND	0.25–14.0	9.8	ND
EVP-FB	0.18–10	ND	ND	0.29–16.5	ND	ND
EVP-NS	0.18–10	ND	ND	0.33–18.7	ND	ND
EVP-0	0.18–10	ND	ND	N/A	N/A	N/A
Nicotine	N/A	N/A	N/A	0.1–1000	80	586

[a]*Concentration in % bPBS and μg/mL nicotine where the cardiotoxicity threshold of 1.025 is crossed. cTP: Cardio Toxicity Potential. N/A: Not appropriate. ND: no effect was detected within the exposure range tested.*

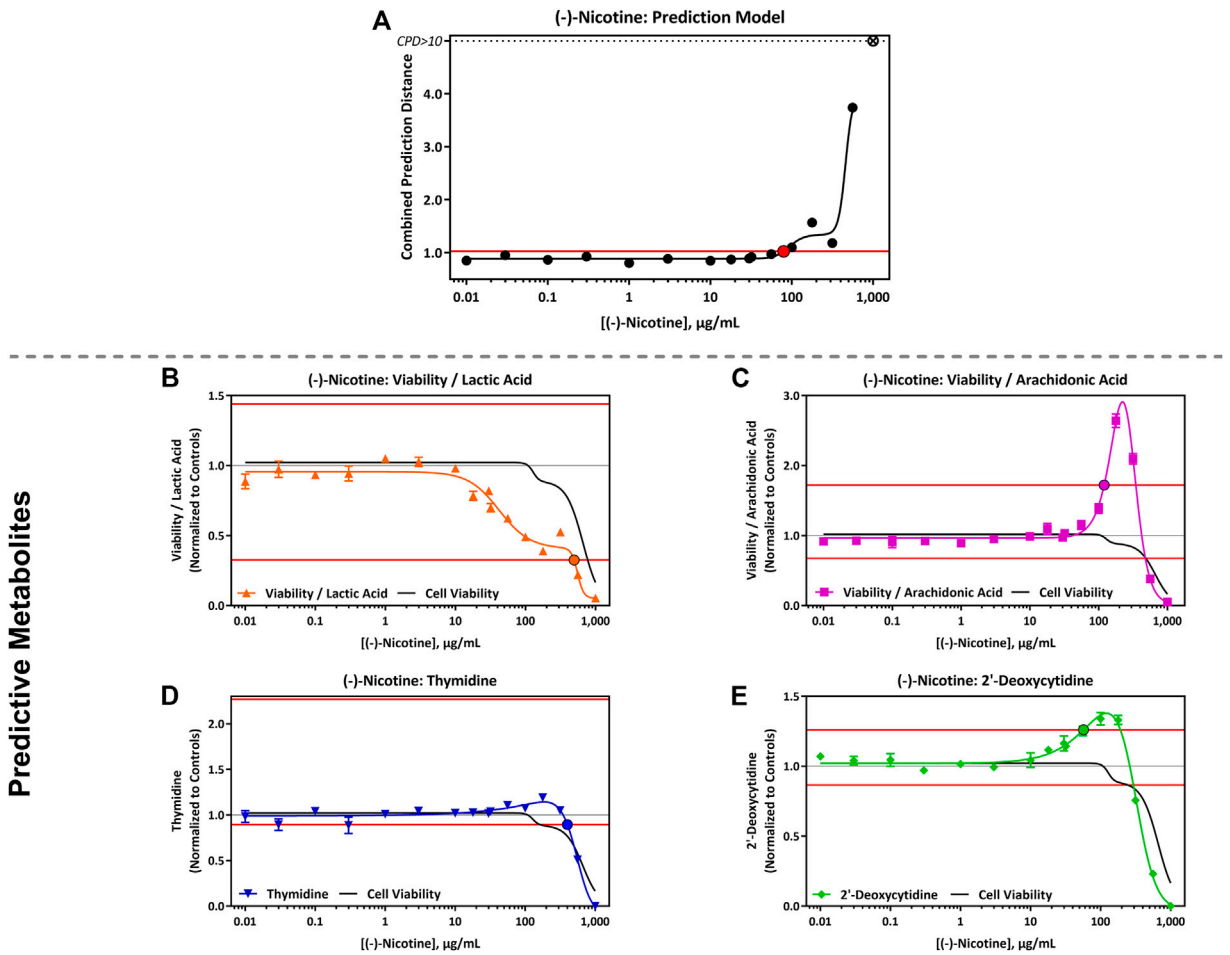

FIGURE 1 | Cardio^{qP} Assay Results Following Exposure to 0.01–1,000 μg/mL (-)-Nicotine. **(A)** (-)- Nicotine dose-response results for the Cardio^{qP} prediction model. The horizontal red line represents the cardiotoxicity threshold and the red filled circle indicates the predicted cardiotoxicity potential concentration (cTP). Concentrations greater than the cTP are predicted to be cardiotoxic. The y-axis is the combined prediction distance, which is determined from each biomarker ratio's response normalized to its predictive threshold (level of change required for a toxic prediction). CPD: Combined prediction distance; ⊗: CPD>10 **(B)** Change in Cell Viability/Lactic Acid; **(C)** Cell Viability/Arachidonic Acid; **(D)** Thymidine, and **(E)** 2′-Deoxycytidine. The horizontal red lines represents the metabolite-specific prediction thresholds. The y-axis is the reference treatment normalized (fold change) value for each metabolite/ratio. The black bordered circles represent the nicotine concentration where the dose-response curve crossed the metabolite-specific prediction threshold.

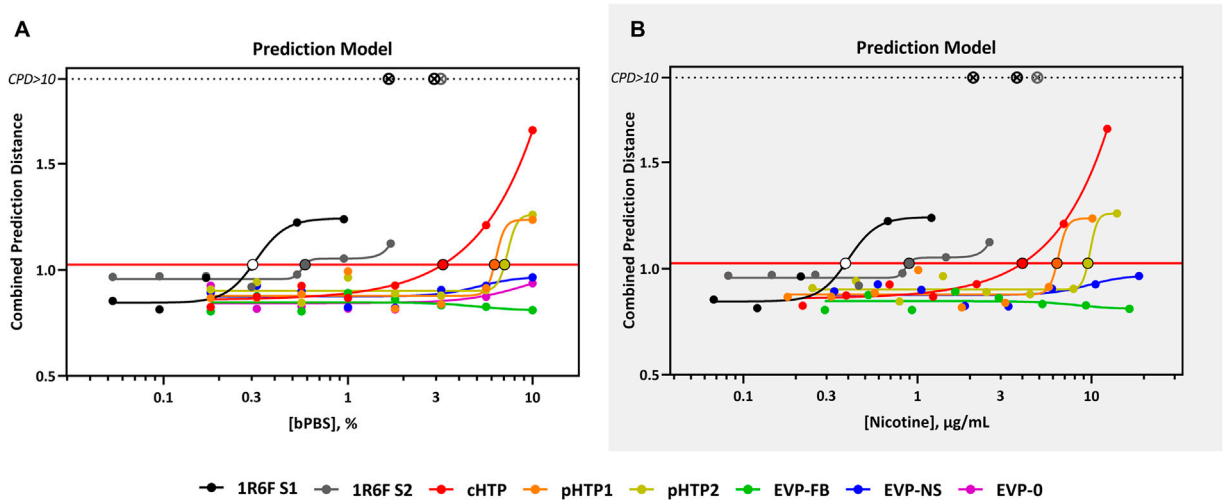

FIGURE 2 | Cardio^{qP} Prediction Model Response Comparison. The horizontal red line represents the cardiotoxicity threshold and the black-bordered, filled circles indicate the predicted cardiotoxicity potential concentration (cTP) for each treatment. The x-axis is the concentration (% bPBS) of the test article **(A)** or nicotine (µg/mL) **(B)**. The y-axis is the combined prediction distance, which is determined from each biomarker ratio's response normalized to its predictive threshold (level of change required for a toxic prediction). CPD: Combined prediction distance; ⊗: CPD>10.

TABLE 6 | Cardiotoxicity potential fold change relative to typical nicotine concentrations following smoking a cigarette.

Sample bPBS	cTP (µg/mL nicotine)	cTP vs C_{max} (0.016 µg/mL [a])
1R6F S1	0.4	25
1R6F S2	0.9	56
cHTP	4.1	256.35
pHTP1	6.3	394
pHTP2	9.8	612.5
EVP-FB	ND	N/D
EVP-NS	ND	N/D
EVP-0	N/A	N/A
Nicotine	80	5000

[a]*D'Ruiz et al.(2015).*

N/A: not appropriate. ND: no effect was detected within the exposure range tested. In vitro *response observed at ≤ 50 × the* in vivo C_{max} *is considered to be relevant for prediction of* in vivo *toxicity (Talbert et al., 2015).*

(0.4 µg/ml of nicotine) and 1R6F S2 at 0.6% (0.9 µg/ml of nicotine). Based on reproducibility testing (data not shown), the variation between S1 and S2 were within the expected level (i.e., within 3-fold, a value determined by Stemina as not biologically different). All three HTP bPSB samples elicited a metabolic response indicative of cardiotoxicity in this assay. However, the predicted cTP concentrations for the HTPs samples were approximately 10-fold higher than the corresponding 1R6F sample, indicating that 1R6F bPBS has the potential to cause cardiotoxicity at a concentration that was 10-fold more potent than the HTP bPBS samples. By contrast, the three EVP bPBS (EVP-0, EVP-FB, and EVP-NS) did not cause a metabolic response indicative of cardiotoxicity potential within the exposure concentrations tested. The cTP (µg/mL nicotine) were then compared to the maximum blood nicotine concentration (Cmax) observed in adult smokers following cigarette smoking (16 ± 9 ng/mL) (D'Ruiz et al., 2015) reported in **Table 6**. An *in vitro* response observed at ≤ 50 × the *in vivo* Cmax was considered to be relevant for prediction of *in vivo* toxicity (Talbert et al., 2015). The prediction model dose-response curves for each test article were statistically compared with one another for the treatments that were conducted within the same study (extra sum-of-squares F Test). When comparing the bPBS curves, both 1R6F bPBS (S1,2) were significantly different from all the NGP bPBS samples ($p < 0.0001$) see **Table 7** and individual values for each product and the four metabolites in **Supplementary Tables S1 to S14**; the only two pairs which were not significantly different to each other were: pHTP1 and pHTP2, and EVP-NS and EVP-0.

3.3.1 Predictive Metabolites

All four predictive metabolites were altered by the 1R6F S1 and S2 bPBS samples **Figures 3**, **4**. Viability/lactic acid was the most sensitive and initially showed a positive dose-response, followed by a decrease, corresponding to decreased cell viability. Viability/arachidonic acid, thymidine and 2′ deoxycytidine decreased as the concentration of bPBS increased. cHTP bPBS caused the same metabolomic response profile as 1R6F bPBS, whereas pHTP 1 and 2 only caused two predictive metabolites (viability/lactic acid and thymidine) to be altered under these study conditions (**Figure 3**). By contrast, EVP-NS, EVP-FB and EVP-0 caused a slight increase in viability/lactic acid and viability/arachidonic acid; however, this increase did not reach the metabolite-specific cardiotoxicity thresholds at the concentrations tested (**Figure 4**). Moreover, the three EVP samples did not alter thymidine and 2′deoxycytidine metabolism (**Figure 4**). Individual viability, lactic acid and arachidonic acid curves can be found in the Supplementary Material (**Supplementary Figures S2, S3**).

The individual dose-response curves within each predictive metabolites were compared on the basis that all parameters were shared (see *Dose-Response Analysis*) and a point of difference (POD) was determined where applicable (detailed results

TABLE 7 | Prediction model of the F test comparison (*p* values) for the product dose response curves, relative to %bPBS or nicotine (μg/mL).

bPBS (%)								Nicotine (μg/ml)					
	1R6F S1	1R6F S2	cHTP	pHTP1	pHTP2	EVP-FB	EVP-NS	1R6F S1	1R6F S2	cHTP	pHTP1	pHTP2	EVP-FB
1R6F S2	<0.0001							<0.0001					
cHTP	<0.0001							<0.0001					
pHTP1		<0.0001							<0.0001				
pHTP2		<0.0001		0.5108					<0.0001		0.4806		
EVP-FB	<0.0001		<0.0001					<0.0001		<0.0001			
EVP-NS	<0.0001		<0.0001			<0.0001		<0.0001		<0.0001			0.0002
EVP-0	<0.0001		<0.0001			0.0011	0.0634						

Treatments that did not have significantly different dose-response curves are highlighted in yellow (p < 0.05). Statistical comparisons were only made on sample run during the same experiment.

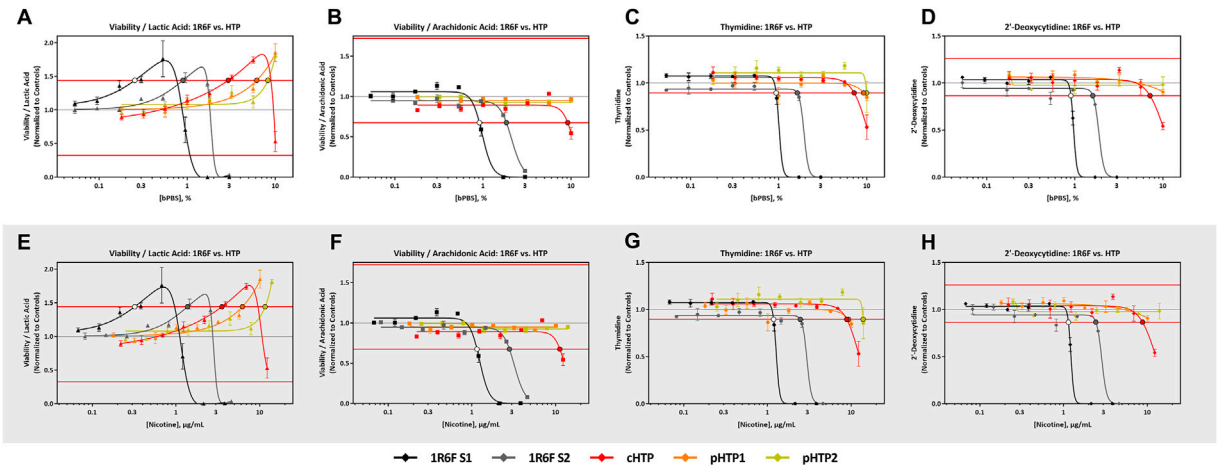

FIGURE 3 | 1R6F vs HTP Cell Viability/Lactic Acid, Cell Viability/Arachidonic Acid Thymidine, and 2′-Deoxycytidine Response Comparison. The x-axis is the concentration (% bPBS) of the test article **(A–D)** or nicotine (μg/mL) **(E–H)**. The y-axes are the reference treatment normalized (fold change) values for each metabolite/ratio. The horizontal red lines represent the metabolite-specific prediction thresholds The black bordered circles represent the nicotine concentration where the dose-response curve crossed the metabolite-specific prediction threshold.

provided in **Supplementary Tables S1–S14**. The dose-response curve for each predictive metabolite were significantly different between 1R6F S1 and S2 bPBS ($p < 0.0001$). which was largely due to the shift in concentration where changes was observed between replicates; however, the same response profile was observed. The dose-response curves for the NGP bPBS samples were all significantly different from the corresponding study 1R6F bPBS sample for each endpoint ($p < 0.0001$, determined by an extra sum-of-squares *F* Test, see **Supplementary Tables S1–S14**). The raw data for nicotine, 1R6f and the NGPS for cell viability and the 4 metabolites are presented in **Supplementary Tables S15–S24**.

4 DISCUSSION

4.1 Quantification of Trapped Carbonyls and Nicotine in Bubbled PBS

The eight carbonyls quantified in this study were selected based on a list described by Buratto et al. (2018), which represent the main carbonyl compounds included in the HPHC lists for tobacco smoke regulatory reporting (FDA, 2012; Health Canada, 2000). Nicotine was used in the present study as a comparative measure of exposure between the test samples. There were clear differences between the quantified constituents form the different NGP aerosol and 1R6F bPBS (**Table 4**). As expected, total carbonyl levels were the highest in the 1R6F bPBS, with substantially lower amounts measured in all HTP bPBS. Limited carbonyls were detected in all EVP bPBS test samples. This is reflective of the whole smoke and aerosol emission profiles typically reported for cigarettes and non-combustible nicotine products (Schaller et al., 2016b; Poynton et al., 2017; Farsalinos et al., 2018; Jaccard et al., 2019; Rudd et al., 2020). For EVP-NS bPBS the formaldehyde level was elevated in comparison to EVP-FB and EVP-0 bPBS, although this was substantially lower than combustible cigarettes based on 120 puffs vs 54 puffs (EVP vs 1R6F).

4.2 The Use of hiPSC in Cardiotoxicity Testing

One of the criticisms of using hiPSC-CMs is the fact that they are in embryonic state of development and additional work needs to be

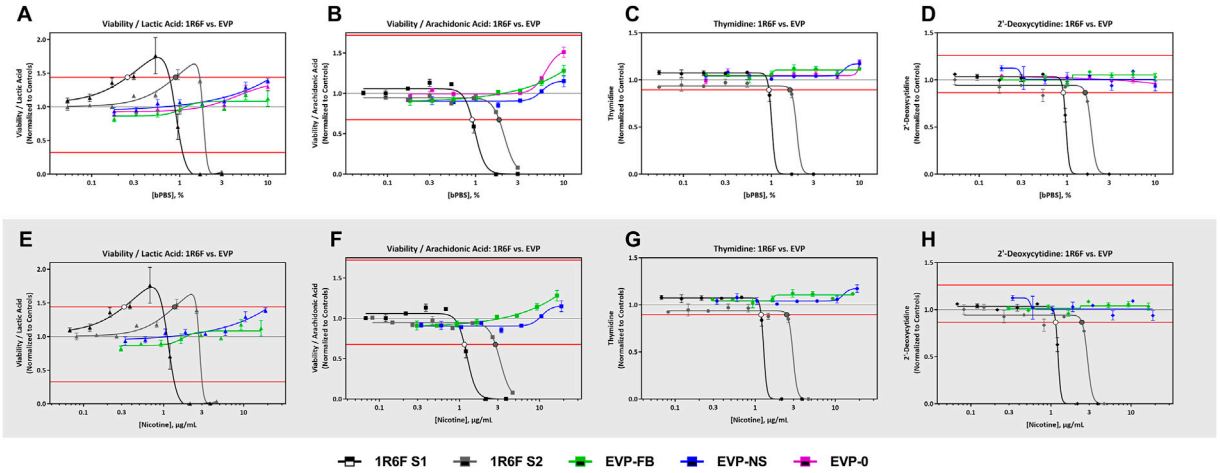

FIGURE 4 | 1R6F vs EVPs Cell Viability/Lactic Acid, Cell Viability/Arachidonic Acid Thymidine, and 2′-Deoxycytidine Response Comparison. The x-axis is the concentration (% bPBS) of the test article **(A–D)** or nicotine (µg/mL) **(E–H)**. The y-axes are the reference treatment normalized (fold change) values for each metabolite/ratio. The horizontal red lines represent the metabolite-specific prediction thresholds. The black bordered circles represent the nicotine concentration where the dose-response curve crossed the metabolite-specific prediction threshold.

done to fully understand the predictive value of this system in the context of preclinical cardiac safety risk assessment. Issues relating to metabolic, structural, and functional maturation are incompletely understood and any of these factors could result in the inconsistent prediction of toxicity across diverse classes of drug candidates when used to screen for new drugs (Pang et al., 2019). However, despite these limitations, due to the increasing prevalence of cardiovascular diseases and the increasing complexity of human exposures to chemicals, it is even more important than ever to consider and adequately test for the potential adverse cardiovascular effects of pharmaceuticals and environmental compounds. However, for drugs and other chemicals, the currently used animal models for cardiovascular endpoints do not provide sufficient information to meet both the high throughput required and human relevance needed, to be able to lower the preventable risks of cardiovascular disease from chemicals (Burnett et al., 2021). The cells used in this assay by Stemina are more metabolically aligned with adult cardiomyocytes, using oxidative phosphorylation instead of glycolysis, which is particularly important as the assay's cardiotoxicity model is based on changes in the metabolism of key metabolites important for cardiomyocyte function, including lactic acid. Previous research has shown that hiPSC-CM cultured in media containing glucose have a mid-fetal state of energy metabolism, generating ATP primarily through glycolysis (Rana et al., 2012; Correia et al., 2017) however, when the media contains galactose, hiPSC-CM energy metabolism shifts to an oxidative phosphorylation energy source more similar to adult cardiomyocytes (Rana et al., 2012; Correia et al., 2017).

4.3 Predictive Metabolites

The four metabolites measured in the Cardio*qP* assay have key roles in modulating oxidative stress, mitochondrial function, and mitochondrial replication, which have been experimentally and clinically associated with cardiotoxicity (described in Palmer et al., 2020) (Adlam et al., 2005; Palmer et al., 2020).

- Lactic acid is known to be associated with cardiotoxicity, especially during cardiac ischemia and heart failure (Sachinidis, 2020). Increased levels of lactic acid in the spent media of hiPSC-CMs signals a change from aerobic respiration to anaerobic respiration and glycolysis.
- 2′-deoxycytidine and thymidine are two of the principal DNA nucleosides and key components of the pyrimidine metabolism pathway. Changes in the metabolism of these metabolites is indicative of mitochondrial toxicity, which is an underlying mechanism of cardiotoxicity (reviewed in Varga et al., 2015)
- Arachidonic Acid is a polyunsaturated, essential fatty acid that is involved in lipid transport, metabolism, and fatty acid metabolism. Arachidonic acid release and oxygenation by enzyme systems leads to the formation of eicosanoids, which is an important group of inflammatory mediators and contributes to the pathogenesis of cardiovascular disease (Sonnweber et al., 2018).

Palmer et al. (2020) tested a panel of known functional and structural cardiotoxicants. In that study, functional cardiotoxicants generally "*caused an increase in lactic acid independent of* (*or prior to*) *changes in the other biomarkers*," whereas "*changes in arachidonic acid, 2′ deoxycytidine, and thymidine prior to changes in lactic acid occurred following exposure to many of the structural cardiotoxicants tested.*" Lactic acid and arachidonic acid are present in the cell culture medium, the change detected indicates that they were secreted (response >1) or taken-up (response <1) by hiPSC-CMs. Thymidine and 2′deoxycytoinde were not media components, therefore elevated levels in response to test article treatment

indicates an increase in the amount secreted by hiPSC-CMs, whereas a decrease indicates that lower levels were secreted.

4.4 Nicotine

Like other mild stimulants, it has been reported that nicotine can cause transient cardiovascular effects in humans at physiological levels. Nicotine acts by binding to nicotine acetylcholine receptors, activating the sympathetic nervous system which leads to vasoconstriction and increases in heart rate, blood pressure, and myocardial contraction (Benowitz and Burbank, 2016). In this study, we assessed whether neat nicotine alters any of the key metabolites assessed in this assay. Low concentrations of nicotine (0.01–10 μg/mL) did not alter the four metabolites; however, all four metabolites were impacted at higher concentrations. Lactic acid was the most sensitive metabolite measured causing a dose-dependent increase at ≥10 μg/ml (**Supplementary Figure S1**). This change was prior to the other biomarkers, therefore, as mentioned previously (*Predictive Metabolites*), at high concentrations (supraphysiological), and under the conditions of test, neat nicotine may have functional cardiotoxic potential. Winbo et al. (2020) demonstrated that when hiPSC-CM monocultures were exposed to nicotine (1 μM = 0.162 μg/mL), the functional endpoint, beating rate, significantly decreased indicating that nicotine can modulate the contractility function of hiPSC-CM (Winbo et al., 2020).

Nicotine exposure induced a small increase in thymidine and 2′-deoxycytidine, indicating that nicotine may cause mitochondrial toxicity at higher concentrations. A recent study by Jia et al. (2020) found that chronic nicotine exposure impaired mitochondrial function, resulting in intrinsic apoptosis, which is consistent with the changes observed in this study prior to decreased cell viability.

Neat nicotine was predicted to be cardiotoxic at 80 μg/mL (493 μM, see **Table 6**) in this specific assay, however the cTP was approximately 5000 times higher than the C_{max} observed after smoking a cigarette (16 ± 9 ng/mL) in adult smokers (D'Ruiz et al., 2015). Based on the results of this study, nicotine is not predicted to be cardiotoxic at physiological levels achieved after smoking a cigarette or use of the assessed NGPs. Moreover, the cTP was not considered relevant *in vivo* as the predicted dose was supraphysiological, and these blood concentrations could not be reached during normal use of the products. The high cTP predicted (80 μg/mL) also suggest that nicotine was not the predominant driver of cardiotoxicity observed for 1R6F (S1 and 2) and HTP (cHTP, pHTP 1 and 2) bPBS as the nicotine concentration tested did not exceed 14 μg/mL.

4.5 Potential Cardiotoxicity Screening for Reference Cigarettes When Compared to NGPs

As reported above, 1R6F altered hiPSC-CM metabolism at lower concentrations and exhibited a different metabolic response compared to either the HTP or EVP variants (**Figures 3**, **4**). The 1R6F bPBS samples caused a significant decrease in all metabolites, with changes to viability/lactic acid being observed prior to changes in other metabolites. Lactic acid is known to be associated with cardiotoxicity, especially during cardiac ischemia and heart failure (Sachinidis, 2020). The 1R6F bPBS potentially acted as a functional cardiotoxicant, based on the predictions of Palmer et al. (2020); however, it is important to note that most of the drugs known to cause functional cardiotoxicity initially caused an increase in lactic acid prior to changes in cell viability, in contrast to the decrease observed here. A similar trend was also seen for the HTP samples but importantly at much higher bPBS concentrations (10-fold). Additionally, these changes in viability/lactic acid were not the same pattern observed with neat nicotine. For nicotine there was no initial uptake of lactic acid at lower concentrations. The cTP of nicotine was 80 μg/mL indicating that nicotine was not the driver of the cardiotoxic potential observed in this assay, and likely it is other smoke constituents generated by tobacco combustion that drive cardiotoxicity. The 1R6F and cHTP bPBS samples both caused a decrease in viability/arachidonic acid at higher concentrations, whereas both the pHTP bPBS samples did not impact this ratio at the concentrations evaluated in this study. The 1R6F bPBS decreased thymidine at concentrations 5–10 times lower than the HTP samples. Lastly, 1R6F and cHTP decreased 2′-deoxycytidine, with cHTP being 4-8-fold less potent than 1R6F. The alterations of these two amino acids thymidine and 2′-deoxycytidine at lower concentrations for 1R6F vs HTP could reflected the higher oxidative stress reported for 1R6F vs HTP and associated mitochondrial toxicity (Malinski et al., 2018).

The three HTP bPBS samples were tested in two separate studies: cHTP in study 1 and pHTP 1 and 2 in study 2. Overall, the cHTP bPBS appeared more potent than both pHTP samples for all endpoints and had a slightly lower cTP concentration; however, the concentrations predicted to be cardiotoxic were within 3-fold of each other and therefore not considered to be biologically different based on expected variability of the assay (Palmer et al., 2020, personal communication, 14th June 2021). Importantly, long term *in vivo* and clinical studies with adult smokers that have compared commercialised HTPs with combustible cigarette smoke exposure, have shown significant improvements in cardiovascular endpoints and related biomarkers upon switching from combustible cigarettes to HTPs (Sakaguchi et al., 2014; Ogden et al., 2015; Smith et al., 2016; Schaller et al., 2016b; Schaller et al., 2016a; Pratte et al., 2017; Gale et al., 2019; Phillips et al., 2019; Shein and Jeschke, 2019; Tran et al., 2020; Wong et al., 2020). A recent systematic review of the health effects of HTP by Zynk et al. (2021) reported favourable improvements in biomarkers related to cardiovascular and respiratory endpoints when comparing continued combustible cigarette smoking adult smokers who switched to using HTPs (Znyk et al., 2021). Improvements in clinically relevant risk markers, especially cholesterol, sICAM-1, 8-epi-PGF2α, 11-DTX-B2, HDL and FEV1 have been observed when adult smokers switched away from combustible cigarettes to HTPs (Zynk et al., 2021).

All three EVP bPBS test samples (EVP-0, EVP-FB, and EVP-NS) did not cause a metabolic response indicative of cardiotoxicity potential at the highest exposure level tested in the current study (10% bPBS) (**Figure 2** and **Table 5**). This may not mean that these products are benign in all biological systems, but the levels of chemicals within the complex mixtures delivered to the cells in this study were not high enough to elicit a response.

However, the EVP samples have been demonstrated as potentially reduced risk for this *in vitro* endpoint when compared to the responses seen from HTP and 1R6F samples. An increase in cell viability/arachidonic acid ratio was observed which was the opposite to that seen for 1R6F and HTP bPBS samples (**Supplementary Figures S2, S3**), suggesting that 1R6F and HTP bPBS may alter the metabolism of hiPSC-CMs differently to that of EVP bPBS. A cross-comparison of the prediction model dose-response curves between the three EVP bPBS identified a significant difference between EVP-FB and the other two EVP samples; however, EVP-NS and EVP-0 were not significantly different.

Malinska et al. (2018) compared 3R4F and THS2.2 (IQOS) Total particulate matter (TPM) exposed to BEAS2B cells for up to 12 weeks, looking for markers of oxidative stress. The authors reported that the amount of TPM that needed to cause a similar extent of oxidative stress and changes in cellular mitochondrial energy production, was 20-fold higher for THS 2.2 (150 μg/mL vs 7.5 μg/ml 3R4F). TPM from THS2.2 at this higher concentration exerted similar effects on protein carbonylation; mitochondrial superoxide levels, and levels of GPx1. Gene expression analysis was also reported to show similar changes in expression patterns of key oxidative stress response genes in BEAS2B cells exposed to either 7.5 μg/mL TPM from reference 3R4F or 150 μg/mL of TPM from THS2.2 aerosol. In the current study, for the three HTP products there was approximately a 10-fold decrease in response when compared to the response to 1R6F. This may also reflect the potential reduction of oxidative stress seen with these products. In this study, The comparison of aqueous extracts was made using 56 puffs of 1R6F vs 120 puffs for the NGPs. This equates to a difference of 20-fold when the different puff numbers are taken in to account. Munakata et al. (2018) also reported reductions in the GSH/GSSG ratio observed in BEAS2B cells exposed to aqueous extracts (AqE) of an HTP product, NTV, and EVP exposures at higher effective concentrations than with observed reductions following 3R4F exposure. This reduction in GSH/GSSG followed the same trend of relative product effects as seen in the current study: 3R4F > HTP > EVP in terms of antioxidant activity.

Oxidative stress and activation of the NRF2/antixodiant response element (ARE) pathways are known to be related to inflammatory responses (Gilmour et al., 2003) and have been seen in human bronchial epithelial cells exposed to cigarette smoke (Taylor et al., 2016). Cigarette smoke AqE exposure has been reported to cause a dose-dependent decrease in the ratio of reduced glutathione to oxidized glutathione (rGSH/GSSG) together with an increased translocation of Nrf2 to the nucleus demonstrated by Western blot analysis. Knock down of the Nrf2 pathway by siRNA completely blocked cigarette smoke AqE -induced IL-8 cytokine release (Lau et al., 2012). In a paper by Czekala et al. (2021), it was demonstrated that repeated exposure of NHBE cells to undiluted EVP aerosol at 30, 60 or 90 puffs for 28 days puffs saw no elevation of IL-8 levels, compared to the strong IL-8 response observed to 90 puffs of 1:17 diluted 3R6F smoke. These relative effects of the two different product categories are in line with the findings of the current study.

Reduced cardiovascular effects have also been reported in longer term *in vivo* and clinical studies with people switching from cigarettes to EVPs, including substantial reductions in biomarkers of exposure to toxicants (including cardiovascular toxicants) in adult smokers who switch from combustible cigarettes to EVP (O'Connell et al., 2016; National Academies of Sciences et al., 2018; Hasan et al., 2020; Abouassali et al., 2021). Moreover, a recent British Heart Foundation funded clinical study found that adult smokers who switched to EVP for 1 month showed favourable improvements in cardiovascular endpoints with significant improvements in endothelial function, arterial stiffness and systolic blood pressure compared with continuing smoking (George et al., 2019). Zynk et al. (2021) stated that HTP has reduced cytotoxicity when compared to smoking but no effect on cytotoxicity was seen with EVPs, which is what was seen with the cell viability/lactic acid graphs. Zynk et al. (2021) reported that cardiovascular risk associated with EVP use is lower than risk associated with combustible cigarette smoking, but may be a higher risk for people with a predisposal cardiovascular disease (Znyk et al., 2021). The results from the present study would support this.

4.6 Use of Cardio*qP* Assay as Part of a Weight of Evidence Approach

This assay was implemented as part of a wider framework of pre-clinical *in vitro* toxicity testing (see Czekala et al. (2021) for a brief outline), which is aligned with the principles of the 3Rs and the 21st Century Toxicology framework, which aim to minimise, or remove where possible, experimental animal use (Berg et al., 2011; Rovida et al., 2015). The framework aims to increase human relevance where possible by using human-derived cell (primary or immortalised) -based *in vitro* assays. These assays include, for example, the DiscoverX BioMap platform to screen for human smoking-related disease relevant biomarkers (Simms et al., 2021). This assay uses an extensive panel of human primary cells, including single cell and co culture systems, and changes in molecular pathways linked to cardiovascular toxicity can be inspected. In the Simms et al. (2021) study, reductions in (cardio) toxicity readouts were observed for NGPs when compared to combustible cigarettes. Another cardiovascular toxicity-related assay within the framework is the scratch wound assay, which looks at the rate of repair of human umbilical endothelial cell layers after a "wound" is cut, and can indicate wound healing rates following exposure to NGP samples relative to combustible cigarette smoke extracts (Simms and Trelles, 2019; Chapman et al., 2021). Results need to be confirmed with human clinical studies, which include assessment of short term safety following use of the test products, including cardiovascular parameters (O'Connell et al., 2019; Chapman et al., 2021; Morris, 2021 under review). Looking at the data from such pre-clinical and clinical assessments in combination can provide weight of evidence, which can be further compared to the scientific literature, to draw conclusions on the potential effects of NGPs relative to combustible cigarette smoking. Observation of the same trends across multiple studies gives scientists more confidence in the reduced risk potential of NGPs when compared to combusted products. It is believed that the

Cardio*qP* assay adds significantly to the battery of information that we collect on potential cardiotoxic effects of NGPs, and in comparison to combustible cigarette.

4.6 Study Limitations

The results of the present study should be viewed within the context of its limitations. The prediction model is currently not able to differentiate functional from structural cardiotoxicity potential with complex mixtures, which may be partially attributed to mixtures having both functional and structural cardiotoxic elements to their toxicity profiles. To date this model has had limited use with complex mixtures. To ensure reproducibility, and therefore valid outcomes in the assay, standardised (ISO) puffing regimes and fixed puff numbers were implemented across studies 1 and 2. All studies were not conducted at the same time, however, the relative difference between the 1R6f and HTP, cTP values were consistent across the studies. This indicates reproducibility of the data outputs for this assay. Furthermore, for trapping of smoke/aerosols, it is difficult to find an ideal medium which could both trap smoke constituents as well as aerosol particulates and gas phase, and be able to be added to the cell culture. For that reason, bPBS was chosen as this could be used in a range of cell culture assays; however its limitations are acknowledged, including the reduced trapping potential of hydrophobic compounds (Smart and Phillips, 2021). It is also important to note that no single *in vitro* assay can fully model the biological complexity of cardiovascular function and disease. However, the Cardio^{qP} assay is a useful screen for short term cardiotoxicity and should complement other pre-clinical and clinical techniques for determining the potential cardiovascular impact of NGPs relative to that seen for combustible cigarettes.

5 CONCLUSION

The aim of the present study was to predict the cardiotoxicity potential of NGPs when compared to cigarettes in a human relevant cardiac cells and to demonstrate the utility of this assay as a potential screening tool for this endpoint. Burnett et al. (2021) stated that the use of hiPSC-CMs fills a critical gap where no routine testing for cardiotoxicity is currently performed (Burnett et al., 2021). The Cardio*qP* assay, which is human relevant, low cost, and high-throughput, was able to detect potential cardiotoxic effects of both the combustible cigarettes samples and all the HTP products when hiPSC-CMs were exposed to bPBS trapped samples for 72 h. However, the cTP responses to the 1R6F bPBS samples were significantly greater, with combustible cigarettes eliciting this response at concentrations 10-fold lower than for HTP. HTP bPBS samples also had a different metabolite response profile when compared to 1R6F bPBS, indicating these non-combustible tobacco-containing products are a different product category to combustible cigarettes. All the EVP bPBS samples were negative for cardiotoxic potential in the assay when tested at concentrations up to 10% bPBS under the assay conditions, this does not mean that they are benign but that the concentrations tested were not reactive enough in this assay. The testing of neat nicotine in isolation suggests that due to the much higher concentrations required, the observed effects following exposure to 1R6F smoke and HTP aerosol samples were not driven by nicotine but more likely by other constituents present in cigarette smoke or HTP aerosol. The nicotine cTP was approximately 100–200 times higher than the cTP 1R6F and 9–13 times higher than cTP for HTPs. In summary, the Cardio*qP* assay was able to differentiate between the these three product categories. Overall, the outcomes of the present study add to this growing body of scientific evidence indicating that NGPs have the potential to be less harmful to the cardiovascular system than continued combustible cigarette smoking and in terms of cardiotoxic potential in this assay Clearly no one *in vitro* assay can model all aspects of cardiovascular disease. However, this assay adds to the information we hold on the *in vitro* toxicity of an acute exposure to bPBS of 1R6F, HTP, and EVP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LS, FY, and KR contributed to conception and design of the study. ES generated samples for testing. JP performed the statistical analysis. LS, FY JP, and KR wrote the first draft of the manuscript reviewing and editing. MS acquired the necessary internal funding. All authors contributed to manuscript revision, read, and approved the submitted final version."

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2022.747508/full#supplementary-material

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Application of Defined Approaches for Skin Sensitization to Agrochemical Products

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Skin sensitization testing is a regulatory requirement for safety evaluations of pesticides in multiple countries. Globally harmonized test guidelines that include *in chemico* and *in vitro* methods reduce animal use, but no single assay is recommended as a complete replacement for animal tests. Defined approaches (DAs) that integrate data from multiple non-animal methods are accepted; however, the methods that comprise them have been evaluated using monoconstituent substances rather than mixtures or formulations. To address this data gap, we tested 27 agrochemical formulations in the direct peptide reactivity assay (DPRA), the KeratinoSens™ assay, and the human cell line activation test (h-CLAT). These data were used as inputs to evaluate three DAs for hazard classification of skin sensitization potential and two DAs for potency categorization. When compared to historical animal results, balanced accuracy for the DAs for predicting *in vivo* skin sensitization hazard (i.e., sensitizer vs. nonsensitizer) ranged from 56 to 78%. The best performing DA was the "2 out of 3 (2o3)" DA, in which the hazard classification was based on two concordant results from the DPRA, KeratinoSens, or h-CLAT. The KE 3/1 sequential testing strategy (STS), which uses h-CLAT and DPRA results, and the integrated testing strategy (ITSv2), which uses h-CLAT, DPRA, and an *in silico* hazard prediction from OECD QSAR Toolbox, had balanced accuracies of 56–57% for hazard classification. Of the individual test methods, KeratinoSens had the best performance for predicting *in vivo* hazard outcomes. Its balanced accuracy of 81% was similar to that of the 2o3 DA (78%). For predicting potency categories defined by the United Nations Globally

Abbreviations: 2o3, 2 out of 3; AOP, adverse outcome pathway; DA, defined approach; DMSO, dimethyl sulfoxide; DPRA, direct peptide reactivity assay; EC1.5, concentration of test substance increasing luciferase activity by 1.5 fold; EC150, concentration of a test substance inducing a relative fluorescence intensity of 150% for CD86; EC200, concentration of a test substance inducing a relative fluorescence intensity of 200% for CD54; FN, false negative; FP, false positive; GHS, United Nations Globally Harmonized System of Classification and Labelling of Chemicals; h-CLAT, human cell line activation test; IATA, integrated approaches to testing and assessment; ITS, integrated testing strategy; KE, key event; LLNA, murine local lymph node assay; MIT, minimum induction threshold; OECD, Organisation for Economic Co-operation and Development; PBS, phosphate-buffered saline; STS, sequential testing strategy; TN, true negative; TP, true positive.

Harmonized System of Classification and Labelling of Chemicals (GHS), the correct classification rate of the STS was 52% and that of the ITSv2 was 43%. These results demonstrate that non-animal test methods have utility for evaluating the skin sensitization potential of agrochemical formulations as compared to animal reference data. While additional data generation is needed, testing strategies such as DAs anchored to human biology and mechanistic information provide a promising approach for agrochemical formulation testing.

Keywords: adverse outcome pathway, alternatives to animal testing, chemical allergy, defined approaches, new approach methodologies, skin sensitization, agrochemicals

1 INTRODUCTION

Safety assessment of agrochemicals, either as single active ingredients or end-use product formulations, which are typically multiconstituent substances with defined compositions, requires extensive testing for hazard characterization and risk assessment purposes. In the agrochemical sector, acute toxicity testing is usually required for hazard classification used to develop appropriate labeling for safe transport, handling, and use (Corvaro et al., 2017). Studies performed for the safety assessment of agrochemical formulations are referred to colloquially as the acute toxicity "6-pack," which includes tests for acute systemic toxicity by the oral, dermal, and inhalation routes; and acute topical toxicities of eye and skin irritation and skin sensitization. Data from these tests are required according to international guidance on chemical safety such as those issued by the United Nations (UN, 2021a; UN, 2021b), which are implemented regionally. Investigating skin sensitization potential is one of the mandatory global requirements for agrochemical formulations that influences labeling and, more recently, quantitative risk assessment (UK, 2008; Sanvido et al., 2018).

Traditionally, guinea pig methods, such as the Buehler or the adjuvant-based maximization test, were used for evaluating sensitization potential and both are included in Organisation for Economic Co-operation and Development (OECD) test guideline (TG) 406 (OECD, 1992). However, the methods described in TG 406 were never formally validated and provide limited information on potency (Van Loveren et al., 2008). They also incorporate experimental procedures that have ethical implications for animal welfare, including a sensitization phase that may use an adjuvant and final elicitation of an adverse allergy event. These limitations, as well as concern for the sensitivity of the assays, have resulted in their reduced acceptance in some jurisdictions specifically for some regulatory contexts within the European Union (e.g., EU for REACH), although they are still preferred in others (e.g., Asian and South Pacific countries) (Daniel et al., 2018). An alternative to the guinea pig methods, the murine local lymph node assay (LLNA) (OECD, 2010a), is the most commonly used *in vivo* method for skin sensitization testing required for agrochemical formulations. The LLNA uses fewer animals and has a shorter exposure duration than the guinea pig methods. Because it measures an early event of the sensitization phase, it reduces the pain and distress that would be produced by an adverse skin reaction. Since the assay measures lymph node T-cell proliferation, it also provides a quantitative assessment of potency. In the United States, the LLNA was formally recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods as a reduction and refinement alternative to the guinea pig test methods (NIH, 1999). The LLNA was internationally accepted via OECD TG 429 (OECD, 2010a), and non-radiolabel modifications were later adopted in TG 442A and TG 442B (OECD, 2010b; OECD, 2014).

Despite the reduction and refinement advantages of the LLNA, it has a number of known limitations, which include false negatives for some metals and clinically relevant allergens and false positives for known irritants (NIH, 2011; Roberts et al., 2016a; Roberts et al., 2016b; Roberts et al., 2016c; Roberts and Api, 2018). These limitations, combined with the greater number of animals required for skin sensitization testing relative to other 6-pack endpoints, have motivated the development and use of non-animal alternatives. Furthermore, advances in knowledge of the mechanisms for skin sensitization makes the use of established alternative methods to address the skin sensitization endpoint an achievable milestone in the overall goal to reduce and ultimately replace animal use in the agrochemical space.

The adverse outcome pathway (AOP) for skin sensitization initiated by covalent binding of chemicals, including agrochemicals, to skin proteins is well-characterized and has been described in an OECD monograph (OECD, 2012). The AOP includes four key events that must proceed for a skin sensitization response to develop. Key event 1 (KE1) is the formation of protein adducts via covalent bonding of a chemical to amino acids in skin cells. KE1 is addressed by three *in chemico* assays described in TG 442C (OECD, 2021c). These assays measure the reactivity of chemicals with synthetic peptides containing lysine and cysteine, which can be used to discriminate between sensitizers and nonsensitizers. Key event 2 (KE2) is the induction of an inflammatory response in keratinocytes, the most common cells in skin. The KeratinoSens and LuSens assays described in TG 442D (OECD, 2018a) address KE2 by measuring the induction of a transfected luciferase gene under the control of the antioxidant response element in a keratinocyte cell line. KE3, dendritic cell activation, is addressed by the human cell line activation test (h-CLAT) and U937 cell line activation test, U-SENS™, covered

in TG 442E (OECD, 2018b). These tests measure changes in the levels of T-cell surface costimulatory proteins CD86 and CD54 as an indicator of dendritic cell activation. Key event 4 (KE4) is the proliferation of T-cells, which depends on activation of keratinocytes (KE2) and/or dendritic cells (KE3) and is measured by the LLNA. Currently, there are no accepted non-animal alternatives to address KE4.

While internationally accepted, the TGs that measure the first three KEs are not designed to be "stand-alone" replacements for *in vivo* assays because the individual outcomes are not necessarily predictive of the overall adverse outcome of skin sensitization. However, this limitation can potentially be overcome by assessing the totality of available experimental and expert-based evidence within the context of integrated approaches to testing and assessment (IATA). However, use of IATA may require expert judgment to reach a conclusion on skin sensitization potential. In addition, the conclusion reached using IATA may not be considered harmonized under the OECD Mutual Acceptance of Data agreement, whereby data developed for a regulatory program in one country is also acceptable in other OECD member countries (OECD, 1981). These limitations may be overcome by the application of defined approaches (DAs). A DA consists of a fixed data integration procedure applied to a defined combination of test results with no expert judgment needed to interpret the outcome (OECD, 2017). Increased acceptance of DAs has resulted in their recent implementation in an OECD guideline, Guideline 497 (OECD, 2021b), that is covered by the Mutual Acceptance of Data agreement. This guideline incorporates three specific DAs. The "2 out of 3" (2o3) DA relies on two concordant tests for hazard classification from the direct peptide reactivity assay (DPRA), KeratinoSens, and h-CLAT. The guideline also describes two versions of an integrated testing strategy (ITS) that uses h-CLAT, DPRA, and an *in silico* hazard prediction. ITSv1 uses Derek Nexus (Lhasa Limited) for the *in silico* prediction and ITSv2 uses OECD's QSAR Toolbox. Both ITSv1 and ITSv2 include a scoring system that can be used to classify substances into the potency categories of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (UN, 2021a).

With the introduction of OECD Guideline 497 on DAs for skin sensitization testing (OECD, 2021b), there is a need for international harmonization in the testing of agrochemical formulations using individual *in vitro* test methods and DAs. For example, in 2018, the United States Environmental Protection Agency started accepting predictions from two DAs for hazard classification of monoconstituent substances: the 2o3 and the KE 3/1 sequential testing strategy (STS) (US EPA, 2018). However, most pesticides are sold as formulated products rather than single compounds. Coformulant compounds range in their purpose, but can include active ingredients, emulsifiers, wetting agents, stabilizers, antimicrobials, safeners, adjuvants, solvents, diluents, binders, fertilizers, and clays. A recent survey described more than 60 agrochemical formulation types, including mixtures of multiple formulation types (Croplife International, 2017). The net result is that most agricultural products come in a variety of complex compositions, which can be roughly divided into liquid solvent-based, liquid water-based, and solid formulations. The *in vitro* tests available to address KE1, KE2, and KE3 have not been validated with complex mixtures, and verification of the applicability domain for agrochemical mixtures has been limited (Settivari et al., 2015). Currently, OECD Guideline 497 is restricted in applicability to monoconstituent substances and excludes formulation products due to lack of data characterizing the reliability of applying DAs to such mixtures (OECD, 2021b).

The purpose of this research was to investigate a potential extension of the applicability domain of three accepted non-animal OECD test methods, the DPRA (TG 442C), the KeratinoSens (TG 442D) and the h-CLAT (TG 442E) and three DAs, the 2o3, STS, and ITSv2, to complex mixtures by testing a balanced selection of agrochemical formulations with known skin sensitization potential. Demonstration of the applicability of these testing approaches to such materials would be expected to support their greater use for agrochemical product registration and result in a decrease in the use of animals for this purpose.

2 MATERIALS AND METHODS

2.1 Formulations Tested

A total of 27 agrochemical formulations were provided to the National Toxicology Program by Corteva Agriscience for evaluation of sensitization potential using *in vitro* approaches. *In vitro* testing was performed by Corteva Agriscience (DPRA and KeratinoSens) and Burleson Research Technologies, Inc. (h-CLAT). These commercial formulations were sourced from several manufacturing sites globally. All formulations were liquids and were selected to cover the most commonly used formulation types. Specifically, 13 formulations were water-based liquids (six suspension concentrates and seven soluble liquids) and 14 were solvent-based (nine emulsion concentrates, three oil dispersions, and three emulsions in water). The complete compositions of these formulations are proprietary and cannot be disclosed. However, identity and percentage weight of each active substance and other selected composition information is reported in **Supplementary File 1**. For the purposes of this publication, the formulations are coded.

As described in the following section, formulations were also selected to provide a balanced representation of nonsensitizers and sensitizers.

2.2 *In Vivo* Reference Data

No new animal tests were conducted to obtain *in vivo* reference data. Instead, data were compiled from previously conducted *in vivo* animal skin sensitization studies used for registration purposes. These data, provided in **Supplementary File 2**, served as the reference data for classification of individual test materials according to GHS skin sensitization potency categories (UN, 2021a). All *in vivo* assays were considered valid; the positive control substances tested in these assays were within expected ranges.

LLNA studies compliant with OECD TG 429 and conducted between 2005 and 2012 were available for 18 formulations (7 positive, 11 negative). CBA/J mice were used for 15/18 formulations and BALB/c mice were used for 3/18

formulations. Formulations were applied to the ears of the mice in 1% L92 solution in water, except for one formulation, Dow1, for which propylene glycol was used as the vehicle. The vehicle of 1% L92 is recommended by the TG and is preferred for agrochemical formulations because it is water-based and simulates the common use condition of dilution with water for distribution via spray tanks. All studies were preceded by an irritancy screening for appropriate selection of the maximum dose. The dose spacing was not always compliant with the recommended dose spacing in TG 429, but this deviation from the guideline did not impact determination of potency. EC3 values, the effective concentration that produced a stimulation index of three, the threshold for a positive response, were interpolated using response data above and below the stimulation index of three. There was no need to rely on an extrapolation procedure which makes assumptions about the slope of the dose-response curve beyond the measured data.

Data from guinea pig maximization tests conducted between 1998 and 2009 in compliance with OECD TG 406 were available for five formulations (two positive, three negative) (OECD, 2021a). Each test included preliminary irritancy screens via both intradermal injection and epidermal application routes. The intradermal induction included Freund's Complete Adjuvant and the epidermal induction used sodium lauryl sulfate.

Data from Buehler tests conducted between 1989 and 2000 in compliance with OECD TG 406 were available for four formulations (three positive, one negative) (OECD, 2021a). Tests included a preliminary irritancy screen and a three-induction experimental scheme.

When multiple tests where available for the same formulation, preference was given to LLNA data over guinea pig data and to guinea pig maximization test data over Buehler test data. LLNA is preferred because it provides a quantitative response measurement and a dose-response assessment. Two formulations had a negative result in the LLNA, with a concordant negative Buehler (Dow2) or guinea pig maximization test (Dow25). One formulation with a positive LLNA but a discordant negative Buehler test was assigned an overall classification of positive (Dow9).

Of the 27 formulations, 15 were GHS Not Classified (nonsensitizers), 11 were GHS Category 1B (other than strong) sensitizers and one was a GHS Category 1A (strong) sensitizer. Note that severe sensitization is a rare outcome in agrochemical formulations (Corvaro et al., 2017).

Finally, no human experimental data (i.e., skin sensitization patch tests) were available for any of the formulations tested. Adverse reporting data were reviewed; however, due to the sparsity of data, no evidence of skin sensitization reactions by any of the formulations in this paper could be inferred. Hence, reference classification was solely based on evidence from existing animal assays.

2.3 Individual Non-Animal Methods for Skin Sensitization

For the *in vitro* assays, the formulations tested were considered to have a purity of 100% and a density of 1 g/ml.

2.3.1 Direct Peptide Reactivity Assay (DPRA)

DPRA predicts the molecular initiating event, KE1, in the AOP for dermal sensitization. The assay was performed at Corteva Agriscience facilities according to TG 442C (OECD, 2021c) with few modifications. While TG 442C considers DPRA to be technically applicable for testing multiconstituent substances and mixtures, testing at the specified molar concentration (100 mM) is not possible. Therefore, we used a modified approach wherein the doses were based on considering each formulation as a single entity rather than as a mixture of multiple components. In this approach, a common molecular weight (MW) of 400 Da was assumed for each formulation, consistent with the approximate MW of the agrochemical active ingredients tested in the present study. The same approach was followed in a recent study testing agrochemical formulations in an *in vitro* KeratinoSens assay (Settivari et al., 2015). The use of a *pro forma* molecular weight for substances with no defined molecular weight was originally proposed in the KeratinoSens protocol (ECVAM, 2019) based on an average molecular weight of 200 Da for cosmetic ingredients.

The positive control used to assess run acceptance in these studies was 100 mM cinnamic aldehyde in acetonitrile. The purity of the synthetic peptides used in the assay, acetylated lysine (Ac-RFAKAA-COOH) or acetylated cysteine (Ac-RFAACAA-COOH) (Celtek Bioscience, Franklin, TN), was 98% or higher.

To conduct the assay, formulations were combined with the cysteine- and lysine-containing peptides at ratios of 1:10 and 1:50, respectively. Three replicates of these solutions were incubated for 24 h in the dark at 25 ± 2.5°C. The concentrations of the cysteine- and lysine-containing peptides were then measured using high performance liquid chromatography with gradient elution and UV detection at 220 nm. The average percent depletion of the cysteine- or lysine-containing peptides replicates was calculated by comparing the concentrations of solutions with and without the respective test materials. Then cysteine and lysine depletion values were averaged together. To confirm potential quantitative interference with the test compound in UV monitoring, we also assayed a preparation containing only test substance without cysteine or lysine peptide stock. Based on the OECD TG 442C criteria, we classified a test substance as positive if the average lysine/cysteine depletion was 6.38% or higher.

OECD TG 442C only describes UV determination of peptide depletion. To improve assay specificity, we also measured peptide depletion using in-line selected ion monitoring for mass spectra of both cysteine and lysine peptides after UV detection. This added step facilitated accurate peptide depletion measurements in the event of co-elution of test chemical and peptide as well as monitoring of cysteine and lysine peptide dimer formation. Intact peptide mass-to-charge ratios were monitored for both the cysteine and lysine peptides. Quantitation was performed on the [M+2H]2+ (376 Da) for the cysteine peptide, while the [M + H]+ (776 Da) was used for the lysine peptide. The retention time was observed to be approximately 12.8 min for the cysteine and 10.2 min for the lysine peptide standards. We report mass spectra results when UV results indicated test chemical interference with the peptide peak determination.

2.3.2 KeratinoSens Test Method

KeratinoSens is an *in vitro* skin sensitization assay addressing the AOP KE2 relevant to keratinocyte responses, including activation of inflammatory cytokines and induction of cytoprotective genes. The KeratinoSens cell line was kindly provided by Dr. Andreas Natsch (Givaudan Schweiz AG, Switzerland). The KeratinoSens test method was performed at Corteva Agriscience facilities as described by Settivari et al. (2015) and adopted by OECD in TG 442D (OECD, 2018a) with minor modifications. Consistent with our approach for the DPRA, a *pro forma* MW of 400 Da was assumed for each formulation. Each test substance was tested at 12 concentrations ranging from 0.4 to 800 μg/ml (instead of 0.2–400 μg/ml suggested in TG 442D). To enable comparison of KeratinoSens data for each formulation in relation to its corresponding active ingredient, the data are presented in μM units (i.e., 1–2,000 μM). The positive control used in these studies was a two-fold dilution in dimethyl sulfoxide (DMSO) of cinnamic aldehyde tested at five concentrations ranging from 4 to 64 μM. The negative control was complete Dulbecco's modified Eagle's medium supplemented with 9.1% fetal bovine serum and 0.55 mg/ml Geneticin® (GIBCO) with 1% DMSO. A no-cell blank was also included. Luminescence was measured with a FLUOstar® Omega (BMG LABTECH, Inc.) multidetection microplate reader to assess luciferase activity. The average maximum fold induction of luciferase activity observed at any concentration of the test substance and the positive control were determined, as well as the concentration of test material that increased luciferase activity to 1.5-fold (EC1.5). In addition, cell viability was determined using the MTT assay in which reduction of the yellow tetrazolium dye [3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product was assessed by measuring absorbance with a spectrophotometer. All agrochemical formulations were tested in two or more wells for each replicate and each experiment was repeated on at least two separate days (independent replicates). Each replicate was considered acceptable when all of the following criteria were met: 1) the positive control induced a dose-dependent increase in luciferase activity with $EC_{1.5}$ between 7 and 30 μM; 2) maximum luciferase induction at 64 μM was between 2- and 8-fold; and 3) average coefficient of variation of the luminescence reading for the solvent-control wells was less than 20%. A test substance was considered positive for skin sensitization when all of the following conditions were met:

- Average maximum fold induction of luciferase activity was at least 1.5-fold over the solvent control value.
- Cell viability was greater than 70% at the lowest concentration with induction of luciferase activity at greater than or equal to 1.5-fold.
- The EC1.5 value was less than 1,000 μM.
- There was a dose-dependent increase in luciferase induction.

2.3.3 Human Cell Line Activation Test (h-CLAT)

The h-CLAT is an *in vitro* skin sensitization assay addressing KE3 of the AOP for skin sensitization, the activation and mobilization of dendritic cells including induction of inflammatory cytokines and surface molecules leading to T-cell priming. The h-CLAT was performed at Burleson Research Technologies, Inc., according to OECD TG 442E (OECD, 2018b). The assay was conducted in the human monocytic leukemia cell line THP-1 and used flow cytometry to measure expression of CD86 (B7.2) and CD54 (intercellular adhesion molecule 1, ICAM-1) cell surface markers associated with dendritic cell activation. The positive control was 2,4-dinitrochlorobenzene prepared in DMSO and diluted to 4.0 μg/ml in culture medium. The negative control was culture medium with the appropriate solvent concentration added. Test substances were prepared based on solubility in either phosphate-buffered saline (PBS) or DMSO at final in-well concentrations up to 0.5% PBS or 0.1% DMSO. An eight-concentration dose range-finder cytotoxicity assay was conducted using propidium iodide staining to identify the concentration that resulted in 75% cell viability (25% cytotoxicity). If test substances prepared in PBS were not cytotoxic, the starting concentration was 0.5%. For the main assay, test substances were prepared in either PBS or DMSO at 100-fold (PBS) or 500-fold (DMSO) of 1.2 x the starting concentration producing 75% cell viability. Eight 1.2-fold dilutions in the appropriate solvents were made to obtain the stock solutions that were further diluted 50-fold (PBS) or 250-fold (DMSO) into the culture medium as working solutions, then diluted 2-fold in the plate to reach final in-well concentrations. CD86 and CD54 expression was measured by flow cytometry using fluorochrome-tagged antibodies. The relative fluorescence intensity for each marker, with respect to solvent controls, was determined at each of eight 1.2-fold dilutions of test material after a 24 h exposure to the test substance.

Each formulation was tested in at least two independent runs to derive a single result based on the CD86/CD54 expression levels. We considered a test substance to be positive if at least one of the following conditions were met in two independent runs:

- The relative fluorescence intensity for CD86 was greater than or equal to 150% in at least one tested concentration (with cell viability at least 50%).
- The relative fluorescence intensity for CD54 was greater than or equal to 200% in at least one tested concentration (with cell viability at least 50%).

For substances classified as positive, we determined the effective concentration that induced a relative fluorescence intensity of 150% for CD86 (EC150) and a relative fluorescence intensity of 200% for CD54 (EC200).

2.3.4 *In Silico* Hazard Predictions

Read-across predictions for skin sensitization hazard were generated using OECD QSAR Toolbox v4.5, which is freely available software (OECD, 2021d). The simplified molecular-input line-entry system (SMILES) specifications of chemical structure and Chemical Abstracts Service registry numbers (CASRNs) for each formulation's ingredients were used as inputs to QSAR Toolbox. Predictions for the ingredients were made using the automated workflow for "EC3 from LLNA or Skin sensitization from GPMT assays for defined approaches (SS AW for DASS)." If the automated workflow could not make a

prediction because an ingredient was a salt, the salt was dissociated, and the automated workflow was applied to the organic portion of the ingredient to make a prediction; Toolbox does not make skin sensitization hazard predictions for inorganic structures. The Toolbox predictions for the formulation ingredients were then used to classify each formulation. Specifically, if a formulation contained one or more ingredients that were positive and the concentration of a positive ingredient in the formulation was at least 0.1%, then the formulation was classified as positive per the GHS guidance for determining the sensitization potential of mixtures (UN, 2021a). Otherwise, the prediction was negative. The Toolbox prediction for a formulation was considered inconclusive if it contained ingredients with no Toolbox prediction and ingredients with negative predictions only. Toolbox does not provide predictions for ingredients with undefined structures (e.g., substances of unknown or variable composition, complex reaction products, or biological materials).

2.4 Defined Approaches (DA) for Skin Sensitization

Data from the DPRA, KeratinoSens, h-CLAT, and Toolbox were used as information sources for multiple DAs. DAs utilize results from multiple non-animal information sources to achieve a predictive capacity for human skin sensitization potential that is equal to or greater than that of animal tests (OECD, 2021b). A DA consists of a fixed data interpretation procedure (e.g., mathematical model or rule-based approach) applied to specific *in silico*, *in vitro*, or *in chemico* data with adequate information to make a prediction on skin sensitization potential without expert judgment. An advantage of DAs for skin sensitization is that they utilize assays that address multiple KEs in the AOP. The limitations of DAs are based on the limitations of the individual data sources or information on a specific test substance.

2.4.1 2 out of 3 DA (2o3)

The 2o3 DA is included in OECD Guideline 497 (OECD, 2021b), United States Environmental Protection Agency interim guidance (US EPA, 2018), and in European Chemicals Agency guidance (ECHA, 2021) for monoconsitutent substances, but is currently not accepted in the United Kingdom (UK HSE, 2021). It predicts skin sensitization hazard based on sequential testing, in no specific order, using the DPRA, KeratinoSens, and h-CLAT methods. The 2o3 DA requires concordant results from two assays to make a prediction. Thus, a test substance is classified as a sensitizer if the outcome in two assays is positive and negative if the outcome in two assays is negative. Borderline results, as defined in OECD Guideline 497 Annex 1, cannot be used as one of the two concordant tests.

We evaluated DPRA, KeratinoSens, and h-CLAT data to identify borderline results according to OECD Guideline 497 with small deviations. If one of the two concordant tests for the 2o3 was a borderline result, we considered the 2o3 prediction to be inconclusive. Because we did not have multiple independent DPRA runs for each substance, we used the single available run to evaluate whether it was a borderline result. For the KeratinoSens evaluation of borderline results, we used the average maximum fold induction of luciferase activity, EC1.5, and viability at the EC1.5. Statistical significance of the average maximum fold induction of luciferase activity compared with controls was not determined because the KeratinoSens data were generated before TG 442D was published and individual well data were no longer available. The evaluation of borderline results for h-CLAT adhered strictly to OECD Guideline 497 because these tests were recently performed, and all test data were available. Typically, negative h-CLAT results for substances with log Kow >3.5 cannot be used with confidence; however, log Kow is not applicable to mixtures such as the formulations we evaluated for this project.

2.4.2 KE 3/1 Sequential Testing Strategy (STS)

The STS is accepted in the United States Environmental Protection Agency interim guidance (US EPA, 2018), but is not included in OECD Guideline 497 (OECD, 2021b) or agrochemical guidance from the European Chemicals Agency (ECHA, 2021) or the United Kingdom (UK HSE, 2021). It was originally described by Nukada et al. (2013) and addresses KEs 1 and 3 in the AOP for skin sensitization using the DPRA and h-CLAT, respectively. The STS provides both skin sensitization hazard and GHS potency classification, as illustrated in **Table 1**. A test substance is evaluated initially in the h-CLAT using the minimum induction threshold (MIT), which is the lowest value of the EC150 for CD86 induction or the EC200 for CD54 induction. A positive h-CLAT with MIT less than or equal to 10 μg/ml is predicted to be a GHS 1A sensitizer, while a substance having a MIT between 10 and 5,000 μg/ml is predicted to be a GHS 1B sensitizer. A negative h-CLAT result requires testing in the DPRA. A positive DPRA result predicts a GHS 1B sensitizer whereas a negative DPRA result yields a negative outcome for the DA with the test substance being considered GHS Not Classified (i.e., a nonsensitizer).

2.4.3 Integrated Testing Strategy v2 (ITSv2)

The ITSv2 is included in OECD Guideline 497 (OECD, 2021b) but not in United States Environmental Protection Agency interim guidance (US EPA, 2018) or in those for agrochemical formulations from the European Chemicals Agency (ECHA, 2021) or the United Kingdom (UK HSE, 2021). The ITSv2 addresses KE3 of the AOP using h-CLAT and KE1 using DPRA (OECD, 2021b). The ITSv2, which predicts both skin sensitization hazard and GHS potency classification, also incorporates an *in silico* hazard classification prediction from QSAR Toolbox. We selected ITSv2 for evaluation over ITSv1 because the *in silico* input is from freely available software. ITSv1 requires *in silico* input from a proprietary source, Derek Nexus v6.1.0.

The data interpretation procedure for the ITSv2 is based on a DPRA score calculated using the mean percent depletion of lysine and cysteine peptides or of the cysteine peptide only (in case of co-elution with the lysine peptide); an h-CLAT outcome based on the MIT; and a QSAR Toolbox skin sensitization prediction (**Table 2**). The scores for the individual inputs are summed and used to predict the skin

TABLE 1 | The KE 3/1 sequential testing strategy (STS).

Testing Order	h-CLAT MIT (μg/mL)	DPRA	Hazard Classification	GHS Potency Classification
1st h-CLAT	≤10	–	Positive	1A
	>10 to 5000	–	Positive	1B
	Negative	–	2nd test in DPRA	–
2nd DPRA	–	Positive	Positive	1B
	–	Negative	Negative	NC

DPRA, direct peptide reactivity assay; h-CLAT, human cell line activation test; MIT, minimum induction threshold.

TABLE 2 | ITSv2 scoring system for individual information sources.

Score	h-CLAT MIT (μg/ml)	DPRA mean cysteine and lysine depletion (%)	DPRA cysteine depletion (%)[a]	Toolbox prediction
3	≤10	≥42.47	≥98.24	-
2	>10 to ≤150	≥22.62 to <42.47	≥23.09 to <98.24	-
1	>150 to ≤5,000	≥6.38 to <22.62	≥13.89 to <23.09	Positive
0	Not calculated	<6.38	<13.89	Negative

[a]*Cysteine-only depletion thresholds for DPRA are used in cases where (a) test substance co-elutes with the lysine peptide and (b) cysteine peptide depletion conforms to test acceptance criteria. There were no such cases in this study.*

TABLE 3 | Interpretation of total ITSv2 scores.

Total score	h-CLAT, DPRA, and Toolbox	h-CLAT and DPRA	h-CLAT or DPRA and Toolbox
6–7	1A	1A	-
5	1B	1[a]	-
3–4	1B	1B	1[a]
2	1B	1B	1B
1	Nonsensitizer	Inconclusive	Inconclusive
0	Nonsensitizer	Nonsensitizer	Inconclusive

[a]*This score is positive and conclusive for hazard, but potency cannot be determined.*

sensitization hazard potential of a test substance (i.e., sensitizer vs. nonsensitizer) and the GHS potency classification (i.e., 1A, 1B, or Not Classified) (**Table 3**). The interpretation of the total score considers partial information situations in which one input is unavailable or out of domain. In some cases, potency category may not be assigned.

2.4.4 Performance Analyses

The performance of the individual test methods and DAs for hazard classification was calculated by counting the number of true positive (TP), true negative (TN), false positive (FP), and false negative (FN) outcomes relative to the *in vivo* data. Accuracy, sensitivity, specificity, and balanced accuracy were calculated as follows:

$$\textbf{Accuracy}\,(\%) = \left[\frac{\mathbf{TP}+\mathbf{TN}}{\mathbf{TP}+\mathbf{TN}+\mathbf{FP}+\mathbf{FN}}\right]^{*}\mathbf{100}$$

$$\textbf{Sensitivity}\,(\%) = [\mathbf{TP}/(\mathbf{TP}+\mathbf{FN})]*100$$

$$\textbf{Specificity}\,(\%) = [\mathbf{TN}/(\mathbf{TN}+\mathbf{FP})]*100$$

$$\textbf{Balanced Accuracy}\,(\%) = \left[\textbf{Sensitivity}\,(\%)+\textbf{Specificity}\,(\%)\right]/\mathbf{2}$$

The performance of the STS or the ITSv2 DAs for GHS potency classification was determined by calculating the overall classification rate based on concordance with *in vivo* reference data and the concordant classification, underprediction, and overprediction rates for each GHS potency category (i.e., 1A, 1B, Not Classified). Inconclusive determinations were not included in performance calculations.

3 RESULTS

3.1 Performance of the *In Vitro* and *In Silico* Methods for Hazard Classification

Supplementary File 3 provides the results from the individual methods–DPRA, KeratinoSens, h-CLAT, and QSAR Toolbox v4.5–as well as predictions from the 2o3, STS, and ITSv2 DAs for all tested agrochemical formulations relative to *in vivo* reference data for hazard and potency. These results were used to determine the performance of each test method or DA. No DPRA data were available for Dow6, due to test chemical interference with the peptide measurements, and for

TABLE 4 | Performance of non-animal methods for GHS hazard classification in comparison with *in vivo* reference data.

Performance statistic	Individual methods				Defined approaches		
	DPRA (*n* = 25)	KeratinoSens (*n* = 27)	h-CLAT (*n* = 27)	QSAR Toolbox (*n* = 21)	2o3 (*n* = 19)	STS (*n* = 27)	ITSv2 (*n* = 24)
Accuracy (%)	64 (16/25)	81 (22/27)	52 (14/27)	48 (10/21)	79 (15/19)	52 (14/27)	54 (13/24)
Sensitivity (%)	45 (5/11)	75 (9/12)	92 (11/12)	100 (10/10)	90 (9/10)	92 (11/12)	91 (10/11)
Specificity (%)	79 (11/14)	87 (13/15)	20 (3/15)	0 (0/11)	67 (6/9)	20 (3/15)	23 (3/13)
Balanced Accuracy (%)	62	81	56	50	78	56	57

Borderline results were used in the assessment of the DPRA, KeratinoSens, and h-CLAT methods because the individual test guidelines do not recommend rejecting borderline results. The n for the 2o3 DA is reduced because borderline results were not used as one of the two concordant tests per OECD Guideline 497.

Dow8, which was not tested in the DPRA. The hazard predictions from QSAR Toolbox were inconclusive for six substances–Dow3, Dow7, Dow20, Dow23, Dow24 and Dow26–because the hazard predictions for the components were out of the domain of the read-across prediction or because a prediction could not be generated (Dow23).

Comparative performance data with respect to animal test results are shown in **Table 4**. Across the individual *in chemico* and *in vitro* methods, accuracy ranged from 52 to 81%, sensitivity from 45 to 92%, specificity from 20 to 87%, and balanced accuracy from 56 to 81%. KeratinoSens had the best overall performance, with accuracy of 81%, sensitivity of 75%, specificity of 87% and balanced accuracy of 81%. The QSAR Toolbox predictions alone had lower accuracy than the *in vitro* test methods (48%), with higher sensitivity (100%), but lower specificity (0%). However, there were no nonsensitizer hazard classifications for formulations based on read-across from QSAR Toolbox because any formulation ingredients that were classified as negative were either outside the applicability domain or combined with positive ingredients at greater than or equal to 0.1%.

3.2 Performance of the Defined Approaches for Hazard Classification

As indicated in **Section 2.4.1**, borderline results for DPRA, KeratinoSens, and h-CLAT were not used for the 2o3 DA. There were three borderline results for DPRA, six borderline results for KeratinoSens, and nine borderline results for h-CLAT (**Supplementary File 3**). Five substances produced borderline results in more than one *in chemico/in vitro* method (Dow7, Dow10, Dow13, Dow16, and Dow22). The 2o3 DA had eight inconclusive results: Dow2, Dow6, Dow18, and the five formulations that had borderline results in more than one method. Dow2 and Dow18 had results that were negative for the DPRA, borderline in the KeratinoSens, and positive for the h-CLAT. Dow6 had no DPRA data and results that were positive for the h-CLAT, and negative for the KeratinoSens.

There were no inconclusive results for the STS DA and three inconclusive results for the ITSv2 DA. The inconclusive results for ITSv2 were for three substances, Dow7, Dow20, and Dow23, which had negative DPRA results and weakly positive h-CLAT results. Because the Toolbox predictions for these substances were inconclusive, the overall evaluation produced ITSv2 scores of 1, which were considered inconclusive based on OECD Guideline 497 (OECD, 2021b).

Across the DAs, accuracy ranged from 52 to 79%, sensitivity from 90 to 92%, specificity from 20 to 67% and balanced accuracy from 56 to 78%. The 2o3 DA had the best overall performance, with accuracy of 79%, sensitivity of 90%, specificity of 67%, and balanced accuracy of 78%. The predictive capacity of the 2o3 was similar to that of the KeratinoSens assay. While accuracy and balanced accuracy of the two approaches were similar (78–81%), the 2o3 had higher sensitivity (90 vs 75%) and lower specificity (67 vs 87%). The STS and ITSv2 results were driven by the h-CLAT outcomes and had similar performance statistics. The STS had exactly the same hazard outcomes for each substance as the h-CLAT, and the ITSv2 outcomes differed from h-CLAT outcomes only for formulations for which the ITSv2 DA produced inconclusive results.

3.3 Performance of the Defined Approaches for GHS Potency Classification

As previously noted, the GHS potency classifications for the *in vivo* reference data and STS and ITSv2 DAs for the agrochemical formulations are shown in **Supplementary File 3**. The 2o3 DA does not provide potency classifications as it only predicts hazard. Although Dow22 was positive for the CD54 marker in the h-CLAT, no EC200, and thus, no MIT, could be calculated due an inadequate dose-response curve. Because it was positive at the lowest dose tested, 1,200 μg/ml, we used this value as a surrogate MIT value. There were no inconclusive results for STS, and four substances had inconclusive results for ITSv2. Dow8 had positive h-CLAT and positive Toolbox results but no DPRA data. This resulted in a total score of 3, which is inconclusive for potency determination when DPRA data are missing. Three substances– Dow7, Dow20, and Dow23–had negative DPRA and positive h-CLAT data with inconclusive Toolbox results that yielded total ITSv2 scores of 1, and thus inconclusive ITSv2 potency results.

The correct overall classification rate, based on concurrence with *in vivo* reference data, for the two potency DAs was 52% for the STS and 43% for ITSv2 (**Table 5**). The overall underprediction rates were 4% for both DAs and the overall overprediction rates were 44% for STS and 52% for the ITSv2. Both DAs correctly classified the single GHS 1A sensitizer, thus there was no underprediction of this class. Neither DA overclassified nonsensitizer substances as GHS 1A sensitizers (**Supplementary File 3**). All misclassified substances were misclassified by one category. The STS was more successful

TABLE 5 | Performance of the defined approaches for GHS potency classification in comparison with *in vivo* reference data.

Performance	STS				ITSv2			
	Overall (*n* = 27)	NC (*n* = 15)	1B (*n* = 11)	1A (*n* = 1)	Overall (*n* = 23)	NC (*n* = 13)	1B (*n* = 9)	1A (*n* = 1)
Correct Classification (%)	52 (14/27)	20 (3/15)	91 (10/11)	100 (1/1)	43 (10/23)	23 (3/13)	67 (6/9)	100 (1/1)
Underpredicted (%)	4 (1/27)	NA	9 (1/11)	0 (0/1)	4 (1/23)	NA	11 (1/9)	0 (0/1)
Overpredicted (%)	44 (12/27)	80 (12/15)	0 (0/11)	NA	52 (12/23)	77 (10/13)	22 (2/9)	NA

NC, GHS Not Classified (nonsensitizer); NA, not applicable.

TABLE 6 | Skin sensitization results[a] for 27 agrochemical formulations.

Code	DPRA hazard	h-CLAT hazard	KS hazard	QSAR TBv4.5 hazard	*In Vivo* hazard	2o3 hazard	ITSv2 hazard	STS hazard	*In Vivo* GHS potency	ITSv2 GHS potency	STS GHS potency
Dow1	0	BL 0	0	1	0	0	0	0	NC	NC	NC
Dow2	0	1	BL 0	1	0	INC	1	1	NC	1B	1B
Dow3	1	1	1	INC	1	1	1	1	1B	1B	1B
Dow4	1	BL 1	1	1	1	1	1	1	1B	1A	1B
Dow5	1	BL 1	1	1	1	1	1	1	1B	1A	1B
Dow6	INC	1	0	1	0	INC	1	1	NC	1B	1B
Dow7	BL 0	BL 1	0	INC	0	INC	INC	1	NC	INC	1B
Dow8	NT	1	1	1	1	1	1	1	1B	INC	1B
Dow9	1	1	0	1	1	1	1	1	1A	1A	1A
Dow10	BL 0	BL 0	0	1	0	INC	0	0	NC	NC	NC
Dow11	0	BL 1	0	1	0	0	1	1	NC	1B	1B
Dow12	0	BL 1	0	1	1	0	1	1	1B	1B	1B
Dow13	0	BL 1	BL 1	1	1	INC	1	1	1B	1B	1B
Dow14	0	1	0	1	0	0	1	1	NC	1B	1B
Dow15	1	1	1	1	1	1	1	1	1B	1B	1B
Dow16	0	BL 0	BL 0	1	1	INC	0	0	1B	NC	NC
Dow17	0	0	0	1	0	0	0	0	NC	NC	NC
Dow18	0	1	BL 0	1	0	INC	1	1	NC	1B	1B
Dow19	0	1	1	1	0	1	1	1	NC	1B	1B
Dow20	0	1	0	INC	0	0	INC	1	NC	INC	1B
Dow21	0	1	1	1	1	1	1	1	1B	1B	1B
Dow22	BL 1	1	BL 0	1	0	INC	1	1	NC	1B	1B
Dow23	0	1	1	INC	1	1	INC	1	1B	INC	1B
Dow24	1	1	BL 1	INC	0	1	1	1	NC	1B	1B
Dow25	0	1	0	1	0	0	1	1	NC	1B	1B
Dow26	1	1	0	INC	0	1	1	1	NC	1B	1B
Dow27	0	1	1	1	1	1	1	1	1B	1B	1B

[a]*Results from three in chemico/*in vitro *assays (yellow), one in silico model (blue), historical animal reference data (green), and three DAs (orange) providing both hazard and potency predictions. 0, negative; 1, positive, BL, borderline; INC, inconclusive; KS, KeratinoSens; NC, GHS Not Classified (nonsensitizer); NT, not tested; TB, Toolbox.*

at classifying GHS Category 1B sensitizers concordantly with *in vivo* tests than the ITSv2, with *in vivo* concordance for the two DAs being 91 and 67%, respectively. The STS misclassified one GHS Category 1B sensitizer while the ITSv2 misclassified three. Underprediction of GHS Category 1B sensitizers ranged from 9 to 11% and overprediction was 0% for the STS and 22% for ITSv2. Both DAs correctly classified nonsensitizers at 20–23%. However, nonsensitizers were overpredicted 77–80% by the DAs. Therefore, both DAs were likely to overclassify a test substance as GHS Category 1B that was GHS Not Classified based on *in vivo* data.

3.4 Comparison Among Methods

As described, multiple non-animal (one *in chemico*, two *in vitro*, one *in silico* and three DAs) and animal-based (LLNA and guinea pig) test methods were used to assess the skin sensitization potential of 27 agrochemical formulations. **Table 6** presents the results for all tested products based on each of the methods. In many cases, several non-animal approaches provided concordant results despite lack of agreement with the animal tests. While new approaches are typically evaluated with respect to the existing animal reference standard, there has been substantial evidence supporting the superior performance of methods and DAs that cover multiple KEs in the adverse outcome pathway for skin sensitization, as compared to human reference data (Kleinstreuer et al., 2018; OECD, 2021b). It is therefore appropriate that each of these methods, non-animal and animal alike, should be considered as potentially equivalent information sources when assessing skin sensitization hazard and potency predictions for a new data set.

4 DISCUSSION

One current limitation to the regulatory application of DAs is that the non-animal methods that comprise them have only been evaluated using monoconstituent substances rather than mixtures or product formulations. With this study, we sought to expand the applicability of internationally accepted OECD test methods and DAs by generating skin sensitization hazard and potency assessments for 27 water-based or solvent-based agrochemical formulations. We evaluated three rule-based DAs: the 2o3, the KE 3/1 STS, and the ITSv2. The 2o3 and the ITSv2 have been adopted for hazard classification and GHS potency classification by OECD (OECD, 2021b), and the 2o3 and the STS are accepted for hazard classification by the United States Environmental Protection Agency (US EPA, 2018), although the STS also classifies substances in GHS potency categories (Nukada et al., 2013). The DAs combine skin sensitization potential information from three non-animal methods that map to key events of the skin sensitization adverse outcome pathway–the DPRA, the KeratinoSens assay, and the h-CLAT–as well as *in silico* hazard predictions from QSAR Toolbox v4.5. There is limited information on the applicability of the individual methods to agrochemical formulations aside from a small proof of concept for KeratinoSens (Settivari et al., 2015).

We evaluated the individual non-animal methods as stand-alone methods for hazard classification of the agrochemical products to compare their performances with the DAs, all with respect to historical reference animal test data. Of the individual methods, KeratinoSens performed best in predicting *in vivo* hazard outcomes (**Table 4**). The best performing DA was the 2o3. However, the 2o3 DA did not outperform the KeratinoSens assay alone with respect to accuracy or balanced accuracy. Both KeratinoSens and the 2o3 DA had accuracy and balanced accuracy of 78–81%. The 2o3 DA had higher sensitivity (90%) than specificity (67%) whereas the KeratinoSens had a better balance of sensitivity (75%) and specificity (87%). The balanced accuracy of the 2o3 DA in this study, 78%, was less than that reported in OECD Guideline 497 for 134 monoconstituent substances (84%), which yielded sensitivity of 82%, and specificity of 85% as compared to LLNA reference data (OECD, 2021b). Relative to animal data, the 2o3 DA had higher sensitivity than specificity for classification of the agrochemical formulations.

For our data set of agrochemicals, the STS and the ITSv2 produced very similar results for *in vivo* hazard classification because both DAs rely on DPRA and h-CLAT results (**Table 4**). These DAs had balanced accuracies of 56–57%, which were lower than that for the 2o3 DA. Sensitivities were 91–92% and specificities were 20–23%. Thus, these DAs were much better at classifying sensitizers than nonsensitizers. The STS and ITSv2 results were driven by the h-CLAT and had similar performance statistics. The STS had exactly the same hazard outcomes for each substance as the h-CLAT and the ITSv2 DA differed with h-CLAT only for formulations for which the ITSv2 had inconclusive results (**Supplementary File 3**). Concordance of the ITSv2 classification with reference animal data was higher for the 156 monoconstituent substances reported in OECD Guideline 497, than that of the current set of agrochemical formulations, especially with respect to balanced accuracy (80%) and specificity (67%), but sensitivity was similar (93%) (OECD, 2021b). It is not unusual for the h-CLAT to perform with higher sensitivity than specificity or accuracy in other datasets (Urbisch et al., 2015; Hoffmann et al., 2018; OECD, 2021b). We speculate that some overprediction could be due to the presence of substances of natural origin, such as lipopolysaccharide, in the formulations. The h-CLAT may be overly sensitive to the stimulation of the CD86 and CD54 cell markers by endotoxins/liposaccharides (Tsukumo et al., 2018; Kobayashi-Tsukumo et al., 2019). It is not uncommon for agrochemical formulations to have active ingredients that are fermentation products or co-formulants/inerts of natural origin (such as methylated seed oil used as surfactant enhancing foliar absorption). This should be a point of caution when using h-CLAT.

Application of the borderline ranges for the 2o3 as described in OECD Guideline 497 improved its performance with respect to the animal data but yielded eight inconclusive results. Before excluding borderline results, the balanced accuracy of the 2o3 was 73%, sensitivity was 75% and specificity was 71% (data not shown). After excluding borderline results, balanced accuracy increased to 78%, sensitivity increased to 90% and specificity decreased slightly, to 67%. The exclusion of borderline values in the OECD evaluation of the 2o3 using single constituent substances had a similar effect (OECD, 2021e). It reduced the reference data set (from 168 to 134 chemicals) and increased balanced accuracy from 79 to 84%, increased sensitivity from 74 to 82%, and left specificity unchanged at 85%, in comparison with LLNA data (OECD, 2021e).

For GHS potency categorization, the performance of the individual test methods could not be compared with that of the STS and ITSv2 because the individual methods have not been validated for potency determination. The performances of the STS and ITSv2 were very similar for predicting *in vivo* GHS potency categories (**Table 5**). Both DAs derive potency information from the h-CLAT. Both also use DPRA, but only the ITSv2 uses DPRA for potency information, although use of these data did not improve the performance of the ITSv2 over the STS for this set of agrochemicals. The overall correct classification rates of the STS and ITSv2 DAs were 52 and 43%, respectively. The overall underprediction rates (4%) were much lower than overprediction rates (44–52%). The overprediction of *in vivo* nonsensitizers as GHS 1B sensitizers contributed greatly to the underprediction rate. The overprediction rate of nonsensitizers was 80% for the STS and 77% for the ITSv2. A previous evaluation of the STS for potency classification of monoconstituent substances relative to LLNA data reported a higher correct classification rate (71%) and more balanced over- and underprediction rates with 12% overprediction and 18% underprediction (Takenouchi et al., 2015). The performance of the ITSv2 against LLNA data reported in OECD Guideline 497 was also better for the 141 monoconstituent substances evaluated by the OECD; correct classification rates were 72% for GHS 1A and 1B sensitizers and 67% for nonsensitizers (OECD, 2021b). For the agrochemicals, the correct classification rates were 100% for GHS 1A (which had only

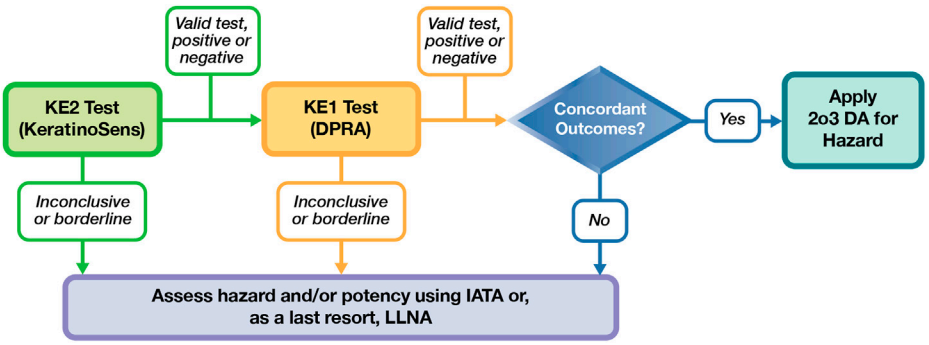

FIGURE 1 | Proposed framework for a non-animal assessment of skin sensitization potential of agrochemical formulations.

one substance), 67% for GHS 1B sensitizers, and 23% for nonsensitizers.

We recognize that the current study has several limitations in its evaluation of the performance of DAs for predicting human skin sensitization potential and potency of agrochemical products. One limitation is the small number of substances evaluated. Another is that all 27 substances were either water- or solvent-based formulations, so we do not know how relevant these results are to other agrochemical formulation types. A third is that our reference data consisted of animal data rather than human data. There is evidence that the animal studies may not accurately predict human endpoints (Kleinstreuer et al., 2018). The OECD evaluation of monoconstituent substances showed that the performance of the LLNA for predicting human skin sensitization hazard yielded 58% for balanced accuracy, 94% for sensitivity, and 22% for specificity (OECD, 2021b). The absence of human data for the agrochemical formulations beyond case studies or adverse event information with questionable reliability presents a challenge to assessing the true performance of the DAs to predict human skin sensitization potential. Although we used the best reference data available, the agreement of the animal data with human responses is uncertain.

A further limitation of our study was the retrospective application of DAs to the data, which resulted in more inconclusive predictions than would be obtained using a prospective approach. Inconclusive predictions for the 2o3 DA were obtained for 30% (8/27) of the formulations due to borderline results of the individual test methods. In a prospective testing situation, an investigator would be able to perform additional tests as borderline results were produced to minimize the number of final discordant results. For example, if two KeratinoSens runs had been conducted, but one produced a borderline result, OECD Guideline 497 would require a third repetition. If the third repetition was not a borderline result, a final positive or negative call could be made, rather than an inconclusive result. Despite this limitation, inconclusive DA predictions may be considered in a weight-of-evidence approach or within IATA to reach a hazard classification decision or develop a risk assessment. Other information considered might include demonstration of exposure to the test system, existing *in vivo* data, clinical data, read-across, and other *in vitro/in chemico/in silico* data (OECD, 2021b).

Our evaluation shows that the 2o3 DA has the most promising performance for predicting the animal-based hazard classification of these particular agrochemicals. KeratinoSens had slightly higher balanced accuracy compared with the 2o3 DA (81 vs. 78%), however, the sensitivity was lower than that of the 2o3 DA (75 vs. 90%) and the specificity was higher (87 vs. 67%). We have more confidence in the results of 2o3 DA because it assesses at least two key events of the AOP. The performance of the 2o3 DA was more similar to its performance in the classification of monoconstituent substances (OECD, 2021b) than the performance of the STS or the ITSv2 (Takenouchi et al., 2015; OECD, 2021b). Given the theoretical advantages of DAs over individual *in vitro* methods and their previously reported success in classifying monoconstituent substances, the low concordance with reference *in vivo* GHS potency classifications for this set of agrochemical formulations was somewhat surprising. However, given the biological and mechanistic relevance of the DAs and their demonstrated superior performance when compared to available human reference data, it cannot be ruled out that the historical animal results may actually be incorrect, and the DAs may provide a more human health protective outcome. Further investigation, including testing of more and additional types of agrochemicals, will be required to determine whether our results with these DAs are applicable to other agrochemical formulations or other mixtures.

In conclusion, based on the limited amount of information available today in the literature and in this study, we identified a potential prospective testing strategy shown in **Figure 1**. This could be considered a first "work in progress" step using the existing methods that demonstrated higher applicability and reliability in this study, KeratinoSens and DPRA. If these two tests are applicable and the results are concordant, the 2o3 approach could be used and additional *in vitro* or *in vivo* testing would not be necessary. In our database, only 11/27 substances had concordant results for the KeratinoSens and DPRA tests. While the number of applicable substances was limited, the performance of the 2o3 was good. Sensitivity was

100% (4/4), specificity was 86% (6/7), balanced accuracy was 93%, and accuracy was 91%. Substances where the KE2 and KE1 test results are not applicable and concordant would undergo additional testing and/or an assessment under IATA. This initial exercise highlights possible routes to reduce animal use and identifies further research needs to characterize test methods and DAs applicable for agrochemical formulations. These may include, for example, KE3 assays that have balanced predictivity, KE1 assays with higher compatibility with agrochemical formulations, data sharing exercises with existing paired *in vitro-in vivo* testing, more complex models (e.g., 3D skin, genomic signatures), and additional testing on multiple formulation types to establish broader applicability.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal studies because all tests were conducted according to the standard OECD test guideline in force at the time of testing to meet regulatory requirements for registration of pesticidal formulations in several geographies; as such, ethical committee approval was not necessary. The vertebrate data used in this manuscript were historical; all animal studies were carried out from 1989 to 2012.

AUTHOR CONTRIBUTIONS

JS, JT, MC, RS and VJ drafted and edited parts of the manuscript. JT curated data and performed analyses. MC, RS, JH, JM, VJ and TG contributed or generated data for the manuscript. SG, DGA, DG and NK participated in planning the project, interpreting the results, and editing the manuscript.

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A New Immortalized Human Alveolar Epithelial Cell Model to Study Lung Injury and Toxicity on a Breathing Lung-On-Chip System

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The evaluation of inhalation toxicity, drug safety and efficacy assessment, as well as the investigation of complex disease pathomechanisms, are increasingly relying on *in vitro* lung models. This is due to the progressive shift towards human-based systems for more predictive and translational research. While several cellular models are currently available for the upper airways, modelling the distal alveolar region poses several constraints that make the standardization of reliable alveolar *in vitro* models relatively difficult. In this work, we present a new and reproducible alveolar *in vitro* model, that combines a human derived immortalized alveolar epithelial cell line (^{AX}iAEC) and organ-on-chip technology mimicking the lung alveolar biophysical environment (^{AX}lung-on-chip). The latter mimics key features of the *in vivo* alveolar milieu: breathing-like 3D cyclic stretch (10% linear strain, 0.2 Hz frequency) and an ultrathin, porous and elastic membrane. ^{AX}iAECs cultured on-chip were characterized for their alveolar epithelial cell markers by gene and protein expression. Cell barrier properties were examined by TER (Transbarrier Electrical Resistance) measurement and tight junction formation. To establish a physiological model for the distal lung, ^{AX}iAECs were cultured for long-term at air-liquid interface (ALI) on-chip. To this end, different stages of alveolar damage including inflammation (via exposure to bacterial lipopolysaccharide) and the response to a profibrotic mediator (via exposure to Transforming growth factor β1) were analyzed. In addition, the expression of relevant host cell factors involved in SARS-CoV-2 infection was investigated to evaluate its potential application for COVID-19 studies. This study shows that ^{AX}iAECs cultured on the ^{AX}lung-on-chip exhibit an enhanced in vivo-like alveolar character which is reflected into: 1) Alveolar type 1 (AT1) and 2 (AT2) cell specific phenotypes, 2) tight barrier formation (with TER above 1,000 Ω cm^2) and 3) reproducible long-term preservation of alveolar

characteristics in nearly physiological conditions (co-culture, breathing, ALI). To the best of our knowledge, this is the first time that a primary derived alveolar epithelial cell line on-chip representing both AT1 and AT2 characteristics is reported. This distal lung model thereby represents a valuable *in vitro* tool to study inhalation toxicity, test safety and efficacy of drug compounds and characterization of xenobiotics.

Keywords: alveolar epithelial cells, distal lung, lung-on-a-chip, SARS-CoV-2, cyclic stretch, lung inflammation, lung toxicity, AT1 and AT2

INTRODUCTION

The impairment of functional gas exchange at the alveolar epithelial barrier is a crucial determinant of the clinical outcome of various acute and chronic respiratory diseases, such as acute respiratory distress syndrome (ARDS) (Sapoznikov et al., 2019), emphysema in chronic obstructive pulmonary disease (COPD) (Moazed et al., 2016) and pulmonary fibrosis (PF) (Kulkarni et al., 2016). Such respiratory conditions are often detected at an advanced stage, when they are more difficult to treat. Hence, there is an urgent need for new investigative strategies to generate more effective therapeutics.

The alveolar microenvironment in the distal lung is a complex and dynamic structure that allows efficient gas exchange, determines immune responses to various environmental stimuli and forms a tight barrier that prevents any fluid accumulation into the lung airspace (Knudsen and Ochs, 2018). The alveolar epithelial lining forms the initial barrier against inhaled pathogens or xenobiotics. In a healthy lung, paracellular and transcellular barriers are formed by alveolar junctions that control the permeability to inhaled particles, toxins and pathogens. Thus, the destruction of the tight alveolar barrier by environmental stressors disrupts the delicate equilibrium of epithelial cells and alters their function and regulation of various signalling pathways (Olivera et al., 2007; Short et al., 2016). This highlights the significance of a concerted interplay between epithelial cell-cell communication and the stability of tight junction components in the alveoli. The alveolar epithelium comprises two distinct cell types: alveolar type I (AT1) and type 2 (AT2) pneumocytes which are tightly connected by intercellular junctions as well as other cell-to-cell connections. Physiologically mimicking parameters, such as culture at ALI, have been shown to significantly improve the alveolar character of lung alveolar primary cells and cell lines (A549) (Ravasio et al., 2011; Wu et al., 2018). On the other hand, breathing exposes the alveoli to continuous and differential levels of mechanical forces since early development (Waters et al., 2012). Experimental evidence has shown that *in vivo* like breathing motion (8–12% linear strain) plays an essential role to regulate alveolar cell differentiation (Liu et al., 2016; Li et al., 2018) and has the potential to induce regenerative responses in wound injury (Desai et al., 2008). In contrast, aberrant mechanical stress is a critical factor in the development of various lung diseases including acute lung injury, ARDS, lung fibrosis (Sehlmeyer et al., 2020) as well as ventilation induced mechanical trauma (Oeckler and Hubmayr, 2007).

Due to the lack of robust *in vitro* alternatives to study distal lung, and the inherent translational limitations of animal models, "new approach methodologies (NAMs)" are becoming increasingly relevant (Russell, 2004). Advanced *in vitro* models recapitulating important aspects of the alveolar niche promise to bridge the gap and provide a reliable alternative for drug safety as well as efficacy studies, toxicity profiling and precision medicine (Low et al., 2020). The organ-on-chip technology replicates key points of the dynamic alveolar microenvironment such as cyclic mechanical strain, perfusion and inflammatory or thrombotic pathomechanisms (Huh et al., 2010; Stucki et al., 2015; Jain et al., 2017; Stucki et al., 2018; Felder et al., 2019; Thacker et al., 2020; Huang et al., 2021; Si et al., 2021). Besides, the recent inclusion of 3D breathing motion (10% linear strain), ALI culture conditions, biological scaffolds and use of primary human alveolar epithelial cells (AECs), has brought the lung-on-chip technology closer to physiological dimensions (Stucki et al., 2015; Stucki et al., 2018; Zamprogno et al., 2021).

Notwithstanding the complexity of organs-on-chip, a critical challenge remains the procurement of a suitable cell source. Primary human AECs continue to be one of the most reliable alveolar cell models reflecting the *in vivo* situation in terms of alveolar phenotype and barrier formation (Elbert et al., 1999), molecule absorption and ion-transport studies (Bove et al., 2010; Bove et al., 2014). Their combination with lung-on-chip models or their use as alveolar organoids has predictive and translational applications, which have been accelerated and successfully put to test in drug screening and pathway studies, particularly due to the COVID-19 pandemic (Zhang et al., 2020; Thacker et al., 2021; Domizio et al., 2022) (Huang et al., 2020; Katsura et al., 2020; Youk et al., 2020). On the other hand, iPSC-derived AECs exhibit highly relevant alveolar features and are fitting on-chip candidates (Katsumiti et al., 2020; Van Riet et al., 2020). However, they present a labour-intensive generation and require highly experienced personnel. Primary AECs have their limitations too, including their restricted access, inability to be expanded and donor-specific response. Thus, alternative models capturing relevant features of these cells, combined with elements of the dynamic alveolar microenvironment, offer an opportunity for studies requiring a stable alveolar phenotype at instances where primary cell material proves limiting.

Alveolar epithelial cell lines have been used since the late 80's. A549 and NCI-H441 are still the most widely used human AEC cell lines in pharmaceutical research for lung cancer, asthma, COPD and fibrosis despite their carcinogenic origin (Giard et al., 1973; Brower et al., 1987; O'Reilly et al., 1988). Conversely, viral transduced primary AT2 cells have led to the generation of AT1-like cell lines, including TT1cells (Kemp et al., 2008; van den Bogaard et al., 2009; Katsumiti et al., 2020), or the recently established hAELVi cells (Kuehn et al., 2016). These cell lines display different features of ATI cells phenotype, such as a

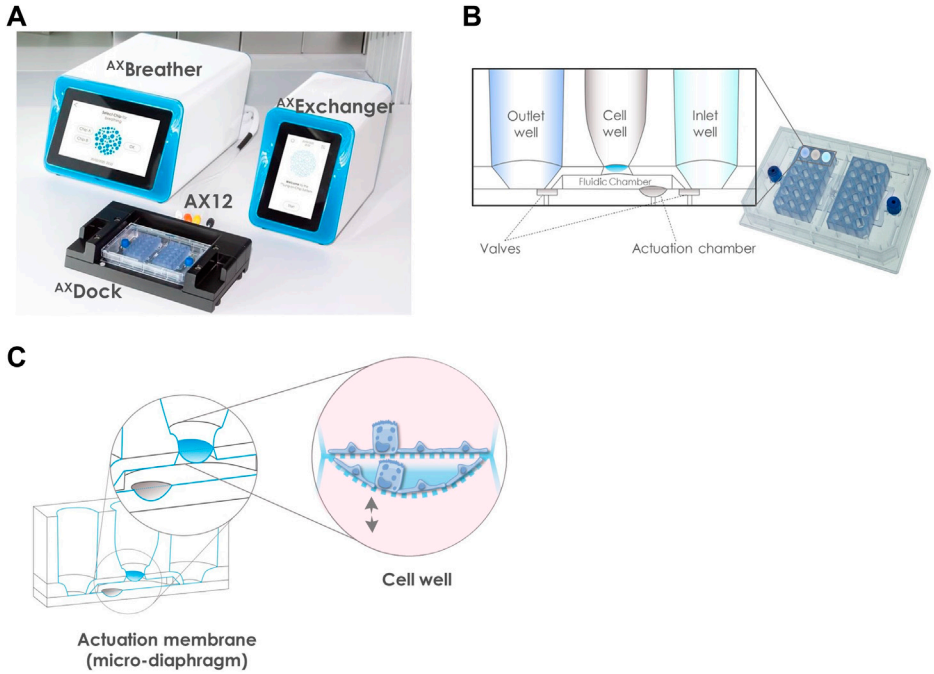


FIGURE 1 | Overview of the AXLung-on-chip System. **(A)** including the Lung-on-chip (AX12), two electro-pneumatic modules: AXExchanger and AXBreather, and the interface platform: AXDock. **(B)** Detail of the AX12 comprised of two chips. Based on a SBS footprint (standard 96-well plate layout 127 mm × 85 mm), the wells are positioned at equal distance of 9 mm. The chip cross-section schematic shows that one unit comprises of an inlet and an outlet well, connected through microfluidic channels and pneumatic valves to the cell compartment with the 3.5 µm ultrathin membrane of porous density 8×10^5 pores/cm^2 (blue). The two valves define the medium flow via the control of the AXExchanger, for which 200 µl of medium are pipetted in the inlet wells. **(C)** Principle of the breathing function. In the fluidic chamber of a volume ~80 µl, the actuation membrane (grey), acting as a micro-diaphragm, is cyclically stretched in 3D by the AXBreather. This movement is transferred to the cells cultured on the cell well membrane (blue). The cell barrier recreated on the ultrathin porous membrane is biomechanically stimulated with a physiological 3D-stretch of 10% linear strain and 0.2 Hz frequency.

flattened and extended morphology (Kemp et al., 2008), or TER-forming barrier properties, respectively (Kuehn et al., 2016; Metz et al., 2020). Besides, both have been successfully implemented in the study of lung inflammation, showing cytokine responses upon proinflammatory triggers such as lipopolysaccharide (LPS), interferon gamma (IFN-γ) or tumor necrosis factor alpha (TNF-α) (van den Bogaard et al., 2009; Metz et al., 2020). Nevertheless, none of these models fully comprised functionally tight barrier, robust long-term alveolar character with AT1 and AT2-like cells, ultra-thin basement membrane and breathing dynamics.

To address this need, we report in this work an *in vitro* system including an immortalized human alveolar epithelial cell line (AXiAEC) combined with the AXlung-on-chip system (AlveoliX, Switzerland). Primary cell-derived AXiAECs were characterized by FACS for typical epithelial and AT1/AT2 markers. AXiAECs cultured on-chip were further inspected for their alveolar cell-specific gene expression profile as well as the presence of cellular host factors required for SARS-CoV-2 (severe acute respiratory syndrome Coronavirus 2) infection. Subsequently, the impact of physiological levels of cyclic strain and ALI culture conditions on cell phenotype was examined by gene expression and protein profiling. Finally, AXiAECs cultured on-chip were exposed to a well-characterized pro-fibrotic growth factor, transforming growth factor β1 (TGFβ1), and a pro-inflammatory stimulus, bacterial LPS, to evaluate the responsiveness and suitability of the model to study different stages of lung injury and its potential application for inhalation toxicology studies and drug safety and efficacy testing.

METHODS

Design and Handling of the AXLung-On-Chip System

The AXLung-on-chip system (AlveoliX, Switzerland) consists of the Lung-on-Chip, called AX12, two electro-pneumatic devices and two interface units (**Figure 1A**). The electro-pneumatic modules, the AXExchanger and AXBreather, are connected to the AX12 via the interface platform, the AXDock, respectively, in the cell culture hood and in the incubator. The AX12 placed on the AXDock is controlled by the touchscreen interface of the AXExchanger and AXBreather. The AX12 has a SBS footprint (96-well plate format) and comprises of two modular chips, supported by a plate, that contains six individual units each. Each unit is composed of an inlet, a cell compartment and an outlet that are connected on the basal side by microfluidic channels and pneumatically controlled valves. The cell compartment comprises an ultrathin (3.5 µm) and elastic porous membrane (3 µm, 8×10^5 pores/cm^2) made of biocompatible silicone. This

membrane separates the cavity into an apical cell well and a basolateral fluidic chamber (**Figure 1B**). The two-part design (modular chips and plate) of the AX12 enables the seeding of the cells directly on either side of the ultrathin membrane.

The chip closing and filling of the basolateral compartment are next performed. Briefly, a drop of cell culture media (with or without cells) is pipetted on the basolateral side of the membrane. Then, each chip is flipped and screwed onto the AX12 plate (blue screw, **Figure 1B**). The AX12 is then inserted into the ^{AX}Dock, and inlet wells are filled up with cell culture media. The chip initial filling is triggered by pressing the "Initial filling" action on the ^{AX}Exchanger: negative and positive pressure is generated to open and close the valves inducing a pumping action, which allows the medium of the inlet to flow into the basal chamber, and subsequently to the outlet well. After basolateral filling, cells are seeded on the apical side of the membrane.

For medium exchange, the AX12 is positioned in the ^{AX}Dock. The inlet, outlet, and cell well are emptied and fresh cell culture media is pipetted into the inlet well. Similarly, as for the initial filling, medium exchange is performed via the "Medium Exchange" function of ^{AX}Exchanger: negative pressure is generated to open the valves and the medium is exchanged by hydrostatic and surface tension forces. The nutrient-exhausted medium on the apical side is replaced with fresh medium by direct pipetting.

To initiate the breathing function, the AX12 is positioned within the ^{AX}Dock within the incubator. 3D cyclic stretch (10% linear strain, 0.2 Hz) is initiated by touchscreen control on the ^{AX}Breather. This automatically leads to the closing of the fluidic chamber and the deflection of the microdiaphragm by the generation of cyclic negative pressure, which recreates the *in vivo* breathing motions (**Figure 1C**). Static (non-breathing) and dynamic (breathing) conditions can be set simultaneously on one AX12, as the two chips are controlled independently.

Cells and Cell Culture

The alveolar epithelial cell line (^{AX}iAECs) was derived from primary human AECs isolated from resected lung tissue, immortalized with InscreeneX' CI-Screen technology (Lipps et al., 2018), and provided by AlveoliX (Switzerland). ^{AX}iAECs were cultured and expanded in AX Alveolar Epithelial Medium (AlveoliX, Switzerland) and used between passages 23 and 30. AX12 were provided precoated with ECM (AlveoliX, Switzerland). On-chip, the cells were maintained in AX Alveolar Barrier Medium (AlveoliX, Switzerland), supplemented with 1% penicillin-streptomycin (ThermoFischer Scientific, Switzerland).

For the co-culture experiments (AX co-culture Biomodel), ^{AX}iAECs were seeded apically, and primary human lung microvascular endothelial cells (hLMVEC) at the basolateral side of the membrane. AX E2 Alveolar Barrier Medium (AlveoliX, Switzerland) was used for both cell types. Peripheral blood mononuclear cells (PBMCs) were introduced at the initiation of the inflammation experiments. All cell manipulations were performed under sterile laminar flow conditions and cells were maintained at 37°C, 5% CO_2. Cell culture medium was replaced every 2–3 days. For monoculture studies on-chip, ^{AX}iAECs cells were seeded at a density of 4×10^5 cells per cm^2 on the apical side of the membrane. The cells were incubated for 24 h allowing them to adhere to the membrane and reached confluence after 48 h. For the co-culture with endothelial cells, hLMVEC were first expanded in AX endothelial medium. Then, harvested hLMVEC were seeded on the basolateral side of each membrane followed by 2 h incubation to promote cell attachment. Chips were consecutively closed and filled with AX E2 Alveolar Barrier Medium using the initial filling function on the ^{AX}Exchanger. ^{AX}iAECs were subsequently seeded on the apical side of the membrane at a density of 4×10^5 cells per cm^2 in medium. ^{AX}iAECs cell seeding is considered as day 0 in the study (D0).

TER Measurement

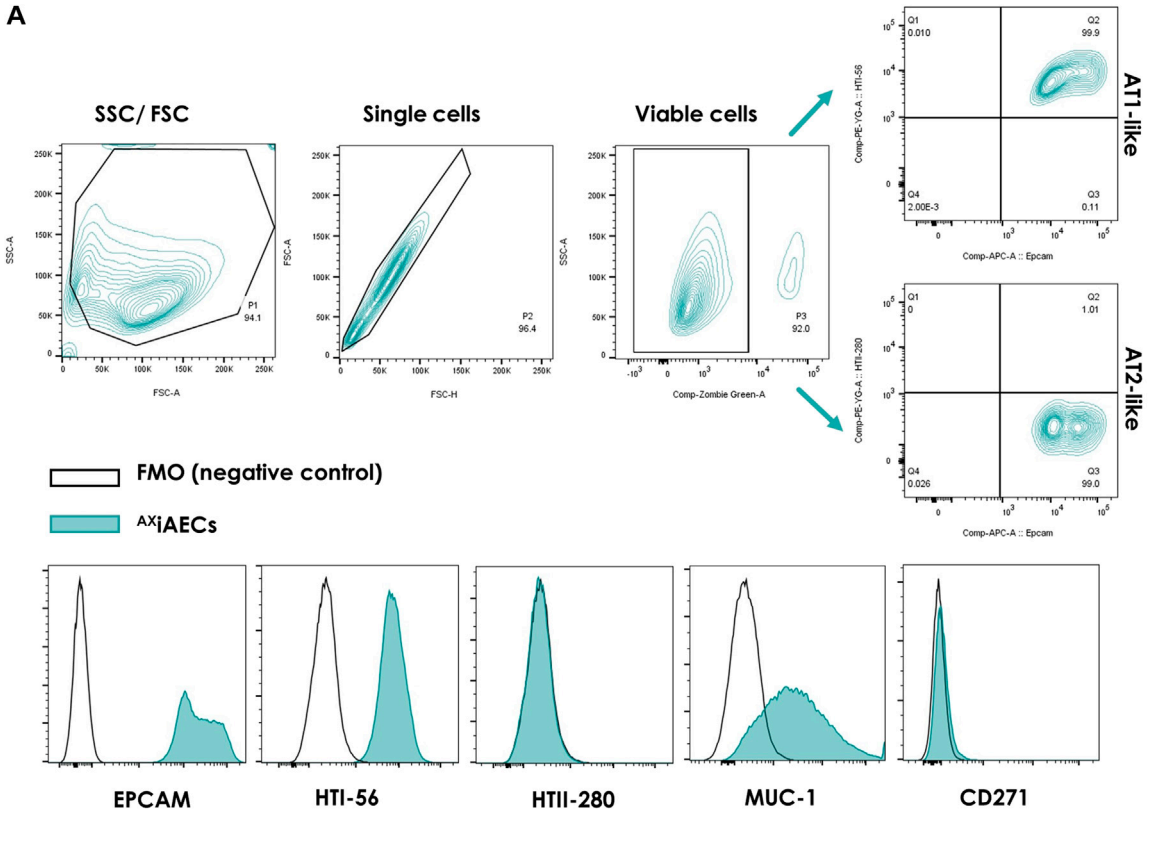
To assess barrier formation, transbarrier electrical resistance (TER) measurements were started 48 h after cell seeding. TER measurements were taken every 2 days using a commercially available 96-well plate electrode (STX100MC96; World Precision Instruments) and an Epithelial Volt/Ohm Meter (EVOM3; World Precision Instruments). TER was measured in mono and co-culture conditions for up to 30 days. The unique semi-open design of the AX12 allows the STX100MC96 electrode to tightly fit in between the outlet well and the cell well which enables precise and consistent positioning of the electrodes and avoid handling variations. The microfluidic channel design: widths between 400 and 600 μm, height of up to 1,000 μm and length of no more than 1,000 μm, enables a low chip induced TER background essential for reliable on-chip measurements.

Before measurement, the electrodes were sterilized with 70% v/v ethanol, rinsed and equilibrated in DPBS (Thermo Fischer Scientific, Switzerland) at room temperature. To measure TER, the AX12 was placed on the ^{AX}Dock in the cell culture hood. The electrodes were properly positioned and the "TER measurement" function was initiated on the ^{AX}Exchanger, enabling valve opening on the microfluidic plate. For cells in ALI, pre-warmed cell culture medium was added 15 min before measuring TER. The background TER (Ω) was measured on a porous membrane with no cells. Background subtracted TER (Ω) values were then multiplied by the surface area of the cell culture well (0.071 cm^2 on-chip) to obtain the final TER reading in Ω cm^2.

Cell Culture Treatments

^{AX}iAECs were stimulated with Transforming Growth Factor β1 (TGFβ1) upon barrier formation. Lyophilized recombinant human TGFβ1 Protein (C# 240-B, R&D) was reconstituted in sterile 4 mM HCl containing 0.1% BSA following the manufacturer's protocol. Cells were stimulated apically at T0 time-point (day 25 on-chip) with a final concentration of 5 or 10 ng/ml diluted in the cell culture medium (AX Alveolar Epithelial medium) for 72 h (T3). Control (vector treated) cells were added with reconstitution medium without TGFβ1. Following treatment, cells were harvested 3 days after TGFβ1 stimulation (T3).

^{AX}iAECs/hLMVEC (AX co-culture Biomodel) were stimulated with 0.1 μg/ml LPS from E-Coli 026:B26 (Sigma Aldrich, Germany) in the presence of peripheral blood mononuclear cells (PBMCs). PBMCs were administered in the basolateral compartment via medium exchange. PBMC and LPS



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AXiAECs	Epcam+	HTI-56+	HTII-280+	MUC1+	CD271+
AXiAECs 1	99.89%	99.25%	0.00%	64.94%	0.63%
AXiAECs 2	99.98%	99.15%	0.07%	66.20%	2.45%
AXiAECs 3	99.97%	99.94%	0.12%	58.00%	1.76%
AT2 cells	100%	n/a	96.65%	91.17%	0.00%
A549	88.08%	100%	0.43%	88.98%	15.38%

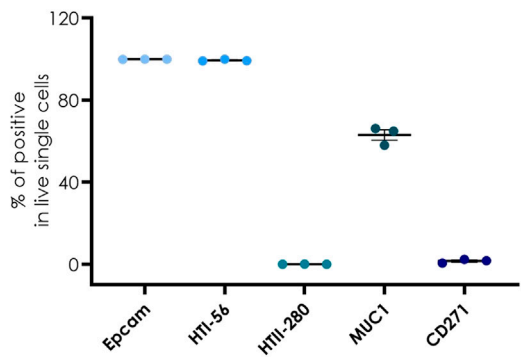

FIGURE 2 | Cell surface phenotype analysis of AXiAECs by flow cytometry at D0 **(A)** FACS gating strategy for identifying distinct alveolar epithelial cell surface markers as indicated in *Methods*. Fluorescence minus one (FMO) controls were used for all quantifications. Data are representative of three independent experiments. **(B)** Expression of cell surface markers is shown from three independent experiments with AXiAECs and one experiment with human AT2 cells and A549 cells. Results are expressed as a percentage of the live single-cell population. Data are represented as mean ± SEM.

instillation was performed at L0 time-point (day 25 on-chip) and maintained for 3 days. TER was monitored every 24 h and ELISA of the apical supernatants were assessed after 72 h of treatment (L3).

Flow Cytometry Analysis

Flow cytometry was performed on cells prior culture on-chip, denoted as D0 in this study. A549 and AXiAECs were expanded in ECM coated T75 flasks (Greiner Bio-One, Switzerland). Expanded cells were harvested before staining and resuspended in FACS staining buffer. Freshly isolated AT2 cells were used as a positive control for alveolar markers. Cells were incubated with the following fluorescently conjugated human monoclonal antibodies from Miltenyi Biotec: CD326 (Epcam)-APC (130-111-117), CD271 (LNGFR)-PE-Vio770 (130-112-792), MUC1- PE-Vio770 (130-106-838). HTI-56 and

HTII-280 from Terrace Biotech were added to the original panel and coupled, respectively, with Goat anti-Mouse IgG-PE (C#12-4010-82, eBioscience, Switzerland) and Goat anti-Mouse IgM-PE (C# M31504, Invitrogen, Switzerland) secondary antibodies in a sequential incubation. Cells were incubated on ice in the dark for 30 min. To exclude dead cells and debris, Zombie Green staining was performed prior to immunostaining. Cell acquisition was achieved using a BD FACS SORP LSRII. For analysis, a minimum of 1×10^4 events were collected and analyzed using FlowJo software version 10.8.

Fluorescence minus one (FMO) controls were used for gating strategy. Forward scatter (FCS) and side scatter (SSC) profiles allowed the exclusion of debris and doublets (P1 and P2, **Figure 2A**). Dead cells were excluded based on the Zombie Green gate. Analysis was performed on live single cell population (P3, **Figure 2A**).

qRT-PCR

Total RNA was isolated and purified using the Direct-zol™ RNA Microprep kit following manufacturer's instructions (Zymo Research, Switzerland). Cells were lysed with the supplied TRI Reagent. RNA concentration and purity were analyzed with a Nano-Drop Spectrophotometer (ThermoFischer Scientific, Switzerland). cDNA preparation was performed using the Super Script III Reverse Transcriptase kit (Life Technologies, Switzerland) according to manufacturer's instructions. qRT-PCR reactions were performed in triplicates with SYBR® Select Master Mix (Thermo Scientific) in an ABI7500 Fast (Applied Biosystems) real-time PCR system. Target gene expression was normalized to housekeeping gene expression (HPRT). The primer sequences are provided in **Supplementary Table S6**.

Immunofluorescence Staining and Imaging

Cells cultured on AX12 were fixed with 4% paraformaldehyde in DPBS. The modular chips were unscrewed from the plate to separate the fluidic and pneumatic parts. Chips were then opened with the ^{AX}Disassembly tool (AlveoliX, Switzerland) to dissociate the chip bottom part with the embedded membrane prior to staining and mounting.

Following permeabilization with 0.1% Triton X-100 (Sigma-Aldrich, Germany), cells were blocked with blocking buffer solution (2% BSA in DPBS- (Sigma-Aldrich, Germany)). Mono-culture staining was performed with: mouse anti-ZO-1 antibody (C# 33-9100, Fisher T Scientific), rabbit anti-mature SP-C antibody (C# WRAB- 76694, Seven Hills Bioreagents), mouse anti-HTI-56 (C# TB-29AHTI-56, Terrace Biotech), rabbit anti-MUC1 (C#NBP1-60046, Novus Biologicals), Epcam conjugated antibody (C# 130-111-117, Milteny Biotec), rabbit anti-HOPX (C# ab106251, Abcam), rabbit anti-ABCA3 (C# ab99856, Abcam), mouse anti-PECAM1 (#3528 Cell Signaling). Human ACE-2 Alexa Fluor 647 conjugate (C# FAB9332R, R&D Systems) and mouse anti-TRMPSS2 (clone P5H9-A3; C# sc-10184, Santa Cruz). The primary antibodies were diluted in 2% BSA/PBS and incubated overnight at 4°C. Secondary antibodies were used as follows: donkey anti-mouse Alexa Fluor 488 (C# A21202, Invitrogen), donkey anti-rabbit Alexa Fluor 568 (C# A10042, Invitrogen), were diluted 1:2,000 in 2% BSA/PBS and incubated 2 h at RT. Nuclei were stained with DAPI (C#D1306, Invitrogen). The actin cytoskeleton of the cells was visualized using the conjugated Alexa Fluor 647 phalloidin stain (C# PHDN1-A, Cytoskeleton, Inc.). Lastly, the stained membranes were sealed between two glass coverslips using mounting medium (C# F6182, Sigma-Aldrich).

Images were obtained using a confocal laser scanning microscope (Zeiss LSM 710), or Nikon Eclipse Ti-E Spinning Disk using appropriate filter settings. For fluorescent intensity calculation, Zen Blue software v2.1 (Zeiss) was used to obtain background corrected mean fluorescence intensity (MFI) for each channel of interest. To obtain comparable results from different area of interests on the membrane, identical settings for the optical and digital gain, area of focus and laser intensity was maintained. Finally, the mean fluorescence intensity (MFI) of the test channel (green channel, ZO-1) was normalized with the MFI of the blue channel (stained with DAPI).

ELISA

Collected supernatants were analyzed for IL-8 secretion using the 4-Plex ProcartaPlex custom (Thermo Scientific, Switzerland) for the Bio-Plex from Bio-Rad following manufacturer recommendations.

Statistical Analysis

All data are presented as mean ± standard error of mean (SEM). For AX12 experiments, "N" represents the experimental repetitions and "*n*" represents the number of individual wells accounted for across all experiments. For gene expression analysis, "*n*" represents the pool of three to four wells per experimental repetition. Two-tailed unpaired Student's *t*-test was used to assess significant differences using GraphPad Prism v8.0 software. Statistical significance was defined as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The exact number of repeats performed for each experiment is indicated in the corresponding figure legends.

RESULTS

Identification of Alveolar Epithelial Subsets by FACS

^{AX}iAECs were investigated for AT1 and AT2 cell-specific protein expression by flow cytometry at D0. In parallel, human primary AT2 cells isolated from lung resections and the tumor derived, alveolar cell line A549, where analysed for comparison (**Figure 2C**). Epcam is an epithelial specific cell adhesion molecule (Litvinov et al., 1997), ^{AX}iAECs and freshly isolated AT2 cells were 100% Epcam+, whereas A549 cells had mixed cell populations (Epcam+, 88.08%). Among the Epcam+ population, most ^{AX}iAECs cells were HTI-56+ cells (~99.44%), an AT1 specific cell marker, indicating a predominant AT1-like character (Dobbs et al., 1999). To investigate the AT2-like population, we screened the cells for the AT2 cell specific marker HTII-280. Our results revealed that it was predominant on primary freshly isolated AT2 cells (HTII-280+, ~96.65%), but could not be detected on ^{AX}iAECs (~0.06%) nor on A549 cells (0.43%) (Gonzalez et al., 2010).

We further investigated Mucin1, a marker expressed by AECs, known to have a higher presence in AT2 cells (Jarrard et al., 1998). Our findings were consistent with the data reported in the literature: 91.17% of the AT2 cells were positive for Muc1, whereas Muc1+ A549 cells represented 88.98% of the total population. More than 2/3 of the Epcam+ ^{AX}iAECs were positive for Muc1 (Muc1; ~63.04%). Finally, we investigated the presence of Nerve growth factor receptor (NGFR/CD271) as a negative control, given that it is a typical marker for upper airway cells. AT2 cells (NGFR, 0%) and ^{AX}iAECs were negative for this marker (~1.61%), whereas a fraction of A549 cells was positive (15.38%). Altogether, our results support the alveolar character of the ^{AX}iAECs, with predominant AT1-like characteristics when compared to AT2 cells (**Figure 2C**). A549, however, consisted in a mixed population comprising alveolar epithelial, Epcam- and NGFR+ cells.

Molecular (Alveolar) Characterization of the ^{AX}iAECs On-Chip

To investigate the cellular identity of the ^{AX}iAECs cultured on-chip in terms of alveolar cell fate, alveolar and epithelial markers were studied at day 7 at the gene and protein level (**Figure 3A**).

Fluorescence imaging revealed that ^{AX}iAECs expressed epithelial cell-specific proteins like Epcam and mucin 1 (MUC1) following 7 days of culture at the protein level. Furthermore, the AT1 cell-specific markers HOP Homeobox (HOPX) (Liebler et al., 2016) and HTI-56 (Dobbs et al., 1999) were also expressed, supporting an AT1-like character (**Figure 3B**). On the other hand, the canonical AT2 cell-specific surfactant protein C (SP-C) (Glasser et al., 1987; Warr et al., 1987) and the ATP-binding cassette sub-family A member 3 (ABCA3) (Mulugeta et al., 2002) were additionally observed on these cells, highlighting the presence and coexistence of both alveolar epithelial types on the AX12.

We further evaluated the alveolar character of the ^{AX}iAECs-on-chip at the gene level by RT-qPCR (**Figures 3C,D**). As a reference, we used A549 as a conventional *in vitro* model of the distal airway often used to assess cytotoxicity and (pro) inflammatory responses (Öhlinger et al., 2019; Barosova et al., 2021). Our results confirmed that both cell types, ^{AX}iAECs and A549, present similar transcript levels of the epithelial genes E-cadherin-1 (CDH1) after 7 days cultured on-chip. However, both the epithelial mucin MUC1 (Relative/Rel. expression ^{AX}iAECs 3.87 vs. A549 0.54; *p*-value 0.0279 and the tight junction protein 1 (TJP1) (Rel. expression ^{AX}iAECs 3.50 vs. A549 0.36; *p*-value 0.0402), demonstrated a higher expression on ^{AX}iAECs than in A549. Genes associated to the alveolar character (Caveolin1; CAV1 and ABCA3) were in a similar range for both cell lines, with surfactant protein C (SFTPC) gene showing a higher expression on ^{AX}iAECs. Besides, SFTPC gene expression increased after culture time on-chip in contrast to A549 cells (D7, **Figure 3D** vs. D0, **Supplementary Figure S1D**). Overall, the gene expression profile of ^{AX}iAECs on-chip showed transcripts levels in range with whole lung tissue and primary AT2 cells for the studied markers, with the exception of the highly AT2-specific SFTPC. A549 cells exhibited a similar gene marker profile to ^{AX}iAECs at D0 (**Supplementary Figures S1C,D**). However, marked differences were observed when comparing A549 cultured on inserts, a widely adopted *in vitro* model (Barosova et al., 2021), with their culture on the AX12 over 7 days (**Supplementary Figures S1E,F**). Culture on-chip led to a general improvement in the alveolar and epithelial character.

At the protein level, A549 cells showed expression of Epcam and HT1-56 with a few cells expressing ABCA3, in line with our qPCR observations (**Supplementary Figure S2A**). These cells, however, quickly led to the formation of thick multilayers (**Supplementary Figure S2B**), whereas ^{AX}iAECs remained stable up to D14 on-chip (**Supplementary Figure S2B**).

The zoonotic transmission of the coronavirus has been a crucial concern in the ongoing SARS-CoV-2 pandemic. Recent studies have highlighted certain key host factors that are crucial for the entry and promotion of SARS-CoV-2 infection in human host cells including Angiotensin I Converting Enzyme 2 (ACE2) and Transmembrane Serine Protease 2 (TRMPSS2) (Baggen et al., 2021). To further characterize our model of the distal airway, we analyzed the expression of ACE2 and TRMPSS2 by immunofluorescence staining and gene expression at D7. Our results showed that ^{AX}iAECs express both ACE2, TRMPSS2 at the protein and gene level (**Figures 3E,F**), and confirmed the gene expression of other genes relevant for SARS-CoV-2 infection such as Neuropilin 1 (NRP1) and Furin/Paired Basic Amino Acid Cleaving Enzyme (FURIN) on-chip over time (**Figure 3F**).

Robust Long-Term Barrier Formation On-Chip

A critical parameter for drug safety and toxicity studies *in vitro* is the recreation of a tight functional alveolar barrier (Zhang et al., 2018; Sapoznikov et al., 2019). To assess the biological impact of the alveolar microenvironment and investigate barrier integrity over time, ^{AX}iAECs were cultured on the AX12 up to 25 days in submerged conditions. We set the threshold for a robust alveolar barrier at 1,000 Ω cm^2, as this value is indicative of a tight monolayer for primary human alveolar cells with active water and ion transport (Fuchs et al., 2003). Our results demonstrated a tight barrier formation that reached the threshold at day 14–16 from cell seeding with a maximum reaching 3,000 ± 500 Ω cm^2 (**Figure 4A**). Furthermore, cells exhibited a robust barrier function (TER) across passages initiating within a similar timeframe (**Supplementary Figure S2D**). ^{AX}iAECs were additionally cultured in ALI conditions on the AX12 to replicate more faithfully the physiologically relevant alveolar barrier. Here, a strong barrier formation was again observed over time in the same time window as for submerged conditions, reaching maximal TER values of 2,300 ± 800 Ω cm^2 (**Figure 4B**).

The alveolar barrier function of the lung epithelium depends on the orchestrated interactions among tight junctions, adherens junctions and the actin cytoskeleton (Fanning et al., 1998; Ivanov et al., 2010). Therefore, we further analyzed barrier formation on-chip by investigating the expression and distribution of tight junction protein ZO-1 by immunofluorescent staining in submerged and ALI conditions for 25 days. F-actin fibres were

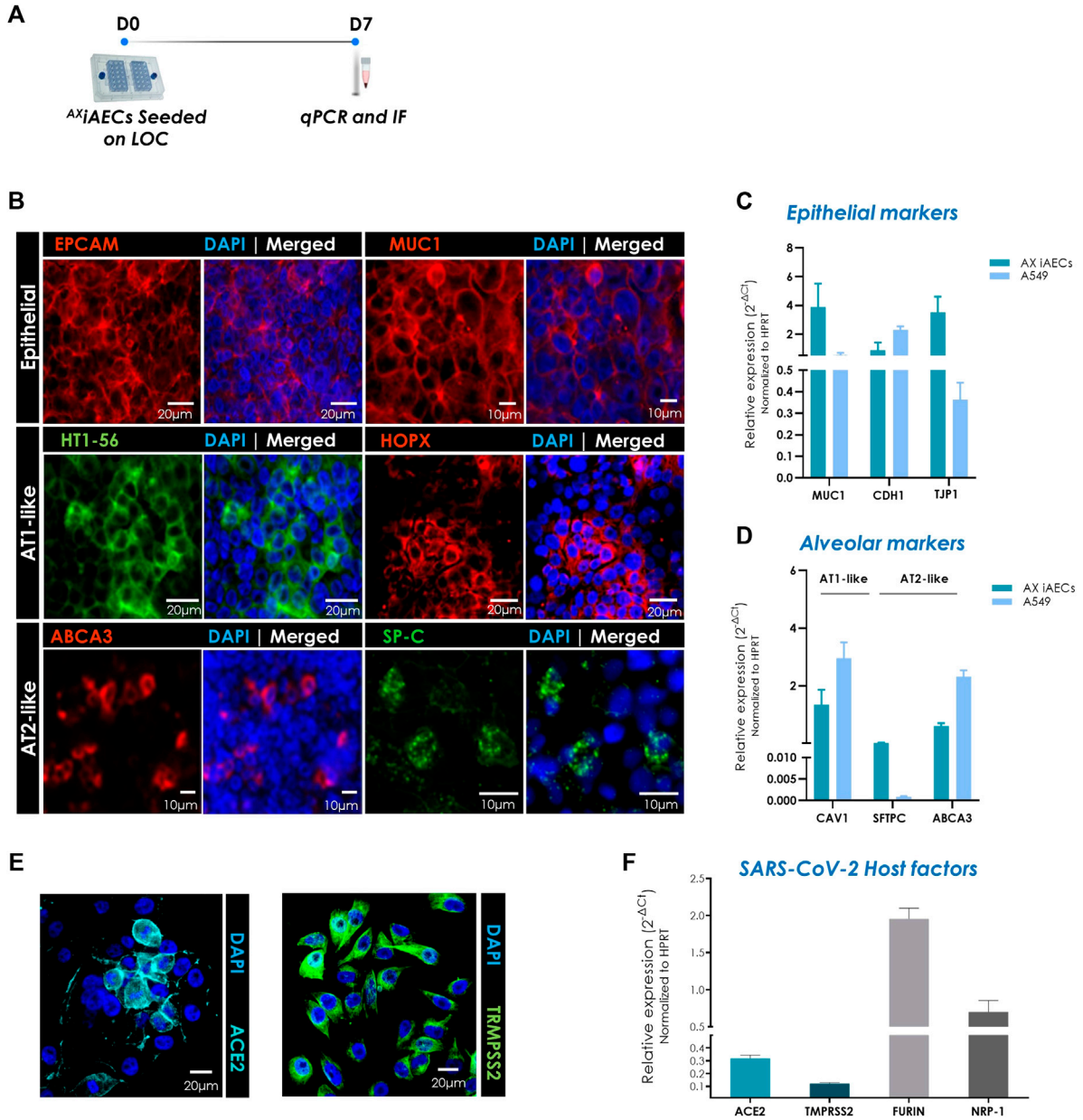

FIGURE 3 | Expression of distinct alveolar epithelial cell markers in ^{AX}iAECs cultured on ^{AX}Lung-on-chip. **(A)** Timeline and schematic of the ^{AX}iAECs cultured on the AX12. **(B)** Representative immunofluorescent staining for ^{AX}iAECs fixed after 7 days cultured in AX12 (D7), probed for epithelial: EPCAM (red), MUC1 (red); AT1-like: HTI-56 (green), HOPX (red). and AT2-like markers: SP-C (green), ABCA3 (red). Nuclei were stained with DAPI (blue). Scale bar is provided with each image. **(C)** Relative gene expression of distinct epithelial and **(D)** alveolar genes in A549 cells (N = 1; *n* = 3) and ^{AX}iAECs cultured on ^{AX}Lung-on-chip (N = 2; *n* = 4) for 7 days. **(E)** ^{AX}iAECs stained for ACE2 (cyan) and TMPRSS2 (green). **(F)** qPCR results show gene expression of SARS-CoV-2 host factors in ^{AX}iAECs (N = 2; *n* = 4) after 7 days of culture in AX12. Data are shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

also examined by phalloidin staining (**Supplementary Figure S2A**). Our results demonstrated a tight ZO-1 network encompassing the ^{AX}iAEC borders in both submerged and ALI conditions on-chip (**Figure 4C**). Interestingly, HTII-280 was detected co-localizing with ABCA3 in ^{AX}iAECs cultured in ALI, indicative of AT2-like phenotype (**Figure 4D** and **Supplementary Figure S3B**). The gene expression analysis in ^{AX}iAECs harvested from D25 on-chip confirmed a significant increase in transcript levels of AT1 cell-associated aquaporin 5 (AQP5) and AT2 cell makers (ABCA3, SFTPC) in ALI conditions compared to submerged cell culture conditions (**Figure 4E**). These results highlight the plasticity of the ^{AX}iAECs-on-chip, which adopt an *in vivo* like phenotype when cultured in physiologically relevant conditions (ALI).

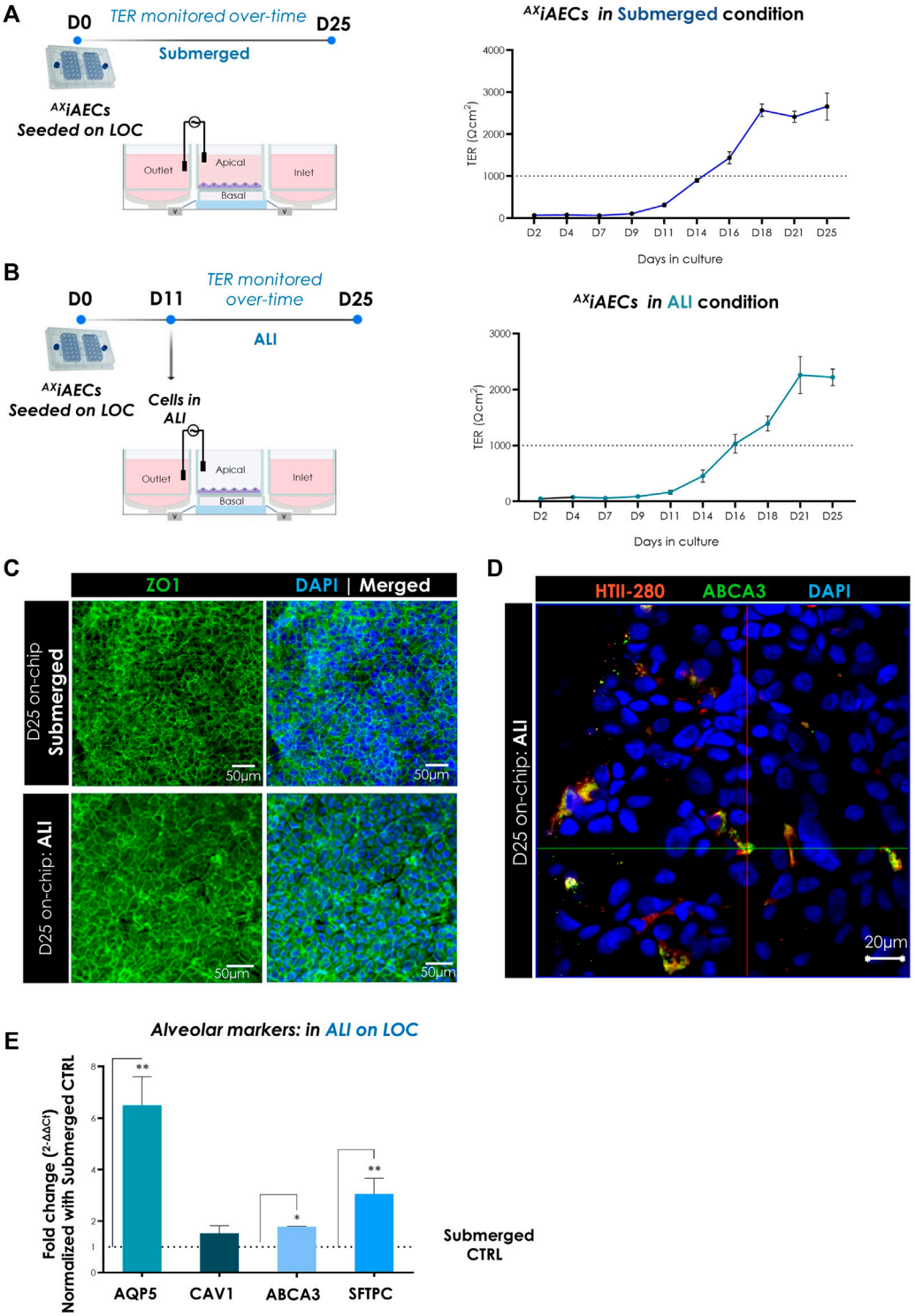

FIGURE 4 | Characterization of barrier formation on-chip. **(A)** Timeline and overall schematic of the ^{AX}iAECs cultured in submerged state on the AX12. TER (Ω cm^2) values of ^{AX}iAECs in submerged conditions on-chip until day 25 (D25) are shown. TER values above 1,000 Ω cm^2 were recorded around day 16 (N = 3; *n* = 10). **(B)** Timeline and overall schematic of the ^{AX}iAECs cultured in ALI condition starting from D5 onwards on-chip. TER (Ω cm^2) values of ^{AX}iAECs in ALI on-chip maintained until day 25 (D25) are shown. TER values above 1,000 Ω cm^2 were recorded around day 16 (N = 3; *n* = 9). **(C)** Representative image for ^{AX}iAECs in submerged and ALI conditions, fixed after 25 days culture in ^{AX}Lung-on-chip (D25) was stained for zonula occludens 1 (ZO-1; green). Nuclei were stained with DAPI (blue). Scale bar = 50 µm. **(D)** Representative staining of ^{AX}iAECs in ALI condition on-chip show co-localization of HTII-280 and ABCA3 proteins on-chip. **(E)** Relative gene expression of distinct alveolar epithelial genes in ^{AX}iAECs cultured in ALI culture compared to cells in submerged conditions (CTRL) on the AX12 (N = 1; *n* = 3). Data shown as mean ± SEM, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Effect of Physiological Cyclic Stretch on Gene Regulation

To investigate the impact of *in vivo* like breathing, ^{AX}iAECs on-chip were exposed to breathing (3D cyclic stretch, 10% linear strain, 0.2 Hz frequency) from D5 until D20 in submerged conditions (**Figure 5A**). One chip was cultured in breathing conditions starting on day 5 and the second chip of the AX12 plate was left in non-breathing control (CTRL) conditions (**Figure 5A**) for each experiment. Fifteen days post-stretch, cells were harvested and relative gene expression levels were compared between breathing and non-breathing conditions. To assess the involvement and reorganization of actin cytoskeletal filaments in response to biomechanical stretch, phalloidin staining was performed on the cells. In breathing conditions, cells displayed enhanced expression of F-actin filaments relative to cells in static culture conditions (**Figure 5B**). To obtain more detailed insight into the molecular alterations triggered by breathing conditions, differential gene expression was analyzed. Among the epithelial markers, the genes CDH1 and MUC1 (p-value 0.0201) were significantly overexpressed in breathing condition, whereas ZO-1 (p-value 0.4091) levels remained same (**Figure 5C**). The AT1 cell associated genes AQP5 (p-value 0.0002) and CAV1 (p-value 0.2492) exhibited an increasing trend in breathing cells (**Figure 5C**). The AT2 cell-specific gene coding for SFTPC (p-value 0.0043) also demonstrated a significant upregulation in breathing conditions relative to CTRL non-breathing cells, while HHIP (Hedgehog-Interacting Protein; p-value 0.992) remained unaltered (**Figure 5C**).

TGFβ1 Pro-Fibrotic Induction in ^{AX}iAECs On-Chip

To evaluate the application of the ^{AX}iAECs on-chip model for lung injury, the pro-fibrotic mediator TGFβ1 was used to induce a fibrogenic response as it would occur during wound healing. For this, ^{AX}iAECs were cultured on-chip until barrier formation and subsequently treated with 5 or 10 ng/ml TGFβ1 on day T0 for 72 h (T3) (**Figure 6A**). Absolute TER (Ω cm^2) measured on day T3 demonstrate significant and dose-dependent decrease in TGFβ1 instilled cells, indicative of a disrupted barrier (**Figure 6B**). Furthermore, significant barrier disruption occurred from 24 h onward (**Supplementary Figure S5B**) for both concentrations of TGFβ1 used. Normalized fluorescence intensity assessment confirmed a trend in the reduction of ZO-1 expression in TGFβ1 treated cells compared to CTRL vector treated cells (**Figure 6C**; **Supplementary Figure S5D**). It is known that TGFβ1 treatment in AECs promotes epithelial-mesenchymal transition (EMT) *in vitro* (Kasai et al., 2005). To investigate this further, differential gene expression analysis was performed with TGFβ1 treated (5, 10 ng/ml) and CTRL cells harvested at day T3. Significant and concentration-dependent reduction of CDH1 gene expression (Rel. fold change for 5 ng/ml = 0.55; for 10 ng/ml = 0.23) (**Figure 6D**) and heightened levels of ACTA2 (α smooth muscle actin; Rel. fold change for 5 ng/ml = 3.47; for 10 ng/ml = 5.69) (**Figure 6D**) and COL1A1 (collagen 1α1; Rel. fold change for 5 ng/ml = 4.60; for 10 ng/ml = 4.69) (**Supplementary Figure S5C**) transcript levels were observed in TGFβ1 treated ^{AX}iAECs on-chip, characteristic of an ongoing EMT mechanism.

Influence of LPS Treatment in ^{AX}iAECs On-Chip

Secondly, we evaluated the suitability of the ^{AX}iAECs on-chip model in context of inflammation. We increased the model complexity to bring the system closer to the *in vivo* situation, where not only inflammation but also vascular leakage and edema were considered. To this end, ^{AX}iAECs were co-cultured with endothelial cells (hLMVEC) and exposed to a commonly used proinflammatory trigger, bacterial LPS, in the presence of immune cells.

The co-culture-on-chip model demonstrated a strong barrier formation with a rapid and sustained TER increase reaching more the barrier integrity threshold at D16 (1,000 Ω cm^2) (**Figure 7A**). This model was characterized by a compact and homogenous epithelial cell layer with defined cell borders (**Figure 7B**), and endothelial cells displaying a typical cobblestone morphology decorated with PECAM-1 (CD31) at the cell membrane (Gaugler et al., 2004) (**Figure 7B**).

To simulate inflammation, the model was challenged with 0.1 μg/ml LPS after barrier formation (**Figure 7C**). At the time of treatment, PBMCs were introduced into the basolateral compartment to simulate cell-cell crosstalk in a proinflammatory setting. The treatment with LPS resulted in a time dependent drop in barrier function up to 72 h (L3) (**Figures 7D,E**). Upon barrier dysfunction, apical supernatants were collected and analyzed by ELISA to evaluate the impact at the epithelial compartment. Our results indicated a significant increase in the secretion of IL-8 cytokine levels for LPS stimulated cells relative to the control condition, pointing out the responsiveness of this co-culture model on-chip to proinflammatory stimuli (**Figure 7F**).

DISCUSSION

Alveolar barrier disruption is a hallmark event in fatal pulmonary conditions like ARDS, emphysema and idiopathic pulmonary fibrosis (Yanagi et al., 2015; Bärnthaler et al., 2017; Hou et al., 2019). The discovery of appropriate pharmaceutical compounds for such chronic diseases requires robust pre-clinical *in vitro* models that can mimic a disrupted alveolar barrier. Primary AECs remain the gold standard in terms of physiological relevance for modelling distal lung. However, they present some limitations, including donor to donor variability, scarcity, spontaneous differentiation *in vitro* and controversial use of "healthy" human tissue obtained from tumor resections. For this reason, continuous cell lines represent an interesting and suitable alternative for high throughput screening of toxic molecules or drugs in a preclinical context. Therefore, to ensure reproducibility, robustness, and stability of culture conditions, we characterized a novel distal airway model consisting of a new immortalized human alveolar epithelial cell line (^{AX}iAECs) cultured in an *in vivo* like alveolar microenvironment (^{AX}Lung-on-chip system).

^{AX}iAECs cultured on-chip developed stable alveolar barrier function preserved across passages with an increased range of

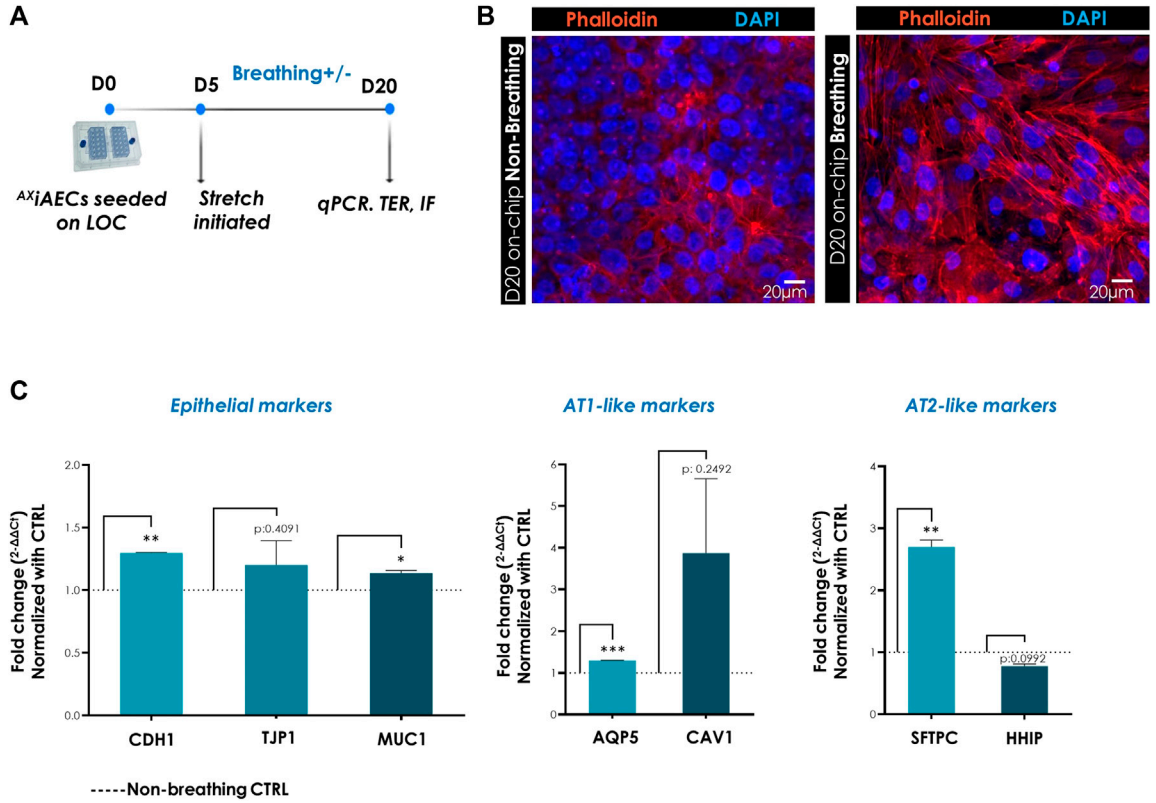

FIGURE 5 | Breathing-induced differential gene expression in ^{AX}iAECS on-chip. **(A)** Timeline and schematic of the ^{AX}iAECs cultured in the ^{AX}Lung-on-chip. Breathing was started at D5 on-chip. **(B)** Immunofluorescent stainings of ^{AX}iAECs in breathing and non-breathing (CTRL) conditions, fixed after 20 days culture on AX12 and stained with phalloidin to visualize F-actin fibers (red). Nuclei were stained with DAPI (blue). Scale bar = 20 µm. **(C)** Normalized gene expression of epithelial cell associated, alveolar type I cell specific and alveolar type II cell specific markers following 20 days of culture in AX12. Data shown as mean ± SEM (N = 2; *n* = 4), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TER values (2,000–3,500 Ω cm^2) in both mono and co-culture with endothelial cells (**Figures 4**, **7**; **Supplementary Figure S2D**). Conversely, barrier forming features are not common for other established alveolar cell lines representative for AECs (van den Bogaard et al., 2009; Nalayanda et al., 2009). The formation of a stable alveolar barrier requires the combined cooperation of various junctional complexes especially tight junctions (like occludins, zonula occludens, claudins) adherens junctions (like cadherins) and actin cytoskeletal filaments (Ivanov et al., 2010; Campbell et al., 2017). Here, both ^{AX}iAECs and A549 cells cultured on-chip (Day 7) demonstrated an increased expression of E-Cadherin (CDH1, **Figure 3C**) and ZO-1 (**Figure 3C**) compared to their levels at the time of seeding (**Supplementary Figure S1C**). Functionally however, ^{AX}iAECs formed a tight barrier characterized by high TER values in contrast to A549, which do not reach barrier formation (Ren et al., 2016; Leibrock et al., 2019). Besides, A549 cells exhibited multilayer formation already from D7 (**Supplementary Figure S2B**), whereas, according to our findings, ^{AX}iAECs retained a more stable structure even at later timepoints (**Supplementary Figure S2C**). Hence, despite both cellular models are promising considering the alveolar markers described here, the carcinoma origin of A549 cells, their inability of forming a TER-tight barrier and their quick multilayer formation, points out ^{AX}iAECs on-chip as a more suitable model for the alveolar epithelial barrier. In terms of alveolar phenotype, several cell lines preserve some of the relevant alveolar epithelial markers at gene and protein levels. The recently established hAELVi cell line exhibits an ATI-like phenotype including high TER and tight junction formation, as well as caveolin-1 expression (Kuehn et al., 2016; Metz et al., 2020). However, hAELVi cells do not exhibit AT2 markers (such as SP-C) (Kuehn et al., 2016). AT2-associated pulmonary surfactant proteins (SP) and lipids play a key role in balancing respiratory dynamics and regulating alveolar inflammation (Cañadas et al., 2020), for instance by inhibiting the JAK/STAT activation pathway (Jin et al., 2018), binding LPS (SP-C) (Augusto et al., 2003) or opsonizing pathogens (SP-A, SP-D) (Crouch and Wright, 2001; Nayak et al., 2012). In this work, the AT1 cell specific HTI-56 and Homeobox only protein x (HOPX), a protein involved in adult lung alveolar injury and fibrosis progression (Ota et al., 2018), were observed on-chip at the protein level (**Figure 3B**). Other AT1 associated genes (AQP5, and CAV1, **Figures 5C**, **3C**), were detected, consistent with the ^{AX}iAECs-on-chip exhibiting an AT1-like phenotype. On the

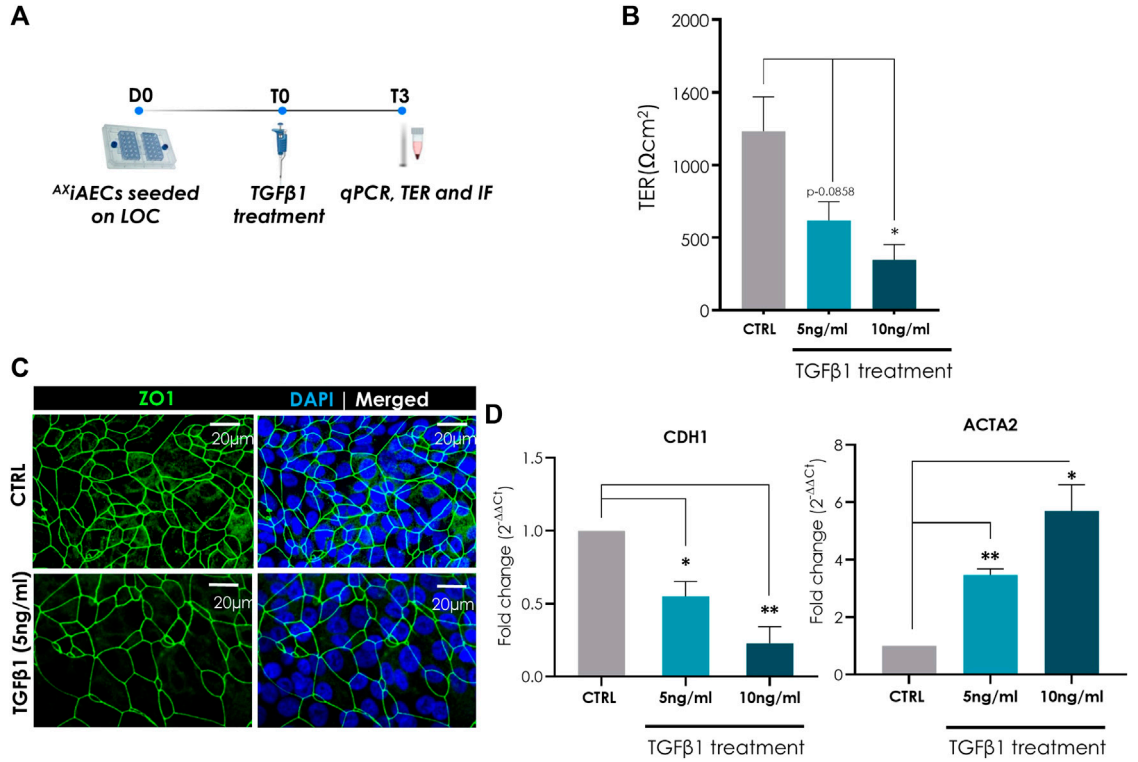

FIGURE 6 | TGFβ1 treatment induced EMT in ^{AX}iAECs on-chip. **(A)** Timeline and schematic of TGFβ1 treatment on day T0 in ^{AX}iAECs. Cells were harvested and fixed for imaging 72 h (T3). **(B)** Significant dose-dependent reduction of TER (Ω cm^2) values recorded on day T3 after treatment with TGFβ1. **(C)** Representative immunofluorescence staining of Control (untreated) and 5 ng/ml TGFβ1 treated cells (N = 2; *n* = 6). Cells fixed on day T3 and stained for ZO-1 (green). Reduced fluorescence intensity of ZO-1 observed in TGFβ1 (5 ng/ml) treated cells on-chip. **(D)** Normalized gene expression of EMT-associated cellular markers assessed following 3 days of treatment with TGFβ1 (N = 2; *n* = 4) on day T3. Data shown as mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

other hand, quantification of gene expression and immunofluorescence staining confirmed the long-term expression of SP-C, and proteins involved in surfactant metabolism (ABCA3) in the ^{AX}iAECs cultured on the AX12 (**Figures 3B–D**). Although, expression of SFTPC in ^{AX}iAECs was low at D0 (**Supplementary Figure S1D**) compared to whole lung and primary AT2 cells (**Supplementary Figure S1B**), a progressive and robust increase was observed over time on-chip at the protein and gene level (**Figures 3B,D**). Besides, breathing dynamics and ALI were also found to further enhance the expression of this AT2 marker (**Figures 4E**, **5C**).

The impact of the lung-on-chip micro-environment as a driver for physiological lung markers was also observed for A549. Our results showed that these cells cultured on the ^{AX}lung-on-chip displayed a significant increase of epithelial (CDH1) and alveolar (CAV1, ABCA3) markers (**Supplementary Figures S1E,F**) compared to culture on inserts within a similar time-frame. Altogether, our findings emphasize the crucial role of integrating physiological parameters in culture conditions.

We also investigated the presence of Mucin1 (Muc1), a membrane associated mucin released mainly by AT2 cells, with a minor presence in AT1 cells (Astarita et al., 2012). Recent studies have established that MUC1 or its released fragment, KL6 are prominent membrane markers mediating the expression of anti-inflammatory genes in interstitial lung diseases, asthma, COPD and lung cancer (Ishikawa et al., 2011; Ishikawa et al., 2012; Milara et al., 2018; Milara et al., 2019). Our results showed that ^{AX}iAECs on-chip model expressed this clinically relevant target, at both protein and gene transcription levels (**Figures 2**, **3B,C**), at higher levels than A549 cells on-chip (**Figure 3C**) indicating its potential applications to model lung disease.

Physiological cues including breathing and inter facial stresses are particularly relevant in the mature and developing lung (Waters et al., 2012; Knudsen and Ochs, 2018), and are crucial to recapitulate *in vivo* like functions in AECs (Singer et al., 2003; Stucki et al., 2018; Van Riet et al., 2020; Diem et al., 2020). In our model, we have demonstrated that physiological breathing conditions (3D stretch, 10% linear strain, 0.2 Hz) and ALI enhanced both barrier properties and the alveolar character of ^{AX}iAECs-on-chip. Breathing-like stretch resulted in an increased expression of the junction complex associated genes CDH1 and ZO-1 (**Figure 5C**), indicating robust barrier formation consistent with previous findings on stretched AECs (Stucki et al., 2018; Huang et al., 2021). In addition, AT1 cell specific gene CAV1 and AT2 cell specific SFTPC transcript levels were also increased under breathing conditions, suggesting that stretch supports alveolar features and barrier function (Sanchez-Esteban et al., 2001; Diem et al., 2020).

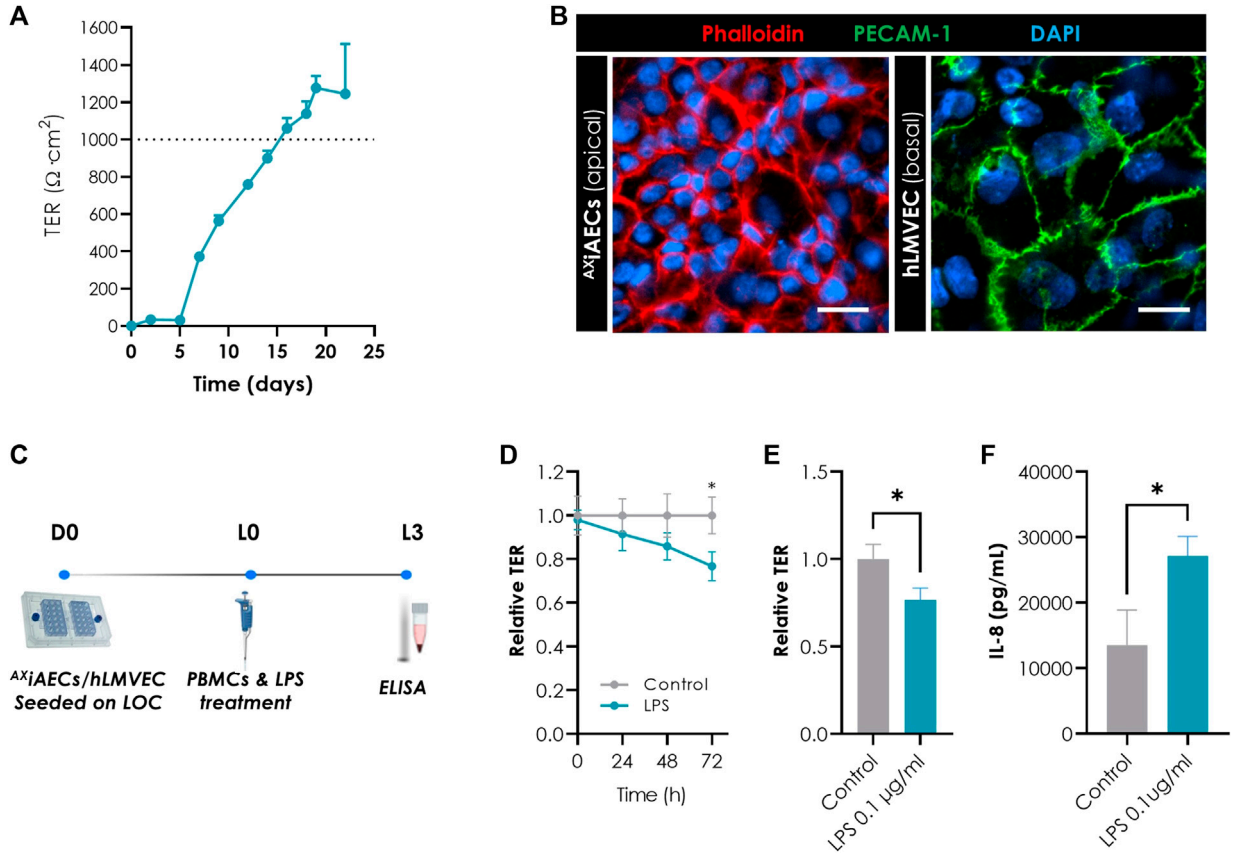

FIGURE 7 | LPS-induced inflammation in ^{AX}iAECs/hLMVEC co-culture on-chip. **(A)** TER values above 1,000 Ω cm^2 (dotted line) were recorded from day 16 (N = 4, *n* = 42). **(B)** Representative image of ^{AX}iAECs/hLMVEC co-culture stained with phalloidin (red) and PECAM-1 (green). Nuclei were stained with DAPI (blue). Scale bar is 20 µm. **(C)** Timeline and schematic of LPS treatment on day L0 in ^{AX}iAECs/hLMVECs/PBMCs. Cells were harvested 72 h after stimulation with 0.1 µg/ml LPS (L3). **(D)** Significant reduction on TER values (relative to L0 and control conditions) recorded on day L3 after treatment with LPS (control, N = 3; *n* = 7; LPS treated: N = 3; *n* = 9). **(E)** IL-8 secretion from control (untreated) and LPS (0.1 µg/ml) treated cells were determined by ELISA on L3 (control, N = 3; *n* = 6; LPS, N = 3; *n* = 7). **(F)** IL-8 secretion from control (untreated) and LPS (0.1 µg/ml) treated cells were determined by ELISA on L3 (control,N = 3; *n* = 6; LPS, N = 3; *n* = 7). Data shown as mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ALI, is known to be relevant for surfactant release and alveolar cell function (Ravasio et al., 2011; Hobi et al., 2012). Therefore, lung models including this physiological parameter represent more faithfully the *in vivo* situation to better understand alveolar function and chronic lung diseases (Pezzulo et al., 2011; Van Riet et al., 2020). To investigate the effect of ALI on our model, ^{AX}iAECs were cultured under ALI conditions for 2-weeks on the AX12 (**Figure 4B**). ALI-cultured ^{AX}iAECs demonstrated a tight functional barrier in long term culture conditions with TER above 1,000 Ω cm^2 (**Figure 4B**) consistent with previous observations on human AECs (Stucki et al., 2018; Huang et al., 2021). Furthermore, we found a significant upregulation of AT1 and AT2 markers (AQP5, SFTPC and ABCA3) in ALI culture compared to submerged conditions (**Figure 4E**), consistent with the preservation of alveolar markers of primary alveolar cells on ALI chips (Van Riet et al., 2020) and similar to the observations from a recent study on A549 (Wu et al., 2018). In this work, ALI conditions enhanced the alveolar character of this cell line, leading to an increased expression of AT1 and AT2 markers (Wu et al., 2018).

Research efforts over the past two decades have focused on streamlining drug development pipeline to reduce animal experimentations according to the 3R principles (replace, reduce and refine) (Ehrhardt and Kim, 2007). NAMs have gained a central role during the recent respiratory disease pandemic (COVID-19), accentuated by the limited recapitulation of critical features of COVID-19 pathogenesis in animal models (Kiener et al., 2021). Besides, it has been shown that life threatening forms of the disease (pneumonia, ARDS) result from the infection of AT2 cells in the distal lung (Mulay et al., 2020). These facts have added to the increasing need for predictive *in vitro* human models of the distal lung, for which conventional models, such as A549, have shown limited suitability in terms of infectivity or viral propagation (Si et al., 2021). On-chip approaches have proven to be efficient when recapitulating relevant COVID-19 hallmarks in the upper (Si et al., 2021) and in the distal lung: alveolar-capillary barrier, differential alveolar epithelial and endothelial host cell responses, replication of viral particles in the alveolar epithelium and increased inflammatory cytokine release (Zhang et al., 2020;

Thacker et al., 2021). These studies have also provided insights into the molecular mechanisms of viral infection and replication relevant for drug repurposing (Si et al., 2021), as well as different cell targets in addition to AT2 cells, including HTII-280- ACE2+ cells (Zhang et al., 2020), or lung microvascular endothelial cells (Thacker et al., 2021). Here, we have demonstrated that our cell line on-chip model expresses key host cell factors for viral infection (ACE2, TMPRSS2, Furin and NRP1) (Hoffmann et al., 2020a; Hoffmann et al., 2020b; Kyrou et al., 2021) after 7 days of culture (**Figure 3E**), underlining the potential for the ^{AX}iAECs on-chip model as a tool to investigate key pathways for productive infection with SARS-CoV-2.

To confirm the application of ^{AX}iAECs on-chip as an *in vitro* model for disease modelling and toxicity screening, we exposed them to TGFβ1 or LPS to simulate key events during lung regeneration and inflammation. After TGFβ1 treatment, the alveolar barrier was significantly disrupted as measured by TER (**Figure 6B**; **Supplementary Figure S5B**) in agreement with previous studies (Pittet et al., 2001; Overgaard et al., 2015). TGFβ1 treatment is associated with the induction of Epithelial-mesenchymal transition (EMT), an intricate and orchestrated mechanism where epithelial cells lose their specific markers and adopt a mesenchymal cell phenotype in response to stress or injury (Kalluri and Weinberg, 2009). Until now, the occurrence of EMT in microfluidic models has only been reported in the context of cancer studies (Dhawan et al., 2018; Liu et al., 2020; Cho et al., 2021). Interestingly, our results suggest EMT occurrence in the ^{AX}iAECs when treated with TGFβ1, which is evidenced by the decrease in the epithelial specific marker, CDH1, and the increase in mesenchymal cell targets such as ACTA2 and COL1A1 (**Figure 6D**; **Supplementary Figure S5C**). In addition, TGFβ1 treatment was found to induce the loss of ZO-1 in human epithelial cells (Zhang et al., 2013). Consistent with this work, reduced protein expression was observed in cells treated with TGFβ1 on-chip (**Figure 6C**). Future studies addressing the signaling pathway mechanism for TGFβ1 response at the alveolar epithelium would benefit from the inclusion of lung fibroblasts, a key player in profibrotic remodelling. These cells can be introduced in co-culture with ^{AX}iAECs on-chip and used as a preclinical model for safety and efficacy testing as previously reported (de Maddalena et al., 2021).

Finally, the response of this cellular model to inflammatory stimuli was investigated. LPS was used as a pro-inflammatory stimulus in an advanced model of the air-blood barrier including ^{AX}iAECs co-cultured with endothelial cells (hLMVECs). LPS treatment in the presence of the immune component (PBMCs), led to a significant disruption of the alveolar barrier after 72 h, which was observed by a decrease in TER as previously described (Roldan et al., 2019). This is consistent with a leakier air-blood barrier as observed in patients with sepsis-associated acute lung injury (Meng et al., 2010; Kobayashi et al., 2016). This decrease in TER was accompanied by an increase in proinflammatory cytokine IL-8 secretion from the alveolar epithelial cell compartment, as previously reported (**Figure 7F**) (Standiford et al., 1990; Witherden et al., 2004; Roldan et al., 2019), reflecting the crosstalk between the different players involved including the immune component and endothelial cells. IL-8 is a neutrophil chemoattractant chemokine (Henkels et al., 2010), suggesting that our *in vitro* observations may precede immune cell recruitment and neutrophil infiltration *in vivo*, which is associated with further barrier damage and inflammation and is a hallmark of various inflammatory lung diseases (Grommes and Soehnlein, 2010; Yang et al., 2021).

Altogether, our results point out that physiologically mimicking cell culture conditions are essential to recapitulate crucial features of alveolar AT1 and AT2 cells *in vitro*. More specifically, we have shown that long term culture, breathing and ALI conditions provided by the chip technology support an enhancement of AT1- and AT2-like phenotypes on the ^{AX}iAECs cultured on-chip, resembling functional coexistence. Recent studies highlight the relevance of simulating the *in vivo* lung microenvironment (structure, cell types and mechanical cues) (Ainslie et al., 2019; Nawroth et al., 2019) and the key role of mechanical forces during lung development and function (Waters et al., 2012). Along with those works, our data reinforces the essential role of easy-to-use organ-on-chip technology combined with well-characterized and physiological functioning proper cell types to tackle the need for predictive preclinical human models. These models are beneficial for basic research, but additionally, have precise importance for industrial applications, including toxicity testing of inhaled molecules, and drug safety and efficacy studies. Particularly in the industrial setting, cell line-based organs-on-chip such as the ^{AX}iAECs on-^{AX}Lung-on-chip represent a path forward to provide qualified models with higher throughput, robustness, and automatization opportunities, which are essential factors for the standardization and validation of this technology with a regulatory scope (Ainslie et al., 2019; Clapp et al., 2021; Rusyn and Roth, 2021).

The future addition of other cellular components like alveolar macrophages (Mubarak et al., 2018), or modelling inhalation exposure *in vitro*, will be of high relevance to further establish this technology as a reference NAM for xenobiotics toxicity assessments, as well as for drug safety and efficacy applications (Artzy-Schnirman et al., 2019; Ding et al., 2020; Sengupta et al., 2021).

CONCLUSION

In summary, the breathing ^{AX}iAECs on-chip model recapitulates critical aspects of the alveolar microenvironment including air-blood barrier function, the breathing motion and long-term culture conditions in ALI for cell differentiation. The new cell line ^{AX}iAECs on-chip, forms a tight barrier with TER values above 1,000 Ω cm^2, expresses key AT1 and AT2 markers and SARS-CoV-2 infection-associated cellular host factors. Besides, we have demonstrated that this model responds to profibrotic and proinflammatory triggers that elicit physiological responses such as EMT and inflammation, respectively.

In addition to simple handling, this model allows reproducible cell culture conditions that are pivotal for drug-development and toxicity screening studies. Altogether, our current results suggest that this alveolar barrier on-chip model will be a valuable tool for precision medicine applications in the future and is a promising

alternative to animal models currently used for respiratory research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AS, NR, NH, and OG conceived and developed the overall pipeline of this study. AS and NR performed and analysed experiments with other authors assisting with experiments and data analysis. MK assisted with characterization of the SARS-CoV-2 host factors and qPCR for A549 cells. LF performed the FACS analysis. GR and TI assisted with the LPS inflammation experiments. JS and AR were involved in the technical development of the AX12 platform and LM assisted in biological validation of the AX12. TM assisted in the cell line (^{AX}iAECs) development. NSD and PC assisted in the cell line (^{AX}iAECs) characterization. AS and NR wrote the manuscript. PC, C-ML, MK-dJ, TM, and TG provided the input in their respective field of expertise and contributed to data interpretation. All authors provided feedback and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2022.840606/full#supplementary-material

Supplementary Figure S1 | Differential gene expression of ^{AX}iAECs and A549 cells. **(A)** Relative epithelial and **(B)** alveolar gene expression of freshly isolated primary AECs (N = 2 donors; *n* = 4) and from resected healthy human lung tissues (Whole lung; N = 1; *n* = 2). **(C)** Comparative qPCR reveals distinct epithelial and **(D)** alveolar gene expression levels in A549 cells (N = 1; *n* = 3) and ^{AX}iAECs at Day 0 (N = 2; *n* = 4). A549 cells cultured on AX12 for 7 days (N = 1; *n* = 3) show improved **(E)** epithelial and **(F)** alveolar marker levels compared to when cultured on inserts for 7 days (N = 1; *n* = 3). Data shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure S2 | Characterization of A549 cells on ^{AX}lung-on-chip. **(A)** Representative immunofluorescent stainings of Epcam (yellow), HT1-56 (green), ABCA3 (red) and DAPI (blue) in A549 cells, fixed after 7 days cultured on ^{AX}Lung-on-chip. **(B)** Orthogonal view for A549 cells on-chip (HT1-56 in green, DAPI in blue) at Day 7, **(C)** Orthogonal view for ^{AX}iAECs (actin in orange, DAPI in blue) at Day 14 on-chip. Scale bar = 20 µm. **(D)** TER progression was measured across increasing passages (P27–P31) of ^{AX}iAECs.

Supplementary Figure S3 | Air-liquid interface culture conditions on-chip improve the alveolar character of ^{AX}iAECs. **(A)** Representative immunofluorescent images for ^{AX}iAECs on-chip stained for phalloidin (red) in ALI conditions show a tight network of actin filaments, fixed after 25 days culture (D25). Nuclei were stained with DAPI (blue). **(B)** Immunofluorescent stainings of ^{AX}iAECs in ALI condition on-chip demonstrate co-localization of HTII-280 (red) and ABCA3 (green) on-chip. Scale bars provided with each image.

Supplementary Figure S4 | Breathing induced gene expression changes of SARS-CoV-2 host factors in ^{AX}iAECs on-chip. **(A)** Timeline and schematic of the ^{AX}iAECs cultured in the ^{AX}Lung-on-chip. Breathing was started at D5 on-chip. **(B)** Normalized qPCR data show gene expression of SARS-CoV-2 associated markers following 20 days of culture in AX12. Data shown as mean ± SEM (N = 1; *n* = 2).

Supplementary Figure S5 | Time-course investigation of alveolar barrier changes in TGFβ1 treated ^{AX}iAECS on-chip. **(A)** Timeline and schematic of TGFβ1 treatment on day T0 in ^{AX}iAECs. TER (Ω cm^2) was measured every 24 h until day T3 after treatment. **(B)** Reduced TER (Ω cm^2) values, normalized to day T0 (before TGFβ1 treatment) were recorded until day T3 in cells treated with 5 and 10 ng/ml TGFβ1 (N = 1; *n* = 3). **(C)** Relative gene expression of collagen 1α1 (COL1A1) was assessed in cells following 3 days of treatment with TGFβ1 (N = 1; *n* = 3). Data shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Table S6 | Primer sequences.

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Use of new approach methodologies (NAMs) to meet regulatory requirements for the assessment of industrial chemicals and pesticides for effects on human health

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New approach methodologies (NAMs) are increasingly being used for regulatory decision making by agencies worldwide because of their potential to reliably and efficiently produce information that is fit for purpose while reducing animal use. This article summarizes the ability to use NAMs for the assessment of human health effects of industrial chemicals and pesticides within the United States, Canada, and European Union regulatory frameworks. While all regulations include some flexibility to allow for the use of NAMs, the implementation of this flexibility varies across product type and regulatory scheme. This article provides an overview of various agencies' guidelines and strategic plans on the use of NAMs, and specific examples of the successful application of NAMs to meet regulatory requirements. It also summarizes intra- and inter-agency collaborations that strengthen scientific, regulatory, and public confidence in NAMs, thereby fostering their global use as reliable and relevant tools for toxicological evaluations. Ultimately, understanding the current regulatory landscape helps inform the scientific community on the steps needed to further advance timely uptake of approaches that best protect human health and the environment.

KEYWORDS

new approach methodologies (NAMs), *in vitro*, *in silico*, risk assesment, toxicity testing, industrial chemicals, pesticides

1 Introduction

Regulatory agencies are tasked with ensuring protection of human health and the environment, and implementing various processes for achieving this goal. Legal frameworks that do not require upfront toxicological testing have relied heavily on chemical evaluations using analogue read across and grouping based on chemical categories, while others with upfront testing requirements have relied on prescribed checklists of toxicity tests, often using animals to fulfill the required testing. However, scientific advancements have led to investments in the development, implementation, and acceptance of reliable and relevant new approach methodologies (NAMs). NAMs are defined as any technology, methodology, approach, or combination that can provide information on chemical hazard and risk assessment without the use of animals, including *in silico*, *in chemico*, *in vitro*, and *ex vivo* approaches (ECHA, 2016b; EPA, 2018d). NAMs are not necessarily newly developed methods, rather, it is their application to regulatory decision making or replacement of a conventional testing requirement that is new.

Regulatory agencies worldwide have recognized the importance of the timely uptake of fit for purpose NAMs for hazard and risk assessment and are introducing flexible, efficient, and scientifically sound processes to establish confidence in the use of NAMs for regulatory decision-making (van der Zalm et al., 2022; Ingenbleek et al., 2020). The use of NAMs has been prioritized because of their ability to efficiently generate information that, once established to be as or more reliable and relevant than the conventional testing requirement, may be used to make regulatory decisions that protect human health. NAMs can mimic human biology and provide mechanistic information about how a chemical may cause toxicity in humans. They can also be used to inform population variability, for example, by rapidly identifying susceptible subpopulations from potential exposures in fence line communities or workers, and by allowing for the consideration of individualized health risks and the generation of data tailored to people with pre-existing conditions or those more sensitive to certain chemicals (EPA, 2020e).

This article describes opportunities for and examples of the use of NAMs in regulatory submissions for industrial chemicals and pesticides in the United States (US), Canada, and the European Union (EU). For industrial chemicals, it includes the US Environmental Protection Agency (EPA)'s Office of Pollution Prevention and Toxics (OPPT), the US Consumer Products Safety Commission (CPSC), Health Canada (HC)'s Healthy Environments and Consumer Safety Branch (HECSB), and the European Chemicals Agency (ECHA). For pesticides and plant protection products (PPP), it highlights the EPA's Office of Pesticide Programs (OPP), HC's Pest Management Regulatory Agency (PMRA), and the European Food Safety Authority (EFSA). This article also provides examples of collaborations, across sectors and borders, to build scientific, regulatory, and public confidence in the use of NAMs for the protection of human health, and to reach the ultimate goal of global acceptance. Tables 1, 2 summarize some of the guidance, strategic plans, and other helpful documentation related to the implementation of NAMs. While this article addresses the assessment of human health effects of industrial chemicals and pesticides in the US, Canada, and the EU, similar collaborative efforts and opportunities to use NAMs in regulatory submissions exist in other sectors and countries. Furthermore, many of the discussed actions and efforts also likely apply to other types of chemicals and to ecotoxicological effects.

2 Overarching activities to advance the implementation of NAMs

2.1 International collaboration

The Organisation for Economic Co-operation and Development (OECD) publishes guidelines for the assessment of chemical effects on human health and the environment. Under the mutual acceptance of data (MAD) agreement among the 38 OECD member countries, which aims to reduce duplicate testing, "...data generated in the testing of chemicals in an OECD member country in accordance with OECD Test Guidelines (TG) and OECD Principles of Good Laboratory Practice (GLP), shall be accepted in other member countries" (OECD, 2019). A portion of the nearly 100 OECD test guidelines describe *in chemico, in vitro,* or *ex vivo* methods that are accepted by certain regulatory agencies for the testing of various types of chemicals. At their discretion, agencies can decide which OECD test guidelines to require and whether to accept non-OECD guideline methods (OECD, 2019). Building toward regulatory implementation of non-guideline methods, parallel OECD efforts are working to advance the development of best practices, guidance, data integration and evaluation frameworks such as Integrated Approaches to Testing and Assessment (IATA) and Adverse Outcome Pathways (AOPs).

The International Cooperation on Alternative Test Methods (ICATM) was originally established in 2009 by the US Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM), HC, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM), and the Japanese Center for the Validation of Alternative Methods (JaCVAM) to facilitate cooperation among national validation organizations. Since its establishment, Korea (KoCVAM) has signed the agreement and China, Brazil (BraCVAM), and Taiwan participate in ICATM activities. In 2019, Canada established the Canadian Centre for the Validation of Alternative Methods (CaCVAM). Each group works in-country and collaboratively to advance NAMs. For example,

TABLE 1 US, Canada, and EU: industrial chemicals and household products.

Agency	Strategic plans, guidance, and other documentation for the implementation of NAMs referenced in this manuscript
EPA OPPT	• Interim science policy: use of alternative approaches for skin sensitization as a replacement for laboratory animal testing EPA, (2018b)
	• Strategic plan to promote the development and implementation of alternative test methods within the TSCA program EPA, (2018d)
	• Utility of *In Vitro* Bioactivity as a Lower Bound Estimate of *In Vivo* Adverse Effect Levels and in Risk-Based Prioritization Paul Friedman et al. (2020) [a]
	• List of alternative test methods and strategies (or new approach methodologies [NAMs]), Second update: 4 February 2021 EPA, (2021d)
	• New approach methods work plan, reducing use of animals in chemical testing EPA, (2021e) [b]
	• A WoE Approach for Evaluating, in Lieu of Animal Studies, the Potential of a Novel Polysaccharide Polymer to Produce Lung Overload Ladics et al. (2021)
CPSC	• Recommended Procedures Regarding the CPSC's Policy on Animal Testing (16 CFR Part 1500)
	• Guidance on Alternative Test Methods and Integrated Testing Approaches CPSC, (2022)
HC HECSB	• Fact sheet series: Topics in risk assessment of substances under CEPA HC, (2016b)
	• Guidance document for the notification and testing of new chemicals and polymers HC, (2021c)
	• Canadian regulatory perspective on next generation risk assessments for pest control products and industrial chemicals Bhuller et al. (2021)
	• Utility of *In Vitro* Bioactivity as a Lower Bound Estimate of *In Vivo* Adverse Effect Levels and in Risk-Based Prioritization Paul Friedman et al. (2020)
	• Science approach documents HC, (2016c), HC, (2021f), HC, (2022c), HC, (2021f), HC, (2022c)
ECHA	• How to use alternatives to animal testing to fulfil the information requirements for REACH registration ECHA, (2016a)
	• Read-across assessment framework ECHA, (2017)
	• 4th report on the use of alternatives to testing on animals for REACH ECHA, (2020)
	• Utility of *In Vitro* Bioactivity as a Lower Bound Estimate of *In Vivo* Adverse Effect Levels and in Risk-Based Prioritization Paul Friedman et al. (2020)
	• Skin sensitization ECHA, (2021)

[a]EPA Office of Research and Development (ORD) involved.
[b]Applicable to all EPA offices.
CEPA, Canadian Environmental Protection Act; CFR, Code of Federal Regulations; CPSC, Consumer Products Safety Commission; ECHA, European Chemicals Agency; EPA OPPT, Environmental Protection Agency Office of Pollution Prevention and Toxics; HC HECSB, Health Canada Healthy Environments and Consumer Safety Branch; NAM, new approach methodologies; TSCA, Toxic Substances Control Act; WoE, weight-of-evidence.

the Tracking System for Alternative Methods (TSAR), an overview of non-animal methods that have been proposed for regulatory safety or efficacy testing of chemicals or biological agents, was established and provided by EURL ECVAM (EURL ECVAM, n.d.).

In 2016, ECHA organized a workshop on NAMs in Regulatory Science, which was attended by 300 stakeholders to discuss the use of NAMs for regulatory decision making (ECHA, 2016b). Since 2016, EPA, HC, and ECHA have held workshops to discuss the development and application of NAMs for chemical assessment as part of an international government-to-government initiative titled "Accelerating the Pace of Chemical Risk Assessment" (APCRA) (EPA, 2021a). EPA and HC further collaborated through the North American Free Trade Agreement (NAFTA; in 2020, NAFTA was replaced by the US-Mexico-Canada Agreement (USMCA)) Technical Working Group (TWG) on Pesticides and through the Canada-US Regulatory Co-operation Council (RCC). The RCC was a regulatory partnership between the pesticides regulating department and offices of HC and EPA that has facilitated the alignment of both countries' regulatory approaches, while advancing efforts to reduce and replace animal tests (ITA, n.d.; HC, 2020). The NAFTA TWG on Pesticides and the RCC included specific work plans and priority areas along with accountability for deliverables (NAFTA TWG, 2016).

The development and implementation of NAMs within regulatory agencies relies heavily on collaboration with a variety of stakeholders, including other offices and departments within the same agency, other national and international agencies, as well as industry representatives, method developers, academics, and non-profit/non-governmental organizations. For example, within EPA, there

TABLE 2 US, Canada, and EU: pesticides and plant protection products.

Agency	Strategic plans, guidance, and other documentation for the implementation of NAMs referenced in this manuscript
EPA OPP	• Guidance for waiving or bridging of acute EPA, (2012); EPA, (2016a); EPA, (2020c) and repeat dose EPA, (2013b) toxicity tests for pesticides (for formulations and single-active ingredients) • Use of an alternate testing framework for classification of eye irritation potential of EPA pesticide products EPA, (2015) • Process for evaluating & implementing alternative approaches to traditional *in vivo* acute toxicity studies for FIFRA regulatory use EPA, (2016b) • Interim science policy: Use of alternative approaches for skin sensitization as a replacement for laboratory animal testing EPA, (2018b) • Recommendation on test readiness criteria for new approach methods in toxicology: Exemplified for DNT Bal-Price et al, (2018a) [a] • Utility of *In Vitro* Bioactivity as a Lower Bound Estimate of *In Vivo* Adverse Effect Levels and in Risk-Based Prioritization Paul Friedman et al. (2020) [a] • New approach methods work plan, reducing use of animals in chemical testing EPA, (2021e) [b] • Retrospective analysis of the dermal absorption "triple pack" data Allen et al. (2021) • Performance of the GHS Mixtures Equation for Predicting Acute Oral Toxicity Hamm et al. (2021) • Integration of toxicodynamic and toxicokinetic new approach methods into a WoE analysis for pesticide DNT assessment Dobreniecki et al. (2022) • ReCAAP: A reporting framework to support a weight of evidence safety assessment without long-term rodent bioassays EPA, (2020f); Hilton et al. (2022)
HC PMRA	• Guidance for waiving or bridging of mammalian acute toxicity tests for pesticides HC, (2013a); Acute Dermal Toxicity Study Waiver HC, (2017) • PMRA's 2016–2021 strategic plan HC, (2016d) • Canadian regulatory perspective on next generation risk assessments for pest control products and industrial chemicals Bhuller et al. (2021) • Guidance for developing datasets for conventional pest control product applications HC, (2021d) • ReCAAP: A reporting framework to support a weight of evidence safety assessment without long-term rodent bioassays Hilton et al. (2022)
EFSA	• Guidance on dermal absorption EFSA et al. (2017) • OECD/EFSA workshop on DNT: The use of non-animal test methods for regulatory purposes Fritsche et al. (2017) • Reconnecting exposure, toxicokinetics and toxicity in food safety: OpenFoodTox and TKplate for human health, animal health and ecological risk assessment Dorne et al. (2018) • Recommendation on test readiness criteria for new approach methods in toxicology: Exemplified for DNT Bal-Price et al. (2018a) • Workshop on *in vitro* comparative metabolism studies in regulatory pesticide risk assessment EFSA, (2019) • Advancing human health risk assessment Lanzoni et al. (2019) • Utility of *In Vitro* Bioactivity as a Lower Bound Estimate of *In Vivo* Adverse Effect Levels and in Risk-Based Prioritization Paul Friedman et al. (2020) • Development of IATA case studies on DNT risk assessment EFSA PPR Panel et al. (2021) • EFSA Strategy 2027 EFSA, (2021) • Development of a Roadmap for Action on New Approach Methodologies in Risk Assessment Escher et al. (2022)

[a]EPA Office of Research and Development (ORD) involved.
[b]Applicable to all EPA offices.
EFSA, European Food Safety Authority; EPA OPP, Environmental Protection Agency Office of Pesticide Programs; FIFRA, Federal Insecticide, Fungicide, and Rodenticide Act; HC PMRA, Health Canada Pest Management Regulatory Agency; NAM, new approach methodologies. ReCAAP, Rethinking Chronic toxicity and Carcinogenicity Assessment for Agrochemicals Project.

is substantial cross-talk between OPP and OPPT (both of which are a part of the Office of Chemical Safety and Pollution Prevention (OCSPP)) as well as the Office of Research and Development (ORD). Agencies also consult with external peer-review panels, such as science advisory boards or committees, which provide independent scientific expertise on various topics. The exchange with external stakeholders provides diverse perspectives and experiences with different NAMs. Several of these collaborations have led to journal publications, presentations at national and international meetings, and webinars. For example, since 2018, EPA has partnered with PETA Science Consortium International e.V.

and the Physicians Committee for Responsible Medicine to host a webinar series on the "Use of New Approach Methodologies (NAMs) in Risk Assessment" which brings together expert speakers and attendees from around the world to discuss the implementation of NAMs (PSCI, n.d.). EPA's OCSPP and ORD also held conferences on the state of the science for using NAMs in 2019 and 2020 and are currently planning the next conference for October 2022 (EPA, 2019a; EPA, 2020b).

2.2 National roadmaps or work plans to guide and facilitate the implementation of NAMs

2.2.1 United States

Several US agencies have roadmaps or work plans to guide and facilitate the implementation of NAMs for testing industrial chemicals or pesticides. For example, following publication of the EPA-commissioned National Resource Council (NRC) report titled "Toxicity Testing in the 21st Century: A Vision and A Strategy" (NRC, 2007), EPA released a strategic plan that provided a framework for implementing the NRC's vision, which incorporates new approaches into toxicity testing and risk assessment practices with less reliance on conventional apical approaches (EPA, 2009). Furthermore, in June 2020, EPA's OCSPP and ORD published a NAM Work Plan (updated in December 2021) that describes primary objectives and strategies for reducing animal testing through the use of NAMs while ensuring protection of human health and the environment (EPA, 2021e). It highlights the importance of communicating, collaborating, providing training on NAMs, establishing confidence in NAMs, and developing metrics for assessing progress.

In 2018, the 16 US federal agencies that comprised ICCVAM (including EPA and CPSC) published a strategic roadmap to serve as a guide for agencies and stakeholders seeking to adopt NAMs for chemical safety and risk assessments (ICCVAM, 2018). The ICCVAM strategic roadmap emphasizes three main components: 1) connecting agency and industry end users with NAM developers to ensure the needs of the end user will be met; 2) using efficient, flexible, and robust practices to establish confidence in NAMs and reducing reliance on using animal data to define NAM performance; and 3) encouraging the adoption and use of NAMs by federal agencies and regulated industries. A list of NAMs accepted by US agencies can be found on the website of the US National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which supports ICCVAM's work (NICEATM, 2021).

2.2.2 Canada

The PMRA's 2016–2021 strategic plan notes how rapidly the regulatory environment is evolving through innovations in science and puts an onus on the Agency to evolve accordingly (HC, 2016d). The strategic plan includes drivers for evolution, the importance of public confidence, the vision, mission, and key principles of scientific excellence, innovation, openness and transparency, and organizational and workforce excellence. The plan further mentions strategic enablers, which include building upon PMRA's success in establishing and maintaining effective partnerships with provinces, territories, and other stakeholders both domestically and internationally.

2.2.3 European Union

The EU is a political and economic union of 27 European countries (Member States) and its operation is guaranteed through various legal instruments. Unlike regulations and decisions that apply automatically and uniformly to all countries as soon as they enter into force, directives require Member States to achieve a certain result by transposing them into national law. In 2010, Directive 2010/63/EU on the protection of animals used for scientific purposes (EU, 2010) was adopted to eliminate disparities between laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Article 4 states that "wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure," which applies to all research purposes including regulatory toxicity testing (EU, 2010). Further, the directive lays the foundation for retrospective analyses of animal experiments, mutual acceptance of data, as well as the European Commission and Member States' contribution to the development and validation of NAMs.

In October 2020, the EU Chemicals Strategy for Sustainability (CSS) Towards a Toxic-Free Environment was published (EC, 2020). It identified a need to innovate safety testing and chemical risk assessment to reduce dependency on animal testing while improving the quality, efficiency, and speed of chemical hazard and risk assessments. However, fulfilling its additional information requirements will more likely lead to an increase in animals used. Also, it is currently unknown whether the implementation of the CSS will open opportunities for the application of more NAMs.

3 Industrial chemicals

3.1 United States

In the US, industrial chemicals are subject to regulation under the Toxic Substances Control Act (TSCA). TSCA was originally signed into law (15 US Code [USC] §2601 *et seq.*) on 11 October 1976 with the intent "[t]o regulate commerce and protect human health and the environment by requiring testing and necessary use restrictions on certain chemical substances,

and for other purposes" (Pub. L. 94-469, Oct. 11, 1976). TSCA was significantly amended in 2016 (Pub. L. 114-182, 22 June 2016). EPA is responsible for implementing and administering TSCA (see 15 USC §2601(c)) and OPPT, within EPA's OCSPP, carries out much of that work.

TSCA provides EPA the authority to regulate new and existing chemical substances under Sections 5 and 6 of TSCA, respectively. Existing chemical substances are those on the TSCA Inventory, either those that were in commerce prior to the enactment of TSCA and grandfathered in, or those that OPPT evaluated as new chemical substances and were subsequently introduced into commerce. Entities that wish to introduce a new chemical substance or an existing chemical substance with a significant new use into commerce must submit a notification to OPPT (*i.e.*, pre-manufacture notice (PMN) or significant new use notice (SNUN)) or an appropriate exemption application, where an application is required for the exemption (e.g., low volume exemption), prior to manufacturing, including importing, the chemical substance.

Prior to the 2016 Amendments, when entities submitted a new chemical notification, no specific action by EPA was required. If EPA did not take regulatory action on the new chemical substance, the entity was allowed to manufacture the chemical substance at the expiration of the applicable review period (e.g., 90 days for a PMN). For existing chemicals, much of EPA's TSCA activity was focused on data collection, including through section 8 rules and issuing test rules on chemical substances, including those identified by EPA's interagency testing committee (ITC). The ITC was established under Section 4(e) of TSCA and was charged with identifying and recommending to the EPA Administrator chemical substances or mixtures that should be tested pursuant to Section 4(a) of TSCA to determine their hazard to human health or the environment. Although allowed by TSCA, EPA's ability to regulate and restrict the use of existing chemical substances under Section 6 of TSCA was significantly impaired following a 1991 ruling by the US Court of Appeals for the Fifth Circuit (*Corrosion Proof Fittings vs. EPA*, 974 F.2d 1201), which vacated much of EPA's TSCA Section 6 rule to ban asbestos, a rule that EPA had first announced as an advanced notice of proposed rulemaking in 1979.

The above issues with TSCA—namely new chemical substances being automatically introduced into commerce if the "clock ran out" and EPA's limited regulation of existing chemical substances under Section 6 of TSCA—garnered Congressional attention, which culminated on 22 June 2016. On that date, then-President Obama signed the Frank R. Lautenberg Chemical Safety for the 21st Century Act into law, thereby amending TSCA (Pub.L. 114-182, 2016). The TSCA amendments placed new requirements on EPA, including requirements to review and publish risk determinations on new chemical substances, prioritize existing chemical substances as either high- or low-priority substances, and perform risk evaluations on those chemical substances identified as high-priority substances. The TSCA amendments also included new requirements for EPA to comply with specific scientific standards for best available science and weight of the scientific evidence (WoE) under Sections 26(h)-(i) of TSCA when carrying out Sections 4, 5, and 6; a new requirement to reduce testing on vertebrate animals under Section 4(h) of TSCA; and a provision giving EPA the authority to require testing on existing chemical substances by order, rather than by rule,[1] under Section 4(a)(1) and (2) of TSCA.

The discussion that follows is focused on EPA's authority under Section 4(h) to reduce testing on vertebrate animals, EPA's use of this authority for new and existing chemical substances, and voluntary initiatives by the regulated community that have advanced the understanding and use of NAMs.

3.1.1 General requirements

TSCA does not contain upfront vertebrate toxicity testing requirements, which allows flexibility for the adoption of NAMs. Since the enactment of the TSCA amendments, EPA has used its authority to order testing on existing chemical substances, while meeting its requirements under Section 4(h) of TSCA (EPA, 2022c). Section 4(h) includes three primary provisions: (1) the aforementioned general requirements placed on EPA for reducing and replacing the use of vertebrate animals; (2) the requirements on EPA to promote the development and incorporation of alternative testing methods, including through the development of a strategic plan and a (non-exhaustive) list of NAMs identified by the EPA Administrator; and (3) the requirements on the regulated community to consider non-vertebrate testing methods when performing voluntary testing when EPA has identified an alternative test method or strategy to develop such information.

3.1.2 Regulatory flexibility

There are several sections of TSCA and the implementing regulations where EPA may use NAMs for informing its science and risk management decisions under TSCA. Data generated using NAMs may trigger reporting requirements on the regulated community. For example, under Section 8(e) of TSCA, it is possible that results generated using NAMs would trigger a reporting obligation for substantial risk for instance, if the data meet the requirements under one of EPA's policies, such as *in vitro* skin sensitization data. In its "Strategic Plan to Promote the Development and Implementation of Alternative Test Methods Within the TSCA Program," OPPT lists criteria

1 The distinction between an order and a rule is that the former may be issued without following the procedural requirements of notice and comment rulemaking under the Administrative Procedure Act (5 U.S.C. §§500 *et seq.*), whereas the latter must comply with these requirements.

that provide a starting point for considering the scientific reliability and relevance of NAMs (EPA, 2018d); however, it has yet to issue official guidance to the regulated community on its interpretation of the criteria for accepting NAMs, as meeting the scientific standards under Section 26(h) of TSCA. In addition, while OPPT has yet to issue official guidance on the criteria it uses to identify NAMs for inclusion on the list of methods approved by the EPA Administrator, the agency has presented a proposed nomination form, which provides some insight on EPA's considerations (Simmons and Scarano, 2020).

3.1.3 Implementation of NAMs

OPPT's activities to implement NAMs have included issuing a "Strategic Plan to Promote the Development and Implementation of Alternative Test Methods Within the TSCA Program" (EPA, 2018d), establishing a list of approved NAMs (EPA, 2018c; EPA, 2019b; EPA, 2021d), and developing a draft policy allowing the use of NAMs for evaluating skin sensitization (EPA, 2018b). The latter is based on EPA's participation in the development of the OECD guideline for Defined Approaches on Skin Sensitisation (OECD, 2021a). EPA has also performed significant outreach and collaboration to advance its understanding of NAMs, as well as educate the interested community about these technologies.

In March 2022, OPPT and ORD presented the TSCA new chemicals collaborative research effort for public comments (EPA, 2022b). This multi-year research action plan to bring innovative science to the review of new chemicals under TSCA includes: 1) refining chemical categories for read-across; 2) developing and expanding databases containing TSCA chemical information; 3) developing and refining Quantitative Structure-Activity Relationship (QSAR) and other predictive models; 4) exploring ways to apply NAMs in risk assessment; and 5) developing a decision support tool that will transparently integrate all data streams into a final risk assessment.

3.1.3.1 Examples of NAM application

Already prior to the 2016 amendments to TSCA, EPA had established numerous methods for assessing chemical substances. For example, EPA has been using structure-activity relationships (SAR) for assessing the potential of new chemical substances to cause harm to aquatic organisms and an expert system to estimate potential for carcinogenicity since the 1980s (EPA, 1994).

In early 2021, OPPT issued test orders on nine existing chemical substances (EPA, 2022c). For each of the substances, OPPT ordered dermal absorption testing using an *in vitro* method validated by the OECD (OECD, 2004) instead of animal testing. After consideration of existing scientific information, EPA determined that the *in vitro* method, which is included on its list of NAMs, could be used. While EPA required the *in vitro* testing on both human and animal skin, a report has since been published analyzing 30 agrochemical formulations, which supports the use of *in vitro* assays using human skin for human health risk assessment because they are as or more protective and are directly relevant to the species of interest (Allen et al., 2021; EPA, 2021f). In reviewing test plans or test data provided to be considered *in lieu* of the ordered testing, EPA consulted with the authors of Allen et al. (2021) and subsequently determined that it would be acceptable for the *in vitro* testing to be conducted on human skin only for the chemicals subject to these particular orders.

The interested community has also been actively developing robust NAMs that can be used for regulatory decision making. For example, an entity performed voluntary *in chemico* testing on a polymeric substance that OPPT had identified as a potential hazard. The substance was classified as a poorly soluble, low-toxicity substance that, if inhaled, may lead to adverse effects stemming from lung overload. OPPT issued a significant new use rule (SNUR) on this substance, which required any entity to notify EPA (submission of a SNUN) if the polymer is manufactured, processed, or used as a respirable particle (i.e., <10 μm) (EPA, 2019c). The SNUR listed potentially useful information for inclusion in a SNUN, which consisted of a 90-day subchronic inhalation toxicity study in rats. However, the entity voluntarily undertook an *in chemico* test *in lieu* of the *in vivo* toxicity study. The *in chemico* test showed the daily dissolution rate of the polymer in simulated epithelial lung fluid exceeded the anticipated daily exposure concentrations and was, therefore, not a hazard concern from lung overload. After evaluating these data, OPPT agreed with the results and issued a final rule revoking the SNUR (EPA, 2020h). These data were subsequently published in the peer-reviewed literature (Ladics et al., 2021).

3.1.4 Consumer products

In addition to the regulation of individual chemical ingredients of household products under TSCA, the Federal Hazardous Substances Act (FHSA) requires appropriate cautionary labeling on certain hazardous household products to alert consumers to the potential hazard(s) that the products may present (15 USC §1261 *et seq.*). However, the FHSA does not require manufacturers to perform any specific toxicological tests to assess potential hazards (e.g., systemic toxicity, corrosivity, sensitization, or irritation). CPSC has the authority with administering FHSA. It issued guidance on the use of NAMs in 2021 (CPSC, 2022). This document lays out what factors CPSC staff will use when evaluating NAMs, IATA, and any submitted data being used to support FHSA labeling determinations. CPSCS, 2012 Animal Testing Policy (16 Code of Federal Regulations [CFR] Part 1500) strongly encourages manufacturers to find alternatives to animal testing for assessing household products.

3.2 Canada

The *Canadian Environmental Protection Act* (CEPA, Statutes of Canada [SC] 1999, c.33) provides the legislative framework for industrial substances, including new chemical substances and those that are currently on the Canadian market (i.e., existing substances on the Domestic Substances List [DSL]), for the protection of the environment, for the well-being of Canadians, and to contribute to sustainable development. The Safe Environments Directorate in the HECSB of Health Canada and Environment and Climate Change Canada are jointly responsible for the regulation of industrial substances under the authority of CEPA.

Existing and new substances have different legal requirements under CEPA. Accordingly, based on respective program areas, the requirements for and use of traditional and NAMs data are considered in various decision contexts including screening, prioritization, and informing risk assessment decisions. Risk assessments consider various types and sources of information, as required or available for new or existing substances respectively, including physico-chemical properties, inherent hazard, biological characteristics, release scenarios, and routes of exposure to determine whether a substance is or may become harmful according to the criteria set out in section 64 of CEPA.

The Chemicals Management Plan (CMP) was introduced in 2006 to, in part, strengthen the integration of chemicals management programs across the Government of Canada (HC, 2022e). Key elements of the CMP have been addressing the priority existing chemicals from the DSL identified through Categorization for risk assessment pursuant to obligations under CEPA and the parallel pre-market assessments of new substances not on the DSL and notified through the *New Substances Notification Regulations* provisions made under CEPA.

Under the Existing Substances Risk Assessment Program (ESRAP), the approximate 4,300 priority substances were assessed over three phases (2006–2021), requiring the development of novel methodologies and assessment strategies to address data needs as the program evolved from a chemical-by-chemical approach to the assessment of groups and classes of chemicals (HC, 2021b). The limited empirical toxicity data available for many of the priority substances necessitated the implementation of fit-for-purpose approaches, including the use of computational tools and read-across. Further, the use of streamlined approaches (HC, 2018) assisted the program to more efficiently address substances considered to be of low concern. Building on experiences and achievements from the CMP to date, the Government of Canada continues to expand on the vision for modernization. This shift takes into consideration new scientific information regarding chemicals to support innovative strategies for priority setting and to maintain a flexible, adaptive and fit-for-purpose approach to risk assessment to manage increasingly diverse and complex substances and mixtures (HC, 2021b; Bhuller et al., 2021).

The New Substances Program (NSP) is responsible for administering the *New Substances Notification Regulations* (NSNR, Statutory Orders and Regulations [SOR]/205-247 and SOR/2005-248) of CEPA (HC, 2022f). These regulations ensure that new substances (chemicals, polymers, biochemical, biopolymers, or living organisms) are not introduced into Canada before undergoing ecological and human health risk assessments, and that any appropriate or required control measures have been taken.

3.2.1 General requirements

Risk assessments conducted under CEPA use a WoE approach while also applying the precautionary principle. For existing substances on the DSL, there are no prescribed data requirements to inform the assessment of a substance to determine whether it is toxic or capable of becoming toxic as defined under Section 64 of CEPA. As such, an essential first step in the risk assessment process is the collection and review of a wide range of hazard and exposure information on each substance or group of substances from a variety of published and unpublished sources, stakeholders, and various databases (HC, 2022d).

The NSNR (Chemicals and Polymers) require information be submitted in a New Substances Notification (NSN) prior to import or manufacture of a new chemical, polymer, biochemical, or biopolymer in Canada. The NSNR (Chemicals and Polymers) also require that a notifier submit all other relevant data in their possession relevant to the assessment. Subsection 15(1) of the NSNR (Chemicals and Polymers) states that conditions and procedures used must be consistent with conditions and procedures set out in the OECD TG that are current at the time the test data are developed, and should comply with GLP.

Information in support of a NSN may be obtained from alternative test protocols, WoE, read-across, as well as from (Q)SARs [calculation or estimation methods (e.g., EPI Suite)]. The NSP may use various NAMs in their risk assessment, and may accept (and has accepted) test data which use NAMs, as discussed in further detail below.

3.2.2 Regulatory flexibility

For existing substances on the DSL under CEPA, there are no set submission requirements prior to an assessment, which inherently presents the need for flexibility and the opportunity to integrate novel approaches. NAM data are often used to support the assessment of the potential for risk from data poor substances. Since these data poor substances are unlikely to have required or available guideline studies, NAMs, including computational modelling, *in vitro* assays, QSAR and read-across, are used as approaches to address data needs offering an

opportunity for a risk-based assessment where this may have been challenging in the past (HC, 2022a). For new substances, the NSP supports ongoing NAM development, as well as monitoring studies, to provide information on levels of substances of interest in the environment; both are used to fill risk assessment data gaps. In 2021, the NSP published a draft updated Guidance Document for the Notification and Testing of New Substances: Chemicals and Polymers (HC, 2021c). Section 8.4 of this Guidance Document lists examples of accepted test methods, which could in the future include NAMs as they are shown to be scientifically valid. Under the NSNR, alternative approaches will be acceptable when, in the opinion of the NSP, they are determined to provide a scientifically valid measure of the endpoint under investigation that is deemed sufficient for the purposes of the risk assessment. NAM data are evaluated on a case-by-case basis and can form part of the WoE of an assessment.

3.2.3 Implementation of NAMs

Given the paucity of data available for many substances on the market, as well as for new substances, there is a long history of using alternative approaches for hazard identification and characterization in support of new and existing substances risk assessment decisions. Over the last 2 decades, a variety of NAMs have been used by different program areas to address information gaps for risk assessment. The approaches implemented have been fit-for-purpose and largely determined by the data need, the timeline, the type of chemical(s), and the level of complexity associated with the assessment (HC, 2016a). Most notably for existing substances, *in silico* models, (Q)SAR, and read-across have been the most widely used methods with the progressive adoption and expanded use of computational toxicology and automated approaches ongoing for both ESRAP and the NSP. More specific details on the evolution of the ESRAP under CEPA are highlighted in the CMP Science Committee meeting report (HC, 2021b).

There are currently no formal criteria that have been published in order to achieve regulatory acceptance for the implementation of NAMs for existing substances in Canada. However, experience and efficiencies have been gained through the strategic development and implementation of streamlined risk-based approaches that support rapid and robust decision-making. To this end, a number of science approach documents (SciAD) have been published describing and demonstrating the implementation of NAMs to evaluate the potential for environmental or human health risk from industrial substances (HC, 2022c). SciADs are published under section 68 of CEPA, and do not include regulatory conclusions; however, the approach and results described within a SciAD may form the basis for a risk assessment conclusion when used in conjunction with any other relevant and available information. Furthermore, the implementation of NAMs as described in SciADs can also be used to support the identification of priorities for data gathering, data generation, further scoping, and risk assessment (HC, 2022c).

In advancing the vision for progressive chemicals management programs, which includes reduced use of animals and integration of NAMs, it is recognized that there is an ongoing need to develop flexible, adaptive, and innovative approaches. Accordingly, the ESRAP continues to expand the use of computational and *in vitro* models as well as evidence integration strategies to identify and address emerging priority substances. Key to successful implementation moving forward are the productive partnerships with the international regulatory and research communities to continue to build confidence and harmonization for the use of alternative test methods and strategies in chemical risk assessment (Krewski et al., 2020; Bhuller et al., 2021).

Data generated using NAMs may be accepted to fulfil any of the NSNR's test data requirements for an NSN when, in the opinion of the NSP, such data are determined to provide a scientifically valid measure of the endpoint under investigation that is deemed sufficient for the purposes of the risk assessment. The NSP will assess if the method has been satisfactorily validated in terms of scientific rigor, reproducibility, and predictability. Guidance is provided to notifiers who wish to submit information using NAMs during Pre-Notification Consultation meetings with NSP staff, or notifiers can consult Sections 5.4 and 8.4 of the respective Guidance Document (HC, 2021c). Alternative methods that may be accepted by the NSP to meet NSNR requirements include any internationally recognized and accepted test methods (e.g., *in vitro* skin irritation, gene mutation, and chromosomal aberration). Data such as (Q)SAR, read-across (greater than 80% structural similarity), and WoE may be accepted on a case-by-case basis.

3.2.3.1 Examples of NAM applications

As noted above, beyond the use of *in silico* models and read-across, examples of NAM applications for existing substances have been published as SciADs outlining NAM-based methods for prioritization and assessment (HC, 2022c). Specifically, the SciAD "Threshold of Toxicological Concern (TTC)-based Approach for Certain Substances" has been applied to evaluate a subset of existing substances on the DSL identified as priorities for assessment under subsection 73(1) of CEPA and/or were considered a priority based on human health concerns (HC, 2016c). More recently, the SciAD "Bioactivity exposure ratio: Application in priority setting and risk assessment approach" was developed outlining a quantitative risk-based approach to identify substances of greater potential concern or substances of low concern for human health (HC, 2021f). This proposed approach for NAM application builds on a broad retrospective analysis under the APCRA (Paul Friedman et al., 2020) and considers high-throughput *in vitro* bioactivity together with

high-throughput toxicokinetic modelling to derive an *in vitro*-based point of departure. As technologies continue to advance and additional sources of data from NAMs emerge, these may also be considered in the ongoing expansion of the approach to support the derivation of molecular-based PODs as part of a tiered testing scheme. Further work is underway to build approaches for the interpretation of transcriptomics data and to enhance the use of QSAR and machine learning to enrich evidence integration and WoE evaluation using IATA frameworks across toxicological endpoints of regulatory relevance.

New substances are inherently data-poor substances and, as a result, the NSP typically accepts a variety of alternative approaches and NAM data to meet data requirements under the NSNR. QSAR data and read-across data using analogues have historically been used to meet data requirements under the NSNR, particularly for physico-chemical data requirements or in combination with other data to provide a WoE for toxicity data. More recently, newly validated *in vitro* methods for skin irritation and skin sensitization (OECD, 2021a) have been accepted to meet data requirements under the NSNR. The NSP participates in active research programs to develop NAMs for complex endpoints, such as genotoxicity and systemic toxicity. Although not a regulatory requirement, *in vitro* eye irritation tests are also frequently received by the NSP.

3.3 European Union

In 2006, a significant number of updates and revisions were introduced into the EU chemicals policy with the introduction of Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (EC, 2006). REACH entered into force on 1 June 2007, and introduced a single system for the regulation of chemicals, transferring the burden of proof concerning the risk assessment of substances from public authorities to companies. The purpose of REACH, according to Article 1(1), is to "ensure a high level of protection of human health and the environment, including the promotion of alternative methods for assessment of hazards of substances, as well as the free circulation of substances on the internal market while enhancing competitiveness and innovation" (EC, 2006). The Regulation established ECHA to manage and implement the technical, scientific, and administrative aspects of REACH. Enforcement of REACH is each EU Member State's responsibility and, therefore, ECHA has no direct enforcement responsibilities (ECHA, n.d.a). In addition to REACH, Regulation (EC) 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP regulation) (EC, 2008b) was introduced to align the EU chemical hazard labeling system with the United Nations Economic Commission for Europe (UNECE)'s Globally Harmonised System of Classification and Labelling of Chemicals (GHS). Both REACH and CLP regulation are currently undergoing extensive revisions at the time of submission of this manuscript.

3.3.1 General requirements

REACH applies to all chemical substances; however, certain substances that are regulated by other legislations (*e.g.*, biocides, PPPs, or medical drugs) may be (partially) exempted from specific requirements (ECHA, n.d.f). Substances used in cosmetic products remain a contentious issue with them being subject to an animal testing ban under the EU regulation on cosmetics products (EC, 2009b), yet ECHA continues to request new *in vivo* testing under certain circumstances such as for risk assessment for worker exposure (ECHA, 2014). The interplay between the two regulations is under review by the European Court of Justice ('Symrise v ECHA' (2021), T655/20, ECLI:EU:T:2021:98 and 'Symrise v ECHA' (2021), T-656/20, ECLI:EU:T:2021:99).

Whilst REACH is not a pre-marketing approval process in the strictest sense of the definition, it works on the principle of no data, no market with responsibility placed on registrants to manage the risks from chemicals and to provide safety information on the substances. Thus, companies bear the burden of proof to identify and manage the risks linked to the substances they manufacture or import and place on the market in the EU. They must demonstrate how the substance can be safely used and must communicate the risk management measures to the users. Companies must register the chemical substances they manufacture or import into the EU at more than one tonne per year with ECHA. The registration requirement under REACH "applies to substances on their own, in mixtures, or, in certain cases, in articles" (ECHA, n.d.c). Registration is governed by the "one substance, one registration" principle, where manufacturers and importers of the same substance must submit their registration jointly. Companies must collect information on the properties and uses of their substances and must assess both the hazards and potential risks presented by these substances. The companies compile all of this information in a registration dossier and submit it to ECHA. The standard information requirements for the registration dossier depends on the tonnage band of the chemical substance (ECHA, n.d.b). The information required is specified in Annexes VI to X of REACH and include physico-chemical data, toxicology information, and ecotoxicological information.

ECHA receives and evaluates individual registrations for their compliance (ECHA, n.d.f). EU Member States evaluate certain substances to clarify initial concerns for human health or for the environment. ECHA's scientific committees assess whether any identified risks from a hazardous substance are manageable, or whether that substance must be banned. Before imposing a ban, authorities can also decide to restrict the use of a substance or make it subject to a prior authorization.

The CLP regulation requires that relevant information on the characteristics of a substance, classification of toxicity endpoints, and pertinent labelling of a substance or substances in mixtures be notified to ECHA when placed on the EU market (EC, 2008b). In this way, the toxicity classification and labeling of the substance are harmonized both for chemical hazard assessment and consumer risk. In cases where there are significant divergences of scientific opinion, further review of scientific data can proceed (EC, 2008b). New testing is normally not requested for CLP purposes alone unless all other means of generating information have been exhausted and data of adequate reliability and quality are not available (ECHA, n.d.e).

The discussion that follows is focused on the EU's efforts under REACH to reduce testing on vertebrate animals to assess human health effects. This concept lies at the very foundation of REACH, which states in the second sentence of the Preamble that it should "promote the development of alternative methods for the assessment of hazards of substances" (EC, 2006).

3.3.2 Regulatory flexibility

According to Article 13(1) of REACH, "for human toxicity, information shall be generated whenever possible by means other than vertebrate animal tests, through the use of alternative methods, for example, in vitro methods or qualitative or quantitative structure-activity relationship models or from information from structurally related substances (grouping or read-across)" (EC, 2006). Further, according to Article 13(2), the European Commission may propose amendments to the REACH Annexes and the Commission Regulation, which lists approved test methods (EC, 2008a), to "replace, reduce or refine animal testing." Under Title III of REACH, on Data Sharing and Avoidance of Unnecessary Testing, Article 25(1) requires that testing on vertebrate animals must be undertaken only as a last resort; however, the interpretation of Articles 13 and 25 of REACH are often matters of dispute in European Court of Justice ('Federal Republic of Germany v Esso Raffinage' (2021), C-471/18 P, ECLI:EU:C:2021:48), ECHA Board of Appeal (*e.g.*, cases A-005-2011 and A-001–2014), and European Ombudsman cases (cases 1568/2012/(FOR)AN, 1606/2013/AN and 1130/2016/JAS).

In addition, to reduce animal testing and duplication of tests, study results from tests involving vertebrate animals should be shared between registrants (EC, 2006). Furthermore, where a substance has been registered within the last 12 years, a potential new registrant must, according to Article 27, request from the previous registrant all information relating to vertebrate animal testing that is required for registration of the substance. Before the deadline to register all existing chemicals by 31 May 2018, companies (i.e., manufacturers, importers, or data owners) registering the same substance were legally required to form substance information exchange fora (SIEFs) to help exchange data and avoid duplication of testing for existing chemicals (EC, 2006).

REACH standard information requirements for registration dossiers contain upfront testing requirements on vertebrate animals with some flexibility to allow the use of NAMs. Registrants are encouraged to collect all relevant available information on the substance, including any existing data (human, animal, or NAMs), (Q)SAR predictions, information generated with analogue chemicals (read-across), and *in chemico* and *in vitro* tests. In addition, REACH foresees that generating information required in Annexes VII-X may sometimes not be necessary or possible. In such cases, the standard information for the endpoint may be waived. Criteria for waiving are outlined in Column 2 of Annexes VII-X, while criteria for adapting standard information requirements are described in Annex XI of REACH (ECHA, 2016a). In addition to the use of OECD test guidelines, data from *in vitro* methods that meet internationally agreed pre-validation criteria as defined in OECD GD 34 are considered suitable for use under REACH when the results from these tests indicate a certain dangerous property. However, negative results obtained with pre-validated methods have to be confirmed with the relevant *in vivo* tests specified in the Annexes. Whether the aforementioned current revision of REACH and CLP regulations will bring about opportunities to include more NAMs in the assessment of industrial chemicals or lead to an increase in animal testing is to be seen.

3.3.3 Implementation of NAMs

The REACH annexes were amended in 2016 and 2017 to require companies to use NAMs for certain endpoints under certain conditions. Following these amendments, the use of non-animal tests have tripled for skin corrosion/irritation, quadrupled for serious eye damage/eye irritation, and increased more than 20-fold for skin sensitization (ECHA, 2020).

REACH requires that robust study summaries be published on the ECHA website. This helps registrants identify additional data for their registrations and facilitates the identification of similar or identical substances (ECHA, 2020). ECHA's public chemical database may also be used to conduct retrospective data analyses and other research efforts, when the level of detailed data needed are present in such reports (Luechtefeld et al., 2016a; Luechtefeld et al., 2016b; Luechtefeld et al., 2016c; Luechtefeld et al., 2018; Knight et al., 2021).

ECHA engages in OECD expert groups and reviews test guidelines for both animal and non-animal methods. For example, ECHA contributed to the *in vitro* OECD test guidelines for skin and eye irritation in 2016 and skin sensitization in 2017. In addition, ECHA was involved in the finalization of the OECD "Defined Approaches on Skin Sensitisation Test Guideline" (OECD, 2021a). In October 2021, ECHA published advice on how REACH registrants can use the defined approaches guideline, and this was the first official guidance outlining how to use *in silico* tools, such as

the QSAR Toolbox, to assess skin sensitization (ECHA, 2021). Furthermore, ECHA also engages in largescale European research projects (e.g., EU-ToxRisk) where they review mock dossiers based on NAMs that have been developed in these projects.

Before registrants conduct higher-tier tests for assessing the safety of chemicals they import or manufacture, Article 40 of REACH requires that they submit details on their testing plans to ECHA (ECHA, n.d.d). In that submission, companies must detail how they considered NAMs before proposing an animal test. ECHA must agree on these proposals before a company can conduct a new animal test under Annex IX or X. ECHA may reject, accept, or modify the proposed test. As required by REACH, all testing proposals involving testing on vertebrate animals are published on ECHA's website to allow citizens and organizations the opportunity to provide information and studies about the substance in question (ECHA, n.d.d). ECHA will inform the company that submitted the testing proposal of the Member State Committee's decision and is required to take into account all studies and scientifically valid information submitted as part of the third-party consultation when making its decision.

3.3.3.1 Examples of NAM application

The most commonly used NAM under REACH is the read-across approach, where relevant information from analogous substances is used to predict the properties of target substances (ECHA, 2020). Before read-across is accepted by ECHA, it must be justified by the registrant and, therefore, to facilitate its use, ECHA developed a read-across assessment framework (ECHA, 2017). Additionally, ECHA is holding different expert meetings with stakeholders including industry representatives and NGOs to enhance and combine knowledge and to avoid overlap and duplication. Thus, ECHA encourages companies to avoid duplicate animal tests and share any data they have on their substance if requested by a registrant of an analogous substance. For example, based on *in vitro* ToxTracker assay results and read-across data from the analogue substance aminoethylpiperazine, ECHA has not requested *in vivo* genotoxicity data for N,N,4-trimethylpiperazine-1-ethylamine, which was registered by two companies in a joint submission (ECHA, 2019).

4 Pesticides and plant protection products

4.1 United States

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA; 7 USC §136) requires all pesticides sold or distributed in the US to be registered with the EPA, unless otherwise exempted. EPA then has authority under the Federal Food, Drug, and Cosmetic Act (FD&C Act; 21 USC §301 *et seq.*) to set the maximum amount of pesticide residues permitted to remain in/on food commodities or animal feed, which are referred to as tolerances. In 1996, both of these statutes were amended by the Food Quality Protection Act (FQPA), which placed new requirements on EPA, including making safety findings (i.e., "a reasonable certainty of no harm") when setting tolerances (Pub.L. 104-170, 1996).

OPP, within EPA's OCSPP, has the delegated authority with administering the above laws and is responsible for pesticide evaluation and registration. This includes registration of new pesticide active ingredients and products, as well as new uses for currently registered pesticides. Additionally, OPP reviews each registered pesticide at least every 15 years as part of the Registration Review process to determine whether it continues to meet registration standards. A pesticide product may not be registered unless the EPA determines that the pesticide product will not cause unreasonable adverse effects on the environment (as defined by 7 USC §136(bb)).

4.1.1 General requirements

Data requirements for pesticide registration are dependent on the type of pesticide (i.e., conventional, biopesticide, or antimicrobial) and use pattern (e.g., food versus non-food, or anticipated routes of exposure) and are laid out in 40 CFR Part 158. Unlike TSCA, FIFRA and its implementing regulations require substantial upfront testing to register a pesticide in the US, such as product chemistry data to assess labeling, product performance data to support claims of efficacy, studies to evaluate potential hazards to humans, studies to evaluate potential hazards to non-target organisms, environmental fate data, and residue chemistry and exposure studies to determine the nature and magnitude of residues. The data are used to conduct comprehensive risk assessments to determine whether a pesticide meets the standard for registration.

4.1.2 Regulatory flexibility

US regulations give EPA substantial discretion to make registration decisions based on data that the Agency deems most relevant and important for each action. As stated in the CFR, under Section 158.30, the studies and data required may be modified on an individual basis to fully characterize the use and properties of specific pesticide products under review. Also, the data requirements may not always be considered appropriate. For instance, the properties of a chemical or an atypical use pattern could make it impossible to generate the required data or the data may not be considered useful for the evaluation. As a result, Section 158.45 permits OPP to waive data requirements as long as there are sufficient data to make the determinations required by the applicable statutory standards.

To assist staff in focusing on the most relevant information and data for assessment of individual

pesticides, OPP published "Guiding Principles for Data Requirements" (EPA, 2013a). The document describes how to use existing information about a pesticide to identify critical data needs for the risk assessment, while avoiding generation of data that will not materially influence a pesticide's risk profile and ensuring there is sufficient information to support scientifically sound decisions. When data from animal testing will not contribute to decision making, OPP has developed processes to waive guideline studies and/or apply existing toxicological data for similar substances (i.e., bridging). Detailed guidance on the scientific information needed to support a waiver or bridging justification has been developed by OPP for acute (EPA, 2012; EPA, 2016a; EPA, 2020c) and repeat dose (EPA, 2013b) mammalian studies.

Interdivisional expert committees within OPP are tasked with considering waiver requests on a case-by-case basis. The Hazard and Science Policy Council (HASPOC) is tasked with evaluating requests to waive most guideline mammalian toxicity studies, except acute systemic lethality and irritation/sensitization studies (which are referred to as the acute six-pack). HASPOC is comprised of toxicologists and exposure scientists from divisions across OPP focused on conducting human health risk assessments and it utilizes a WoE approach described in its guidance on "Part 158 Toxicology Data Requirements: Guidance for Neurotoxicity Battery, Subchronic Inhalation, Subchronic Dermal and Immunotoxicity Studies" (EPA, 2013b). This includes consideration of multiple lines of evidence, such as physico-chemical properties, information on exposure and use pattern, toxicological profiles, pesticidal and mammalian mode of action information, and risk assessment implications. Although this guidance was developed to address particular toxicity studies, the same general WoE approach is applied by HASPOC when considering the need for other toxicity studies for pesticide regulatory purposes. Between 2012 and 2018, the most common studies requested to be waived were acute and subchronic neurotoxicity, subchronic inhalation, and immunotoxicity studies (Craig et al., 2019). For the acute six-pack studies, the Chemistry and Acute Toxicology Science Advisory Council (CATSAC) was formed to consider bridging proposals and/or waivers using the aforementioned waiving and bridging guidance documents. For example, following a retrospective analysis, the agency released guidance for waiving acute dermal toxicity tests (US EPA, OCSPP, and OPP, 2016). The progress of HASPOC and CATSAC is continuously tracked and reported on an annual basis (Craig et al., 2019; EPA, 2020a, 2021b).

Beyond waiving studies that do not contribute to regulatory decision making, OPP has the ability to use relevant NAMs to replace, reduce, and refine animal studies. The CFR provides OPP with considerable flexibility under Section 158.75 to request additional data beyond the Part 158 data requirements that may be important to the risk management decision. NAMs can be considered and accepted for these additional data, when appropriate.

4.1.3 Implementation of NAMs

Several documents describe OPP's strategies to reduce reliance on animal testing and incorporate relevant NAMs. For example, in addition to overarching EPA strategic plans (see Section 2.1.2.1.), OPP consulted the FIFRA Scientific Advisory Panel (SAP) on strategies and initial efforts to incorporate molecular science and emerging *in silico* and *in vitro* technologies into an enhanced IATA (EPA, 2011). The long-term goal identified for this consultation was a transition from a paradigm that requires extensive *in vivo* testing to a hypothesis-driven paradigm where NAMs play a larger role.

Unlike TSCA that requires OPPT to maintain a (non-exhaustive) list of NAMs that are accepted, OPP does not have a similar statutory requirement. However, OPP does maintain a website with strategies for reducing and replacing animal testing based on studies and approaches that are scientifically sound and supportable (EPA, 2022a). For many of these strategies, OPP has worked closely with other EPA offices, including OPPT and ORD, to develop and implement plans and tools that advance NAMs. Additionally, OPP works with a wide range of external organizations and stakeholders, including other US federal agencies, international regulatory agencies, animal protection groups, and pesticide registrants.

These collaborations have resulted in several agency documents for specific NAM applications. As mentioned in previous sections, there have been national and international efforts to develop defined approaches for skin sensitization in which OPP participated, along with OPPT, PMRA, ECHA, and other stakeholders. In 2018, a draft policy document was published jointly by OPP and OPPT on the use of alternatives approaches (*in silico*, *in chemico*, and *in vitro*) that can be used to evaluate skin sensitization in lieu of animal testing with these approaches accepted as outlined in the draft policy upon its release (EPA, 2018b). As international work develops through the OECD, this policy will be updated to accept additional defined approaches as appropriate. OPP also has a policy on the "Use of an Alternate Testing Framework for Classification of Eye Irritation Potential of EPA Pesticide Products," which focuses on the testing of antimicrobial cleaning products but can be applied to conventional pesticides on a case-by-case basis (EPA, 2015).

Collaborative efforts have also resulted in numerous publications in scientific journals that allow for communication of scientific advancements and analyses, while building confidence in NAM approaches that can support regulatory decisions. For example, analyses have been published demonstrating that many of the *in vitro* or *ex vivo* methods available for eye irritation are equivalent or scientifically superior to the rabbit *in vivo* test (Clippinger

et al., 2021). Additionally, OPP established a pilot program to evaluate a mathematical tool (GHS Mixtures Equation) as an alternative to animal oral inhalation toxicity studies for pesticide formulations. After closing the submission period in 2019, OPP worked with NICEATM to conduct retrospective analyses, which demonstrated the utility of the GHS Mixtures Equation to predict oral toxicity, particularly for formulations with lower toxicity (Hamm et al., 2021). Furthermore, OPP participated in a project to rethink chronic toxicity and carcinogenicity assessment for agrochemicals (called "ReCAAP"). The workgroup, consisting of scientists from government, academia, non-governmental organizations, and industry stakeholders, aimed to develop a reporting framework to support a WoE safety assessment without conducting long-term rodent bioassays. In 2020, an EPA Science Advisory Board meeting was held to discuss reducing the use of animals for chronic and carcinogenicity testing, which included comment on the ReCAAP project (EPA, 2020f), and feedback from the consultation was incorporated into a published framework (Hilton et al., 2022).

4.1.3.1 Examples of NAM application

OPP has recently used NAMs to derive points of departure for human health risk assessment. For isothiazolinones, which are material preservatives that are known dermal sensitizers, NAMs were utilized to support a quantitative assessment (EPA, 2020g). *In chemico* and *in vitro* assays were performed on each chemical to derive concentrations that can cause induction of skin sensitization and were used as the basis of the quantitative dermal sensitization evaluation. The NAM approaches used in the assessment have been shown to be more reliable, human-relevant, and mechanistically driven, and able to better predict human sensitizing potency when compared to the reference test method, the mouse local lymph node assay (EPA, 2020d).

In addition, as part of a registration review, a NAM approach was used to evaluate inhalation exposures for the fungicide chlorothalonil, which is a respiratory contact irritant (EPA, 2021c). The approach utilizes an *in vitro* assay to derive an inhalation point of departure in conjunction with *in silico* dosimetry modeling to calculate human equivalent concentrations for risk assessment (Corley et al., 2021; McGee Hargrove et al., 2021). The approach, which was reviewed and supported by a FIFRA Scientific Advisory Panel (EPA, 2018a), provided an opportunity to overcome challenges associated with testing respiratory irritants, while also incorporating human relevant information.

Further, OPP has been shifting its testing focus from developmental neurotoxicity (DNT) guideline studies to more targeted testing approaches. In addition to evaluating life stage sensitivity with studies based on commonly accepted modes of action, such as comparative cholinesterase assays and comparative thyroid assays, researchers from ORD have participated in an international effort over the past decade to develop a battery of NAMs for fit-for-purpose evaluation of DNT (Fritsche et al., 2017; Bal-Price et al., 2018a; Bal-Price et al., 2018b; Sachana et al., 2019). As part of this effort, ORD researchers developed *in vitro* assays using microelectrode array network formation array (MEA NFA) and high-content imaging (HCI) platforms to evaluate critical neurodevelopmental processes. Additional *in vitro* assays have been developed by researchers funded by EFSA and, together with the ORD assays, form the current DNT NAM battery. The FIFRA SAP supported the use of the data generated by the DNT NAM battery as part of a WoE for evaluating DNT potential and recognized the potential for the battery to continuously evolve as the science advances (EPA, 2020i). The OECD DNT expert group, which includes staff from OPP and ORD as well as representatives from other US agencies (e.g., NTP, FDA), is also considering several case studies on integrating the DNT battery into an IATA. Furthermore, data from the battery along with toxicokinetic assessment and available *in vivo* data were recently used in a WoE to support a DNT guideline study waiver (Dobreniecki et al., 2022).

OPP also collaborated with NICEATM to complete retrospective analyses of dermal penetration triple pack studies (Allen et al., 2021). A triple pack consists of an *in vivo* animal study and *in vitro* assays using human and animal skin and are used to derive DAFs applied to convert oral doses to dermal-equivalent doses to assess the potential risk associated with dermal exposures. The retrospective analyses demonstrated that *in vitro* studies alone provide similar or more protective estimates of dermal absorption with limited exception. The use of human skin for human health risk assessment has the added advantage of being directly relevant to the species of interest and avoiding overestimation of dermal absorption using rat models. These analyses are being used by OPP to support its consideration of results from acceptable *in vitro* studies in its WoE evaluations to determine an appropriate dermal absorption factor (DAF) for human health risk assessment on a chemical-by-chemical basis.

4.2 Canada

In Canada, pest control products and the corresponding technical grade active ingredient are regulated under the *Pest Control Products Act* (PCPA; SC 2002, c.28). The PCPA and its associated Regulations govern the manufacture, possession, handling, storage, transport, importation, distribution, and use of pesticides in Canada. Pesticides, as defined in the PCPA, are designed to control, destroy, attract, or repel pests, or to mitigate or prevent pests' injurious, noxious, or troublesome effects. Therefore, these properties and characteristics that make pesticides effective for their intended purposes may pose risks to people and the environment.

PMRA is the branch of Health Canada responsible for regulating pesticides under the authority of the PCPA.

Created in 1995, PMRA consolidates the resources and responsibilities for pest management regulation in Canada. PMRA's primary mandate is to prevent unacceptable risks to Canadians and the environment from the use of these products. Section 7 of the PCPA provides the authority for PMRA to apply modern, evidence-based scientific approaches to assess whether the health and environmental risks of pesticides proposed for registration (or amendment) are acceptable, and that the products have value. Section 16 of the PCPA provides the legislative oversight for PMRA to take the same approach when regularly and systematically reviewing whether pesticides already on the Canadian market continue to meet modern scientific standards. PMRA's guidance document "A Framework for Risk Assessments and Risk Management of Pest Control Products" provides the well-defined and internationally recognized approach to risk assessment, management, and decision-making. This framework includes insights on how interested and affected parties are involved in the decision-making process. It also describes the components of the risk (health and environment) and value assessments. For example, the value assessment's primary consideration is whether the product is efficacious. In addition, this assessment contributes to the establishment of the use conditions required to assess human health and environment risks (HC, 2021a).

4.2.1 General requirements

In Canada, many pest control products are categorized as conventional chemicals, and include insecticides, fungicides, herbicides, antimicrobials, personal insect repellents, and certain companion animal products such as spot-on pesticides for flea and tick control. Non-conventional chemicals, such as biopesticides (e.g., microbial pest control agents) and essential oil-based personal insect repellents, are also regulated under the PCPA.

The scope of the information provided in this section is most applicable to the health component of the risk assessment for domestic registrations of conventional chemicals (the end-use product and active ingredient). The information provided hereafter excludes the value and environment components along with products, such as food items (e.g., table salt), which are of interest to the organic growers in Canada. Biopesticides and non-conventionals are also outside the scope of this paper.

PMRA relies on a system that links the data requirements (data-code or DACO tables) to proposed use-sites, which are organized using three categories: Agriculture, Industry, and Society (HC, 2006). Given that pest control products can be used on more than one use-site, these sites are further sub-categorized. For example, PMRA's use-site category 14 is for "Terrestrial Food Crops" and includes crops grown outdoors as a source for human consumption (HC, 2013b). The system of linking DACOs with use-site categories is similar to what is used by the US EPA and internationally by the OECD (HC, 2006). The PMRA DACO tables include required (R) and conditionally required (CR) data that are tailored for each use site and take into consideration potential routes, durations, and sources of exposure to humans and the environment. It is important to note that the CR data are only required under specified conditions. In addition, PMRA will consider a request to waive any data requirement, but such waiver requests must be supported by a scientific rationale demonstrating that the data are not required to ensure the protection of human health. In particular, PMRA published a guidance document for waiving or bridging of mammalian acute toxicity tests for pesticides in 2013 (HC, 2013a). This document served as the starting point for the development and subsequent release of the 2017 OECD technical document on the same subject (OECD, 2017).

4.2.2 Regulatory flexibility

The specific data requirements for the registration of pest control products in Canada are not prescribed in legislation under the PCPA. PMRA, therefore, has greater flexibility in either adopting or adapting methods under the PCPA in comparison to other jurisdictions where these data requirements are established in law. Therefore, while the PCPA provides the overarching components for the assessments (i.e., health, environment, and value) it also provides for the flexibility to use policy instruments along with guidance documents to provide the details on the data requirements to satisfy these legislative components. This approach also provides the opportunity for PMRA to engage all stakeholders through webinars, meetings, and public consultations when developing or making major changes to these documents. This open and transparent approach is aligned with PMRA's strategic plan (HC, 2016d), which includes incorporating modern science by building scientific, regulatory, and public confidence in these approaches through collaborative processes. The ability to rely on policy instruments and guidance documents does not preclude PMRA from making regulatory changes, when necessary; however, the experience, thus far, with NAMs supports the current approach of relying on multi-stakeholder collaborations that result in the development of guidance documents, science policy notes, and/or published articles in reputable scientific journals.

4.2.3 Implementation of NAMs

PMRA's 2019-2020 annual report highlights the 25th anniversary of this branch of Health Canada while noting a major transformation initiative of the pesticide program (HC, 2021e). Building upon the strategic plan (see Section 2.2.2), the program renewal project considers the changing landscape and the need for PMRA to keep pace with this change. The 2019-2020 and 2020-2021 reports include a section on evaluating new technologies, which includes opportunities to reduce animal testing wherever possible. Specifically, the use of NAMs, including *in vitro* assays, predictive *in silico* models,

mechanistic studies, and existing data for human health and environmental assessment of pesticides is noted (HC, 2021e; Hc, 2022b).

Bhuller et al. (2021) provides the first Canadian regulatory perspective on the approach and process towards the implementation of NAMs in Canada for pesticides and industrial chemicals (Bhuller et al., 2021). It acknowledges foundational elements, such as the 2012 Council of Canadian Academies (CCA, 2012) expert panel report, "Integrating Emerging Technologies into Chemical Assessment," used to establish the overall vision. The process for identifying, exploring, and implementing NAMs emphasizes the importance of mobilizing teams and fostering a mindset that enables a regulatory pivot towards NAMs. In addition, the importance of engagement and multi-stakeholder collaboration is identified as a pillar for building regulatory, scientific, and public confidence in NAMs along with the broader acceptance of the alternative approaches.

PMRA collaborates with stakeholders on the development of NAMs and their potential implementation for regulatory purposes. For example, PMRA is collaborating with the interested community through several ongoing multi-stakeholder initiatives designed to explore NAMs, at the national and international levels (Bhuller et al., 2021). Another example is several academic-led initiatives along with research and consulting firms that are immersed in developing models, including open-source models. This includes the University of Windsor's Canadian Centre for Alternatives to Animal Methods (CCAAM) and CaCVAM. Within Health Canada, voluntary efforts amongst regulatory and research scientists have resulted in the publication of NAM-relevant documents, such as the current Health Canada practices for using toxicogenomics data in risk assessment (HC, 2019).

4.2.3.1 Examples of NAM application

Multiple NAMs and alternatives to animal testing have been co-developed, adapted, or adopted by the PMRA. Examples include the OECD defined approach for skin sensitization (OECD, 2021a), use of a WoE framework for chronic toxicity and cancer assessment (Hilton et al., 2022), and PMRA's "Guidance for Waiving or Bridging of Mammalian Acute Toxicity Tests for Pesticides" (HC, 2013a). In addition, PMRA no longer routinely requires the acute dermal toxicity assay (HC, 2017), the one-year dog toxicity test (Linke et al., 2017; HC, 2021d), or the *in vivo* dermal absorption study (Allen et al., 2021) in alignment with the US EPA. PMRA will consider these and other NAMs *in lieu* of animal testing for specific pesticides by applying a WoE approach to ensure that the available information is sufficient and appropriate for hazard characterization and the assessment of potential human health risks.

Building upon the strategic plan and the importance of staying current with scientific advancements in an open and transparent manner, PMRA's DACO guidance document for conventional pesticides includes a document history table that enables PMRA to demonstrate the "evergreen" nature of the DACOs while providing an overview of the changes and the corresponding rationales (HC, 2021d). For example, PMRA's science-policy work, resulting in no longer routinely requiring the acute dermal toxicity study, is captured in this table with a reference to the science-policy document (SPN 2017-03) (HC, 2017). The latter then provides details on public consultation processes and the robust retrospective analysis that was undertaken under the auspices of the RCC (HC, 2017).

4.3 European Union

In the EU, the term "pesticides" includes (1) active ingredient and PPPs, which are intended for use on plants in agriculture and horticulture, and (2) biocides, which are used in non-botanical applications, such as rodenticides or termiticides. PPPs and their active ingredients are regulated under Regulation (EC) No 1107/2009 (EC, 2009a). Commission Regulation (EU) No 283/2013 lists the data requirements for active ingredients (EU, 2013c), and Commission Regulation (EU) No 284/2013 lists the data requirements for PPPs (EU, 2013d). Biocides, however, are regulated separately under Regulation (EU) No 528/2012 and are not discussed in this paper (EU, 2012). In addition, the CLP regulation (see Section 3.3) applies to both PPPs and biocides.

The EU is a diverse group of countries as it relates to food consumption, agricultural pests, weather, and level of development, thus, the risk assessment and management procedures were developed to account for the varied needs of different Member States. First, an evaluation of the active ingredient dossier is conducted by a Rapporteur Member State. Then, EFSA peer reviews the dossier evaluation. The peer reviewed risk assessment of the active ingredient is considered by the European Commission, who makes a proposal on whether to authorize the active ingredient, followed by the EU Member States, who vote on final risk management decisions. Once an active ingredient is authorized, individual Member States consider applications for approval of PPPs containing that active ingredient and propose maximum levels of pesticide residues permitted to remain in/on food commodities or animal feed. Finally, the European Commission (often with input from EFSA) will decide whether to approve those maximum residue levels.

Regulation of biocidal active ingredients and products proceeds via a similar route; however, peer review of the Member State assessments of the active ingredients are conducted by ECHA rather than EFSA. In 2017, ECHA and EFSA signed a memorandum of understanding to enhance cooperation between the agencies to facilitate coherence in scientific methods and opinions, and to share knowledge on

matters of mutual interest. As a consequence, both agencies will evaluate the toxicological data package for a PPP.

4.3.1 General requirements

Similar to the US and Canada, there are a large number of up-front data requirements required to register a plant protection active ingredient in the EU, including studies to assess potential hazards to humans and non-target organisms. The toxicology data requirements for support of an active ingredient or PPP are listed in Commission Regulation (EU) No 283/2013 and Commission Regulation (EU) No. 284/2013, respectively, and can be fulfilled using OECD test guideline studies or other guidelines (such as US EPA guidelines) that address the toxicological endpoint of concern. A number of data requirements, such as *in vivo* neurotoxicity studies or two-year rodent cancer bioassays in a second species, are only required when triggered or with scientific justification.

4.3.2 Regulatory flexibility

Article 62(1) of Regulation (EC) No 1107/2009 requires that "testing on vertebrate animals for the purposes of this Regulation shall be undertaken only where no other methods are available." Article 8(1)(d) and Article 33(3)(c) of the same Regulation requires applicants to justify, for each study using vertebrate animals, the steps taken to avoid testing on animals or duplication of studies. Similarly, for biocides, Article 62(1) of Regulation (EC) No 528/2012 states that "[i]n order to avoid animal testing, testing on vertebrates for the purposes of this Regulation shall be undertaken only as a last resort."

The Commission Regulations, which list the data requirements for plant protection active ingredients and products, and their respective Communications (EU, 2013a; EU, 2013b) were published in 2013 and therefore only refer specifically to a limited number of NAMs (e.g., *in vitro* and *ex vivo* methods to assess skin irritation and eye irritation).

Although point 5.2 in the Annex of both Commission Regulation (EU) No 283/2013 and 284/2013 allow for the use of other NAMs, as they become available, to replace or reduce animal use, the outdated list of methods to fulfil data requirements in the Commission Communications may encourage animal use where NAMs should be used. For example, the methods listed to fulfil the requirements for skin sensitization do not include any of the available *in chemico* or *in vitro* methods and do not refer to the OECD Guideline on Defined Approaches to Skin Sensitization (EU, 2013a; EU, 2013b; OECD, 2021a). Therefore, the Commission Communications need to be updated urgently and regularly to avoid the use of animals.

As outlined above, the regulatory landscape of the EU is one of specific regional considerations and interpretation of legislation by individual Member States. For example, some Member State regulatory authorities responsible for PPPs, including those from the Czech Republic (SZU, n.d.), Sweden (KEMI, 2021), and Slovenia (Republika Slovenija, 2022), publicly align themselves with the legal requirement to justify the conduct of studies using vertebrate animals. Other Member States regulatory authorities, including those from the Netherlands (Ctgb, n.d.) and, pre-Brexit, the United Kingdom (HSE, n.d.), interpret the regulation more rigorously and state that applicants or dossiers will not be considered if they are found to have breached Article 62 (testing on vertebrate animals as a last resort).

4.3.3 Implementation of NAMs

EFSA has been proactive in reducing animal testing and implementing reliable NAMs. For example, in 2009, EFSA published a scientific opinion covering the key data requirements for evaluation of pesticide toxicity that were amendable to NAMs (EFSA, 2009). In 2012, EFSA initiated a series of scientific conferences to create a regular opportunity to engage with partners and stakeholders. Following its latest conference in 2018 and the break-out session "Advancing risk assessment science—human health," Lanzoni et al. have emphasized that the human health risk assessment based on animal testing is challenged scientifically and ethically (Lanzoni et al., 2019). They further mention the need for a paradigm shift in hazard and risk assessment and more flexible regulations.

EFSA has developed a chemical hazards database "OpenFoodTox 2.0" and funded collaborative research to develop generic toxicokinetic and toxicodynamic human and animal models to predict the toxicity of chemicals (Dorne et al., 2018; Benfenati et al., 2020). Further, in 2019, EFSA published their opinion on the use of *in vitro* comparative metabolism (IVCM) studies for use in pesticide risk assessment (EFSA, 2019). Currently, the IVCM study is a data requirement for new and renewal data packages being submitted in the EU. This study is intended to identify unique human metabolites as it compares to OECD TG 417, the toxicokinetic study currently performed in rats. Most recently, EFSA published their 2027 Strategy in which they state their goal, to develop and integrate NAMs for regulatory risk assessment (EFSA, 2021). To help achieve this, EFSA launched a contract to develop a roadmap for action on NAMs to reduce animal testing (Escher et al., 2022). The roadmap aims to define EFSA's priorities for the incorporation of NAMs as well as to inform a multi-annual strategy for increasing the use of NAMs in human health risk assessment with a goal of minimizing animal testing (EFSA, 2021). In addition, EFSA is in the process of developing guidance on the use of read-across and has launched several projects to evaluate NAMs in the context of IATA frameworks.

4.3.3.1 Examples of NAM application

EFSA has funded the development of *in vitro* assays that, together with the assays from ORD, form the current DNT NAM testing battery (see Section 4.1.3.1). In partnership with the OECD, EFSA held a workshop in 2017 on integrated

approaches for testing and assessment of DNT (EFSA and OECD, 2017), commissioned an external scientific report on the data interpretation from *in vitro* DNT assays (Crofton and Mundy, 2021), and recently held a European stakeholder's workshop on NAMs for DNT (EFSA, 2022). In 2021, the EFSA Panel on Plant Protection Products and their Residues (PPR) developed AOP-informed IATA case studies on DNT risk assessment (EFSA PPR Panel et al., 2021). The development of a new OECD Guidance Document on DNT *in vitro* assays is being co-led by EFSA, the US, and Denmark (OECD, 2021b).

In 2017, EFSA updated its guidance on dermal absorption that was initially published in 2012. The guidance presents elements for a tiered approach including "*in vitro* studies with human skin (regarded to provide the best estimate)" (EFSA et al., 2017), thereby reducing the use of animals while also increasing the relevance of the data for human risk assessment.

Furthermore, *in silico* modeling software, data mining, and read-across can be used for a variety of applications in support of pesticide registrations within the EU. Specifically, OECD-Toolbox, Derek Nexus, and OASIS TIMES are often used for the evaluation of toxicological significance of metabolites and impurities, and in support of active ingredient conclusions, especially related to genotoxicity (Benigni et al., 2019).

5 Conclusion

Due to widespread interest in the use of testing approaches that are reliable and relevant to human biology, NAMs for hazard and risk assessment are being rapidly developed. It is important to understand the existing regulatory frameworks, and their flexibility or limitations for the implementation of fit for purpose NAMs. This article provides an overview of the regulatory frameworks for the use of NAMs in the assessment of industrial chemicals and pesticides, in the US, Canada, and EU. However, similar collaborative efforts and opportunities to use NAMs in regulatory submissions exist in other sectors and countries. In general, replacing animal use is an important goal for regulatory agencies and, as such, regulators continue to explore the potential of NAMs to efficiently provide more reliable and relevant information about whether and how a chemical may cause toxicity in humans. The regulations reviewed in this paper highlight the many existing opportunities for the use of NAMs, while also showing potential to introduce further flexibility in testing requirements to allow the maximum use of fit for purpose NAMs.

For example, it is important to provide continuing educational opportunities for regulators and stakeholders on the conditions under which application of a certain NAM is appropriate and on how data from that NAM is interpreted. Conferences and webinars, as mentioned in Section 2, are examples of such opportunities. There are also ongoing discussions on how to streamline and accelerate validation processes and gain scientific confidence in the use of robust NAMs, including an ongoing effort within ICCVAM to publish a guidance on this topic. Updating these processes is foundational to timely uptake of fit-for-purpose, reliable, and relevant NAMs (van der Zalm et al., 2022). Also key to the advancement of NAMs is the opportunity to discuss proposed NAM testing strategies with the agency. This allows for the wise use of resources and ensures that data needs of the regulatory agencies are being addressed by the proposed approach. Each regulatory agency has varying ability and instructions on meeting with stakeholders to discuss proposed testing strategies, with some agencies (notably the EPA and HC's NSP) strongly encouraging these meetings, resulting in examples of successful submissions. Additional measures to instate incentives, such as expedited review, would further facilitate innovation and the use of more modern, reliable NAMs.

In addition, national and international communication and collaboration within and across sectors and geographies is of the utmost importance to minimize duplicative efforts and efficiently advance the best science. Ultimately, regulatory frameworks that allow for the timely uptake of scientifically sound toxicology testing approaches will facilitate the global acceptance of NAMs and allow the best protection of human health.

Author contributions

All authors contributed important intellectual content and helped in the conceptualization, writing and revisions of the article. All authors read and approved the final manuscript.

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Conflict of interest

Author JH was employed by the company Corteva Agriscience. Authors CH and JM-H were employed by the company JT International SA. Authors EN and TS were employed by the law firm Bergeson & Campbell PC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

AOP Adverse Outcome Pathways

APCRA Accelerating the Pace of Chemical Risk Assessment

BraCVAM Brazilian Centre for the Validation of Alternative Methods

CaCVAM Canadian Centre for the Validation of Alternative Methods

CATSAC Chemistry and Acute Toxicology Science Advisory Council

CCA Council of Canadian Academies

CCAAM Canadian Centre for Alternatives to Animal Methods

CEPA Canadian Environmental Protection Act

CFR Code of Federal Regulations

CLP Classification, labelling and packaging of substances and mixtures

CMP Chemicals Management Plan

CPSC US Consumer Products Safety Commission

CR Conditionally required

CSS EU Chemicals Strategy for Sustainability

DACO Data-code

DAF Dermal absorption factor

DNT Developmental neurotoxicity

DSL Domestic Substances List

EC European Commission

ECHA European Chemicals Agency

EFSA European Food Safety Authority

EPA US Environmental Protection Agency

ESRAP Existing Substances Risk Assessment Program

EU European Union

EURL ECVAM EU Reference Laboratory for alternatives to animal testing

FD&C Federal Food, Drug, and Cosmetic Act

FHSA Federal Hazardous Substances Act

FIFRA Federal Insecticide, Fungicide, and Rodenticide Act

FQPA Food Quality Protection Act

GHS Globally Harmonised System of Classification and Labelling of Chemicals

GLP Good Laboratory Practice

HASPOC Hazard and Science Policy Council

HC Health Canada

HCI High-content imaging

HECSB Healthy Environments and Consumer Safety Branch

IATA Integrated Approaches to Testing and Assessment

ICATM International Cooperation on Alternative Test Methods

ICCVAM US Interagency Coordinating Committee for the Validation of Alternative Methods

ITC Interagency Testing Committee

IVCM *In vitro* comparative metabolism

JaCVAM Japanese Centre for the Validation of Alternative Methods

KoCVAM Korean Centre for the Validation of Alternative Methods

MAD Mutual Acceptance of Data

MEA NFA Microelectrode array network formation array

NAFTA TWG North American Free Trade Agreement Technical Working Group

NAM New Approach Methodologies

NICEATM US National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods

NRC National Resource Council

NSN New Substances Notification

NSNR New Substances Notification Regulations

NSP New Substances Program

NTP US National Toxicology Program

OCSPP Office of Chemical Safety and Pollution Prevention

OECD Organisation for Economic Co-operation and Development

OPP Office of Pesticide Programs

OPPT Office of Pollution Prevention and Toxics

ORD Office of Research and Development

PCPA Pest Control Products Act

PMN Pre-Manufacture Notice

PMRA Pest Management Regulatory Agency

PPP Plant Protection Products

PPR EFSA Panel on Plant Protection Products and their Residues

QSAR Quantitative Structure-Activity Relationship

RCC Canada-US Regulatory Co-operation Council

REACH Registration, Evaluation, Authorisation and Restriction of Chemicals

ReCAAP Rethink Chronic toxicity and carcinogenicity Assessment for Agrochemicals

SAP Scientific Advisory Panel

SC Statutes of Canada

SciAD Science Approach Documents

SIEFs Substance Information Exchange Fora

SNUN Significant New Use Notice

SNUR Significant New Use Rule

SOR Statutory Orders and Regulations
TG Test Guidelines
TSAR Tracking System for Alternative Methods
TSCA Toxic Substances Control Act
TTC Threshold of Toxicological Concern
UNECE United Nations Economic Commission for Europe
US United States
USC US Code
USMCA US-Mexico-Canada Agreement
WoE Weight of (scientific) Evidence

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Use of new approach methodologies (NAMs) to meet regulatory requirements for the assessment of tobacco and other nicotine-containing products

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Regulatory frameworks on tobacco and other nicotine-containing products (TNCP) continue to evolve as novel products emerge, including electronic nicotine delivery systems (e.g., electronic cigarettes or vaping products), heated tobacco products, or certain smokeless products (e.g., nicotine pouches). This article focuses on selected regulations for TNCPs that do not make health claims, and on the opportunities to use new approach methodologies (NAMs) to meet regulatory requirements for toxicological information. The manuscript presents a brief overview of regulations and examples of feedback from regulatory agencies whilst highlighting NAMs that have been successfully applied, or could be used, in a regulatory setting, either as stand-alone methods or as part of a weight-of-evidence approach to address selected endpoints. The authors highlight the need for agencies and stakeholders to collaborate and communicate on the development and application of NAMs to address specific regulatory toxicological endpoints. Collaboration across sectors and geographies will facilitate harmonized use of robust testing approaches to evaluate TNCPs without animal testing.

KEYWORDS

tobacco and tobacco product, nicotine-containing products, new approach methodologies (NAMs), regulatory flexibility, toxicity testing, *in vitro*, *in silico*

1 Introduction

The health risks from combustible tobacco products (e.g., cigarettes) have been known for decades. In the 20th century, converging lines of evidence were available that began to shed light on the increasing incidence of lung cancers amongst smokers around the world. These evidence streams included population studies, studies in experimental

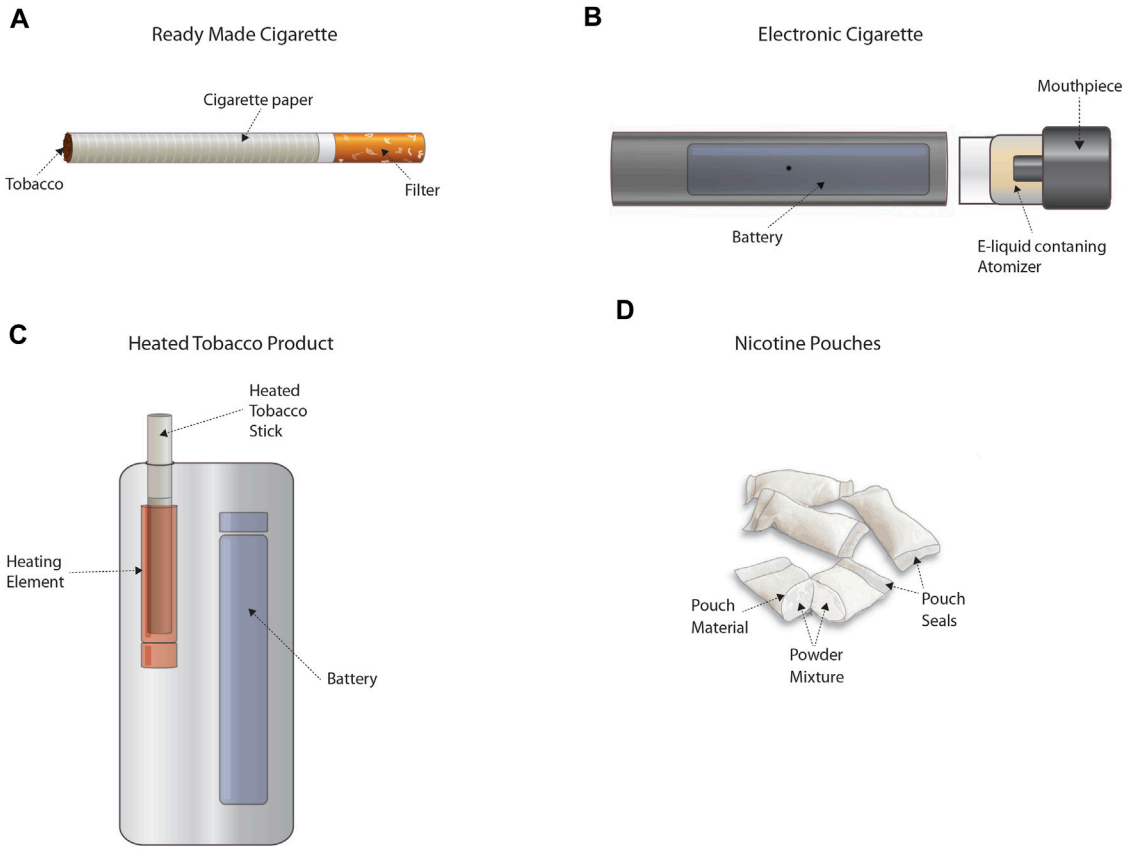

FIGURE 1
Representative tobacco and other nicotine-containing products (TNCP). Panel **(A)** provides an example of a ready-made cigarette that delivers nicotine in smoke formed through combustion. Panel **(B)** provides an example of an electronic cigarette that delivers nicotine in a heated vapor/aerosol. Panel **(C)** provides an example of a heated tobacco product that delivers nicotine in a heated vapor/aerosol. Panel **(D)** provides an example of oral nicotine pouches that deliver nicotine from a powder mixture. TNCPs are defined differently throughout the various jurisdictions and may or may not include other products. Please consult the regulations of the respective geographical area for the exact definitions.

animals, cellular pathology, and the identification of cancer-causing chemicals in cigarette smoke (Proctor, 2012).

Decades later, cigarette smoking remains one of the leading preventable causes of morbidity and mortality. Today, novel tobacco and other nicotine-containing products (TNCP)—including electronic nicotine delivery systems (ENDS) (e.g., electronic cigarettes or vaping products), heated tobacco products, or certain smokeless products (e.g., nicotine pouches) (Figure 1)[1]—are available that may facilitate nicotine addicted smokers to transition to potentially lower risk alternatives and/or cessation. Regulatory frameworks around the world have mechanisms for evaluating novel TNCPs but unlike combustible cigarettes, the available data on these technologies are comparatively limited. Indeed, substantial *in vitro* and *in vivo* data have been generated for combusted tobacco products using a variety of assays to address a number of toxicological endpoints. An overview of studies previously published or provided to authorities is documented in a recent report from the German Institute for Standardisation (DIN, 2021). As regulators provide feedback to industry or as guidelines change and methodologies develop, it is clear that data from multiple assays considered in a weight-of-evidence approach will be useful to fully address the broad spectrum of health effects normally attributed to tobacco smoke. This creates challenges for developers, as well as for regulators who are tasked with determining whether these technologies may advance the decline in smoking prevalence and population harm, without unintended side effects.

In 2007, the United States (US) National Research Council (NRC, 2007) issued its groundbreaking report titled "Toxicity Testing in the 21st Century—A Vision and a Strategy". Since this

1 TNCPs are defined differently throughout the various jurisdictions and may or may not include additional products. Please consult the regulations of each geographical area for the exact definitions.

time, scientists across government, academia, non-governmental organizations, and regulated sectors have been diligently researching and applying novel testing strategies, commonly known as New Approach Methodologies (NAMs). NAMs are any technology, methodology, approach, or combination that can provide information on chemical hazard and risk assessment that replaces or reduces the use of animals, including *in silico*, *in chemico*, *in vitro*, and *ex vivo* approaches (ECHA, 2016; EPA, 2018b). NAMs are not necessarily newly developed methods, rather, it is their application to regulatory decision making or replacement of a conventional testing requirement that is new. They may be based on human cells or cell components that avoid the use of vertebrate animals and the uncertainties associated with extrapolating findings from experimental animals to humans. Therefore, NAMs may offer a unique and more efficient means of informing key events from adverse outcome pathways and exploring potential toxicological effects during the research and development (R&D) process and in regulatory filings (Peitsch et al., 2018; Luettich et al., 2021).

With the growth and increasing variants of non-combustible TNCPs, regulatory requirements have changed or are under development in several nations. In many countries, the provision of toxicological information is obligatory and can vary between specific submissions in the case of substantive product changes or for new product launches (e.g., US) and/or an annual submission for all products (e.g., Canada and the EU).

This article focuses on selected legal and regulatory frameworks for nicotine control in the US, Canada, and the European Union (EU) and the guidance, if available, for using data from NAMs in lieu of data from animals. This perspective provides select examples of opportunities where data from NAMs may be used to evaluate R&D products, as well as to inform data requirements amongst the required toxicological endpoints in certain regulatory frameworks for TNCPs. The authors acknowledge that other countries (e.g., Japan and South Korea) and organizations (e.g., World Health Organisation) are also very active in this area and have published several reports (WHO, 2021). However, this perspective focuses on information available in the US, Canada, and the EU that highlight the need for alignment throughout the world with regard to regulatory acceptance and use of NAMs.

2 Overview of regulatory status

2.1 United States

In 2009, President Barack Obama signed into law the Family Smoking Prevention and Tobacco Control Act (Tobacco Control Act; Pub.L. 111–31), giving the US Food and Drug Administration (FDA) authority to regulate TNCPs under the Federal Food Drug and Cosmetic Act (FD&C Act, 21 USC §301 et seq.; Subchapter IX, 21 USC §387). As a result, the Center for Tobacco Products (CTP) was created within FDA and requires CTP to review and approve or deny new TNCPs before market entry.

For any new TNCP, under FD&C Section 910, an application is mandated, along with the requirement to obtain an FDA marketing authorization order. Marketing authorization may be acquired using one of the following pathways (21 USC §387j):

- Substantial equivalence (SE) report for new TNCPs that have the same characteristics as another product with market approval or its exemption when applicable
- Premarket Tobacco Product Application (PMTA)

The information to be included into SE reports is judged on a case-by-case basis, primarily depending on the changes proposed to the new product compared to a predicate product in a report. The current regulatory impact analysis on SE reports indicates that whatever data are submitted, it should include test protocols, quantitative acceptance criteria, and test results as well as clearly identify when national or international standards are used to test the new and predicate products in addition to explaining any deviations from the standard or stating if no testing standards were used. In this respect, the guidelines are sufficiently broad to allow the submission of information from NAMs (FDA, 2021a).

In the guidance for industry on PMTAs for ENDS, CTP documented that a data package might require the following, non-clinical investigations, if available (FDA, 2019a):

- *In vitro* toxicology studies (e.g., genotoxicity studies or cytotoxicity studies)
- Computational modeling of the toxicants in the product (to estimate the toxicity of the product)
- *In vivo* toxicology studies (to address unique toxicology issues that cannot be addressed by alternative approaches)

Throughout this guidance document, CTP states its support for reducing, replacing, and/or refining the use of animal testing where adequate and scientifically valid NAMs can be substituted (FDA, 2019a). CTP encourages sponsors to meet with them early in the development process to discuss the suitability and acceptability of NAMs for informing potential hazards from the new TNCP. Furthermore, the finalized 2019 guidance document states "[i]n the absence of toxicological data for a particular toxicant of concern, we recommend that you consider computational modeling using surrogate chemical structures" (FDA, 2019a). The guidance goes on to state "[i]f you plan to conduct any computational modeling, we suggest that you meet with CTP to specifically address this issue".

In its final rule, CTP provides information on the types of investigations that applicants must submit as part of a PMTA, if published, known to, or which should reasonably be known to an

applicant including, but not limited to, genotoxicity, carcinogenicity, respiratory toxicity, cardiac toxicity, reproductive and developmental toxicity, and chronic (repeated dose) toxicity of the new TNCP relative to other TNCPs. It includes human exposure studies, *in silico* computational toxicology techniques, risk assessments, *in vitro* toxicology studies, published reports of *in vivo* toxicology studies, and, if necessary, new *in vivo* toxicology studies. Additionally, CTP reconfirms in the final rule its support of reducing reliance on animal testing where adequate and scientifically valid NAMs can be substituted (FDA, 2021b). While the final rule and guidance for industry suggest that NAMs are generally deemed acceptable, CTP did not provide detailed information on specific NAMs and the extent to which they can be used.

CTP's flexibility with considering the use of NAMs is representative of broader initiatives at FDA that have been ongoing for more than a decade. For example, in its 2011 strategic plan titled "Advancing Regulatory Science at FDA," FDA reiterated its general support for the use of NAMs where transformation of toxicology was identified as a key scientific priority that offers enormous opportunities (FDA, 2011). In its 2017 Predictive Toxicology Roadmap, which was published to guide FDA's six product centers—including CTP—in the development and use of new technologies, FDA reiterated its support for the use of NAMs (FDA, 2017). More recently, FDA updated the strategic plan with a report on focus areas of regulatory sciences in which they identified that NAMs will likely provide enhanced prediction of the risk and/or safety outcomes (FDA, 2021c). The FDA is also included in the Interagency Coordinating Committee on the Validation of Alternative Methods which published a strategic roadmap in 2018 to serve as a guide for agencies and stakeholders seeking to adopt NAMs for chemical safety and risk assessments (ICCVAM, 2018).

2.2 Canada

The Tobacco and Vaping Products Act (TVPA) regulates the manufacture, sale, labelling, and promotion of TNCPs (S.C. 1997, c.13). TVPA stipulates that Canada's Governor in Council may make additional regulations prescribing information that manufacturers must submit to the Minister about R&D related to TNCPs and their emissions, including health effects and hazardous properties.

The Tobacco Reporting Regulations (TRR; Statutory Orders, and Regulations [SOR]/2000–273, CDC, 2000) requires an annual report be generated on the toxicity of cigarette emissions. The Regulations refer to the following *in vitro* toxicity tests for Mainstream Tobacco Smoke: Bacterial Reverse Mutation, Neutral Red Uptake Assay, and *In Vitro* Micronucleus Assay, and an annual report on R&D activities as indicated in Section 15 of the TRR in addition to sales, emissions, and contents of the product.

The TRR currently includes testing requirements for tobacco smoke (i.e., combustible tobacco products), but not other nicotine-containing products. The amendment of the TRR as well as implementing vaping reporting regulations were initiated in 2017 and are part of Health Canada's Forward Regulatory Plan 2021–2023 and 2022–2024 but have not been published yet (HC, 2021, 2022).

2.3 European Union

In the EU, the Tobacco Products Directive (TPD; Directive 2014/40/EU, 2014) sets the minimum requirements (e.g., health warnings and reporting) for TNCPs to be placed on the market in EU, 2015a Member States.

For tobacco products, Article 5 (3) requires the submission of a list of ingredients with relevant toxicological data for the ingredients in burnt and unburnt form, as appropriate. In particular, any effects on human health or addictiveness of the ingredients shall be reported. Similar information is required for ENDS, per Article 20. For novel TNCPs, Article 19 requires the submission of any available scientific studies on toxicity, addictiveness, and attractiveness of the novel TNCP. Additionally, Article 6 (2) requires manufacturers and importers of cigarettes and Roll-Your-Own tobacco that contain an additive from the priority list established by Commission Implementing Decision (EU, 2015b) 2016/787 to carry out comprehensive studies (EU, 2016).

The field descriptors for toxicological data submission are documented in Commission Implementing Decision 2015/2186 for tobacco products and 2015/2183 for electronic cigarettes.

The toxicological data reporting indicates that any type of study may be submitted, implying the possibility to submit NAMs for informing potential hazard concerns for genotoxicity, carcinogenicity, respiratory toxicity, reproductive toxicity, cardiopulmonary toxicity, and any other type of toxicity. Several EU Member States, such as Belgium, Estonia, Germany, and Slovakia, have banned the use of animals for the development and testing of tobacco products.

Individual Member States "may also require manufacturers or importers to carry out studies as may be prescribed by the competent authorities in order to assess the effects of ingredients on health, taking into account, inter alia, their addictiveness and toxicity". Member States also require any new or updated information to be submitted to their competent authorities or may require additional tests or information for novel TNCPs.

In the recently published "Support study to the report on the application of Directive 2014/40/EU", Member State feedback indicated that there was a "lack of guidance on how e.g. toxicological studies should be assessed" (EC, 2021). It was also implied that there was a range of scientific approaches submitted by different manufacturers and, as such, when

TABLE 1 Examples of available NAMs currently applied in regulatory settings and NAMs under development or other resources, that could be used to develop Integrated Approaches to Testing and Assessment (IATA) for tobacco and other nicotine containing products. This table features only select examples and serves as conversation starter. Mentions of specific NAMs are neither an endorsement of this particular method nor does it mean that no other NAMs exist to fulfil the information requirements for this particular endpoint.

Endpoint	NAM/Subject matter	Regulatory relevance, recognition or potential application
Examples of NAMs currently or soon expected to be applied in a regulatory setting		
Dermal Toxicity (topical)	Defined Approaches on Skin Sensitisation (DASS)	OECD MAD (TG 497, 442C, 442D, 442 E); EPA TSCA List of Alternative Test Methods and Strategies (or New Approach Methodologies), —The List; EPA OCSPP Skin Sensitization Policy
	DASS automated workflow (AW)/OECD QSAR Toolbox 4.5	
	Derek Nexus v.6.1.0/Derek KB 2020 1.0	
	In Chemico Skin Sensitisation—Assays Addressing the AOP Key Event on Covalent Binding to Proteins (Direct Peptide Reactivity Assay and Amino Acid Derivative Reactivity Assay)	
	In Vitro Skin Sensitisation—Assays Addressing the AOP Key Event on Keratinocyte Activation (KeratinoSens™ and LuSens)	
	In Vitro Skin Sensitisation—Assays Addressing the AOP Key Event on Activation of Dendritic Cells (h-CLAT, U-SENS™, and IL-8 Luc assays)	
	In Vitro Skin Irritation—Reconstructed Human Epidermis Test Method	OECD MAD (TG 439, 431, 435; GD No. 203);; EPA TSCA List of Alternative Test Methods and Strategies (or New Approach Methodologies), —The List
	In Vitro Skin Corrosion—Reconstructed Human Epidermis Test Method	
	In Vitro Membrane Barrier Test Method for Skin Corrosion (Corrositex)	
Carcinogenicity	OncoLogic™ (version 8.0) (fibers, metals, polymers)	EPA TSCA List of Alternative Test Methods and Strategies (or New Approach Methodologies), —The List
	OncoLogic™ (version 9.0) (organic chemicals)	
	In Vitro Cell Transformation Assays (non-genotoxic carcinogens)	EURL ECVAM TM 2004–07 (EU); OECD GD No. 214 & 231; EPA TSCA List of Alternative Test Methods and Strategies (or New Approach Methodologies), —The List
Mutagenicity	Bacterial Reverse Mutation Test (Ames Test) (	OECD MAD (TG 471, 473, 476, 487, 490); EPA TSCA List of Alternative Test Methods and Strategies (or New Approach Methodologies), —The List
	In Vitro Mammalian Chromosomal Aberration Test	
	In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt Genes	
	In Vitro Mammalian Cell Micronucleus Test	
	In Vitro Mammalian Cell Gene Mutation Tests using the Thymidine Kinase Gene	
	Validation of the 3D reconstructed human skin Comet assay, an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays (Pfuhler et al., 2021a)	Accepted into the OECD, 2022 test guideline development program (OECD, 2021)
	Validation of the 3D reconstructed human skin micronucleus (RSMN) assay: an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays (Pfuhler et al., 2021b)	
Pulmonary Toxicity	Reconstructed airway epithelium (MucilAir) evaluated using multiple endpoints for acute irritation (EPA, 2018a; McGee Hargrove et al., 2021)	Applied to EPA FIFRA SAP 2018; EPA 2021, Draft Risk Assessment 2021; EPA TSCA List of Alternative Test Methods and Strategies (or New Approach Methodologies), Appendix B—Other Information or Strategies
	Computational fluid dynamics for exposure assessment combined with reconstituted airway epithelium (MucilAir) (Corley et al., 2021; McGee Hargrove et al., 2021)	
	A weight-of-the-evidence (WoE) approach for evaluating, in lieu of animal studies, the potential of a novel polysaccharide polymer to produce lung overload (Ladics et al., 2021)	Was used by EPA in a WoE approach to revoke a significant new use rule (SNUR)
Examples of work supporting NAMs expansion into additional organs and endpoints		
Cardiotoxicity	PBK based NAM for the prediction of cardiotoxicity (Shi et al., 2021)	May be used as part of WoE approach
	Cardio quickPredict (metabolites-based assay utilizing human induced pluripotent stem cells) (Simms et al., 2022)	
	PBK model-guided evaluation of methadone on human-induced pluripotent stem cell-derived cardiomyocytes, comparison to *in vivo* data (Shi et al., 2020)	

(Continued on following page)

TABLE 1 (*Continued*) Examples of available NAMs currently applied in regulatory settings and NAMs under development or other resources, that could be used to develop Integrated Approaches to Testing and Assessment (IATA) for tobacco and other nicotine containing products. This table features only select examples and serves as conversation starter. Mentions of specific NAMs are neither an endorsement of this particular method nor does it mean that no other NAMs exist to fulfil the information requirements for this particular endpoint.

Endpoint	NAM/Subject matter	Regulatory relevance, recognition or potential application
Cardiovascular toxicity	INSPIRE: A European training network to foster research and training in cardiovascular safety pharmacology (Guns et al., 2020)	Overview of cardiovascular toxicity testing (not specifically NAMs)
Reproductive/ developmental toxicity	devTOX quickPredict (metabolomics biomarker-based assay that utilizes human induced pluripotent stem cells) (Simms et al., 2020)	May be used as part of a WoE approach
	Rethinking Developmental Toxicity Testing (Scialli et al., 2018)	NAM Applicability Reviews
	Beyond AOPs: A mechanistic evaluation of NAMs in DART Testing (Rajagopal et al., 2022)	
Pulmonary Toxicity	Multi-path Particle Dosimetry (https://www.ara.com/mppd/)	May be used as part of an assessment instead of computational fluid dynamics
	Mucociliary Clearance (ciliary beat frequency) (Luettich et al., 2021)	May be used to develop NAMs specific to this human adverse outcome relevant for TNCPs
	Human air-liquid-interface organotypic airway tissue models derived from primary tracheobronchial epithelial cells-overview and perspectives (Cao et al., 2021)	3D Test System review that may be used to develop NAMs
	Eurofins SafetyScreen44 and BioMap Diversity 8 Panel; ToxCast data, an *in vitro* cell stress panel and high-throughput transcriptomics; *in silico* alerts for genotoxicity were followed up with the ToxTracker tool (Baltazar et al., 2020)	May be used as part of a WoE approach
	In vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials (Wiemann et al., 2016)	
Multi-endpoint/ Approach-based	An FDA/CDER perspective on nonclinical testing strategies: Classical toxicology approaches and NAMs (Avila et al., 2020)	Review of NAMs and target organ toxicity by FDA/CDER
	Assessment of *in vitro* COPD models for tobacco regulatory science (Behrsing et al., 2016)	Best Practices Recommendations
	In vitro exposure systems and dosimetry assessment for NAMs (Behrsing et al., 2017)	
	Recommendations for the optimal generation and use of *in vitro* assay data for tobacco product evaluation (Moore et al., 2020)	

submitting information from NAMs, it is recommended that companies provide clear guidance on how to interpret the data.

3 Application of NAMs

For the *in vitro* evaluation of inhaled TNCP, aerosols or smoke are generated and particulates are captured on a filter pad (total particulate matter, TPM), and the remaining gas-vapor phase (GVP) bubbled through a liquid or the cells are directly exposed at an air-liquid interface. Examples of methodologies can be found in the references from Table 1 (e.g., Moore et al., 2020; Smart and Phillips, 2021; Bishop et al., 2020).

The below examples illustrate the current challenges in the application of NAMs for the risk assessment of TNCPs. On the one hand, manufacturers are encouraged to use and submit data from NAMs and not to conduct animal testing for these products (example 3.1). On the other hand, regulatory agencies seem to struggle to interpret some of the data generated with NAMs for risk assessment purposes. As described in Section 2, the guidance documents are unclear in regards to NAMs that are currently readily used and accepted (as in example 3.2) and which NAMs may require

the generation of additional information to fulfil the requirements for regulatory acceptance (as in example 3.3). Ambiguous guidance further leads to increased testing on animals because applicants tend to submit more information if the requirements are unclear (example 3.3). This illustrates the need for industry and regulatory agencies to collaborate and develop Integrated Approaches to Testing and Assessment (IATA) without the use of animals for TNCPs. Table 1 lists examples of available NAMs that could be included in a regulatory submission, as well as NAMs under development that could be incorporated as part of a regulatory submission package.

3.1 Example highlighting acknowledgement of the need to accept NAMs

In December 2020, a Joint Action for Tobacco Control (JATC) review panel set-up to aid EU Member States in the evaluation of data submitted for priority additives issued a report. A recurring comment throughout the report was that "the *in vitro* tests included in the newly performed industry studies are not sufficient to perform an evaluation of the CMR (carcinogenic, mutagenic or reprotoxic) properties, since *in vivo* studies are required to address this issue. Nevertheless, the review panel acknowledges that new *in vivo* studies regarding tobacco products are neither appropriate nor allowed for ethical reasons". The panel went on to comment "we do not have a proposed scientific methodology for fulfilling our request for evidence..." and indicated that if there is a revision of the TPD "the possible use of some assessment methodologies (e.g. Mode of Action and Adverse Outcome Pathway), which do not necessarily need new animal studies, should be considered" (JATC, 2020).

3.2 Example demonstrating how NAMs can be used to inform regulatory decision making

In the decision summary of a PMTA, the CTP Technical Project Lead (TPL) stated "Results from the *in vitro* toxicology studies demonstrated that combusted cigarette smoke fractions (total particulate matter (TPM), gas vapor phase (GVP), or both) were mutagenic, cytotoxic, and genotoxic. By contrast, even at the maximum dose levels tested, neither the TPM nor GVP from any of the aerosols of all the new products or ENDS market comparisons was mutagenic, cytotoxic, or genotoxic under the test conditions." (FDA, 2019b).

3.3 Example reiterating the importance of involving the regulatory agency early in the experimental development process and to clearly describe the NAMs

In the decision summary of a PMTA where five separate *in vitro* organotypic studies were submitted, it was noted "The experimental approach taken in these studies included using methods that are exploratory, have not been independently validated, and have unknown utility for regulatory use. The applicant attempts to extrapolate from acute exposure studies with naïve tissues that have little or no genetic variability to predict toxicity in a diverse population with a history of cigarette smoking. This limits the use of these data." In the same decision summary, a nicotine pharmacokinetic (PK) study with rats was dismissed stating "[t]his study does not provide relevant information for determining the health effects of [the product]; however, human PK studies were submitted and are more informative" (FDA, 2019c).

4 Discussion and next steps

For ethical and scientific reasons, there is a need to use reproducible and human-relevant testing approaches without the use of animals to better understand the potential adverse effects of TNCPs on humans. Investments have been made in the development of NAMs for assessing endpoints of relevance to TNCPs, as well as in the development of adverse outcome pathways that demonstrate the biological relevance of the NAMs. Human relevant NAMs have the potential to efficiently determine whether pre-market technologies should be abandoned due to specific hazard concerns, thereby avoiding unintended human health hazards that may only be identified during post-market surveillance. In addition to their use in product development, fit-for-purpose NAMs may be used to generate human relevant data that fulfill regulatory data requirements.

Section 2 highlighted the differences in the current regulatory frameworks of three exemplary regions and shows the need for more aligned regulations and clear guidance for the use of NAMs. In addition, to facilitate regulatory acceptance, applicants should clearly describe their NAM, including the context in which the NAM will be used (e.g., for screening/prioritization or quantitative risk assessment), the relevance of the model system to human biology and mechanisms of toxicity, and how the NAM informs the toxicological issue/gap. These are important aspects to communicate to regulators because the use of NAM-based data may not provide information that is typically used in regulatory risk assessments, e.g., no-observed-adverse-effect concentration. Rather, the NAM-based data may inform whether a particular substance should be included amongst a

category of chemical substances with known hazard concerns or the data may inform a specific key event in an adverse outcome pathway.

It is also important to keep in mind that a NAM may generate data that are different than data generated from vertebrate animals but are more relevant and mechanistically informative for predicting potential hazards to humans. A representative example in the context of inhalation toxicology is that rodents are obligate nose breathers, whereas humans are oronasal breathers and primarily mouth breathers when using inhalable products (e.g., cigarettes). Species-specific differences in dosimetry of substances can be accounted for using currently available NAMs [e.g., Multiple-Path Particle Dosimetry (MPPD)], which may also be used to inform the most relevant regions of the respiratory tract for investigation in human cells. Especially because regulators may not be as familiar with NAM data, it is essential for the regulated community to provide scientific justification for using NAM data in lieu of vertebrate animal data in a regulatory filing.

The above considerations emphasize the need for early engagement between regulators and the regulated community when utilizing NAMs as part of regulatory filings. These interactions will allow for discussion about how NAM data can be used to answer regulatory questions and foster further optimization of testing approaches to meet regulatory needs and best predict human health effects. Ultimately, the advancement of NAMs will require engagement from both sides to ensure a mutual understanding that the proposed NAMs are providing information in the context of the current paradigm for regulatory risk assessments or explaining how the NAMs may be incorporated to inform an alternative approach for assessment. In addition, consistency and transparency in how agencies consider and use submitted data in their decision making will allow the regulated community to more rapidly meet agency expectations.

Overall, there is a need for broader collaboration between regulatory authorities across geographical areas, between regulatory authorities and their stakeholders, and across sectors (e.g., industrial chemicals, agrochemical, or cosmetics) in order to harmonize best scientific practices. To help eliminate the use of animals in the risk assessment of TNCPs, it is recommended to:

- encourage pre-submission meetings between the agency and applicants to get early feedback on proposed NAMs
- provide consistent and transparent evaluations of application dossiers
- establish an international government-to-government collaboration initiative (similar to e.g., Accelerating the Pace of Chemical Risk Assessment [APCRA] for industrial chemicals)
- establish an open exchange forum between agencies, the regulated community, and other experts to discuss NAMs and how they can be applied for risk assessment purposes
- conduct retrospective reviews of submitted applications (such as PMTAs) with an aim to identify clear information needs for risk assessments
- organize regular workshops and webinars on the use of NAMs in risk assessment
- publicly share data to avoid duplicative testing and aid in read-across (e.g., the INTERVALS database)

Collaborative efforts will advance the harmonized use and acceptance of reliable, fit-for-purpose NAMs that inform human biology. Regulatory alignment on such approaches will not only aid with the development of novel TNCPs but will also aid other industries with evaluating their chemistries and developing products that are of lower risk to human health and the environment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

All authors discussed, wrote, and reviewed the manuscript.

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Conflict of interest

CH and JM-H are employed by JT International SA and HB is an employee of IIVS, a not-for-profit contract research organization that offers assays using in vitro and ex vivo models referred to in this manuscript.

The remaining authors declare no commercial or financial relationships that could be construed as a potential conflict of interest.

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