ALTEX, accepted manuscript
published November 10, 2022
doi:10.14573/altex.2209262

Meeting Report

Report on 2021 International Workshop for Non-animal Approaches in the Food Sector (Japan): Current Status and Avenues for Further Research

doi:10.14573/altex.2209262

1 Introduction

The 2021 International Workshop for Non-animal Approaches in the Food Sector was held by the International Life Sciences Institute (ILSI) Japan's[1] Alternative Animal Testing Promotion Project in the Food Sector (AAT project), in collaboration with ILSI Europe (EU), on October 21 and 22, 2021, and was conducted as a webinar.

The ILSI, founded in 1978, is a global nonprofit organization whose mission is to provide science that improves the health and well-being of humans and safeguards the environment. The ILSI's goal is to further the understanding of scientific issues relating to food safety, nutrition, and health as well as risk science and toxicology by bringing together scientists from academia, government, and industry. ILSI Japan, established in 1981, launched the AAT project in 2018. The AAT project engages in promoting research and collecting and disseminating information. Sixteen beverage and food companies participate in the project's activities in collaboration with various related academic bodies, such as the Japanese Society for Alternative Methods in Animal Experiments, the National Institute of Health Sciences, the Tokyo University of Agriculture, and ILSI EU.

With the emergence of new approach methodologies (NAMs), which are highly predictable, high-throughput, or high-content methodologies, the adoption of alternatives to animal testing is accelerating in various industrial sectors (ECHA, 2017). Although there is a growing understanding that NAMs should be actively incorporated in evaluating food safety and functionality, specific policies have not been shared within the food industry. This workshop provided a platform for researchers from industry, government, and academia in the food sector to discuss the status quo on the use of alternatives to animal testing and suggest avenues for further research.

In the workshop (Meeting Chair: **Prof. Dai Nakae**, ILSI Japan, Tokyo University of Agriculture, currently the Teikyo Heisei University, JP), lectures were held on the activities of ILSI Task Forces in each region; the regulatory status in Japan, US, and EU in the food safety and efficacy assessment area; and the latest research trends in NAMs. Experts from Japan, EU, US, China, Singapore, and India discussed important issues to focus on, such as promoting NAMs, prerequisites for the application of novel NAMs, points of concern in creating case reports, and the possibility of international cooperation for setting a guideline or standard for new methods to assess the safety and efficacy of food and food ingredients (e.g., functional food). A workshop statement regarding future efforts to promote alternatives to animal testing (AAT) in the food sector was prepared in collaboration between ILSI Japan and ILSI EU and is presented in this report.

2 Introduction of ILSI Entity's Task Force activities

In this session, the ILSI Entity's Task Force activities were reported, with **Dr Yoko Hirabayashi** (National Institute of Health Sciences, JP) and **Dr Siméon Bourdoux** (ILSI Europe, BE) as moderators.

Dr Tomohiro Kawaguchi (ILSI Japan, JP) presented on "Novel approaches for food safety risk assessment: Efforts of ILSI Japan." ILSI Japan launched the AAT Project to promote the utilization of NAMs in the food sector. The project aims to establish an animal-independent, highly predictive approach to evaluate systemic toxicity in humans and improve the precision and efficiency of food safety and efficacy evaluation. A related aim is to improve the prediction of the toxicity of food ingredients by adding data on food ingredients from the literature to the existing toxicity prediction system, which mainly contains data on general chemicals and cosmetic ingredients. For example, scientists have examined whether *in silico* models predicting intestinal absorption/blood levels could predict internal exposure to food ingredients (see Section 4). In addition, case reports using NAM-based assessment strategies have been prepared to identify opportunities to refine the safety assessment of food ingredients without using animals (see Section 5).

Dr Siméon Bourdoux presented on the topic "Alternatives to animal testing: ILSI Europe's efforts." He introduced the current status and approaches to alternatives to animal testing in the ILSI EU. These activities are conducted in collaboration with laboratories of food companies and public institutions in the EU and related organizations worldwide. The objectives are to review recently developed methods; to provide evidence-based science and evaluate potential strategies for alternatives to animal testing, and to disseminate this information to the public. These activities are conducted by working and expert groups by organizing webinars, scientific events in the EU, and joint workshops with ILSI Japan, and outcomes are published in international scientific journals. The ILSI EU promotes the use of organoids and organs-on-a-chip as an urgent strategy in food safety assessment and aims to establish a roadmap and/or advice paper on understanding and using human tissues for this purpose.

Dr Boindala Sesikeran (ILSI India, IN) presented on "Alternatives to animal experiments. ILSI India's initiatives." In India, approximately 115.3 million animals are used annually in experiments. However, animal testing has been banned in cosmetic research, and animal testing protocols were minimized by the Department of Biotechnology for all recombinant DNA-

[1] http://www.ilsijapan.org/English/

derived molecules. All predictive *in vitro* processes were prioritized as evidence of efficacy and safety, and the National Centre for Alternatives to Animal Experiments (NCAAE) was established in Tiruchirappalli. ILSI India established the task force on "Alternatives to Animal Testing with Special Reference to Food Safety." This project is expected to increase expertise, foster changes in attitude in the food industry and administrative agencies toward non-animal approaches, balance the need for reliable data with the urge to adapt to modern methods, and provide alternative strategies. Ultimately, it is anticipated that this project will decrease the use of animal testing in food applications.

3 Current situation in regulations of each region

In this session, with **Dr Akihiko Hirose** (National Institute of Health Sciences, JP) and **Dr Siméon Bourdoux** as chairpersons, the status quo of regulations in Japan, EU, and US were reported.

Dr Toru Kawanishi (Food Safety Commission (FSC) of Japan) held the keynote lecture "3Rs in animal testing for ensuring food safety: Current situation in Japan." Data compiled by the Japanese Society for Laboratory Animal Sources show that the number of experimental animals has gradually decreased over the last 15 years (2004–2019). The EU has reported that, of the animals used for regulatory purposes, the fractions used for food and feed legislation were 2.3% and 3.2%, respectively. Although detailed data on the number of laboratory animals used in food-related experiments in Japan are not available, it is expected to be similar to that of the EU. In "Growth Strategy 2017," the Japanese government's policy document, screening tests using artificial intelligence (AI) are referred to as alternatives to toxicity screening tests of chemical substances using animals. The management of testing facilities based on the 3R principle is promoted in the Health Care Policy Document of the Japanese government. The application of the 3Rs principle to animal tests for safety assessment in the food sector is expected to be applied to other sectors in Japan. Some 3Rs approaches for risk assessment in the FSC guidance documents were described. For example, the Guidelines for the Risk Assessment of Food Additives suggest the threshold of toxicological concern (TTC) and quantitative structure-activity relationships (QSAR) as approaches for the safety assessment of flavoring substances and processing aids. To assess the allergenicity of food additives, a combination of *in vitro* tests in accordance with the integrated approaches to testing and assessment (IATA) protocol based on the adverse outcome pathway (AOP) model may be used. In addition, although the allergenicity of enzyme food additives has been assessed mainly by *in vitro* physicochemical stability tests, *in silico* methods (i.e., homology search of amino acid sequences) and IgE-binding activity are also available as options. TTC and QSAR are also used for risk assessment of food contact materials in Japan. For safety assessment of "Foods for Specified Health Uses," which are a type of novel food, limited animal tests are mandatory: genotoxicity, single-dose oral toxicity, and 28-day or 90-day oral repeated-dose toxicity tests; when needed, additional tests such as 1-year chronic toxicity, carcinogenicity, and reproductive and developmental toxicity tests are performed. In the case of genetically modified foods, safety assessment is carried out mainly on the history of use, physicochemical properties, and *in vitro* and *in silico* analyses. When safety cannot be confirmed by the above data, additional animal toxicity data are required. In the near future, grouping/read-across will be introduced for the risk assessment of metabolites and impurities of pesticide residues. This approach allows the prediction of endpoints for target chemicals with no data, using data of source chemicals in the same group classified based on the similarity of chemical structures and/or physicochemical properties. Currently, alternatives to animal tests in the food industry are limited. There are limitations to the regulatory use of alternative methodologies in food safety assessment: (1) Test methods used in food safety assessment must be standardized and their reliability must be assured by validation. (2) Assessment using alternative methodologies should be equal to or better than the present methods using animals. (3) Current regulations are formed based on animal test results, and the criteria may not fit alternative methods. Despite these hurdles, it is vital to advance the 3Rs movement using new technologies.

Prof. Dai Nakae presented on the topic "Alternatives to animal experiments in the regulation of food: The Japanese situation." As concrete examples of the efforts of Japanese regulatory agencies to promote the 3R principle, he introduced the activities of the FSC of Japan. The FSC announces a roadmap of its research plan "Strategic direction for promoting research and survey to ensure food safety" every five years. The roadmap states that the substitution of animal experiments by *in vitro* and *in silico* NAMs is important, and that testing methods should be further improved, which will in turn promote animal welfare. According to the roadmap, research has been conducted on using databases and/or *in silico* methods for the prediction of toxicity and the *in silico* risk assessment of pesticide metabolites. He pointed out that further time and effort are, however, needed in regulatory, academic and industrial fields in this context, and that the efforts of ILSI Japan's AAT projects are important. He remarked that it is currently impossible to assess the safety and efficacy of food and other chemicals using only NAMs. He emphasized that the development and validation of NAMs should be promoted as well as their integration into new strategies such as the AOP-based IATA process and AI-generated procedures.

Dr Alie de Boer (Maastricht University, NL) presented on the topic "Animal-free strategies in food safety and nutrition in the EU" on behalf of the ILSI EU Expert Group. In the EU, based on Article 13 of Directive 2010/63/EU, animal tests should not be performed if alternative non-animal methods can be used. Therefore, it is necessary to update legislation and guidance as soon as alternative methods become available. One of the activities of the ILSI EU Expert Group is the transformation of toxicity testing from whole-animal testing to understanding critical biological pathways that lead to adverse outcomes. Blaauboer et al. (2016) described a stepwise roadmap using non-animal strategies to evaluate the risk of food and ingredients based on an understanding of the mechanisms of toxicity in humans in an exposure-driven approach rather than based on apical endpoints of toxicity in animal models. This requires specifications of chemical structures (QSARs), exposure scenarios, kinetics, *in vitro* and computational methods for toxicity evaluations, a focus on mechanisms of toxicity, and physiological based pharmacokinetic modeling for *in vitro–in vivo* extrapolation. Thus, risk assessment and determination of safety levels can be conducted without

using animal data, which is already enabled in different regulatory procedures implemented in the EU. Various alternative methods with potential for application in foods were emphasized. These promising methods, which avoid animal testing, should be further promoted and applied.

Dr Suzanne C. Fitzpatrick (US Food and Drug Administration (FDA), US) presented on the topic "New approach methodologies: The FDA perspective." The federal family, including the FDA and Environmental Protection Agency, are committed to alternative testing, and communication and collaboration with their stakeholders. Recently, roadmaps dealing with federal acceptance of alternatives were released by the FDA[2], EPA[3], and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)[4]. The 3Rs and 3Cs (commitment, communication, and collaboration) are themes that run through all roadmaps in the US. Clarification of regulatory gaps and needs will help in the development, qualification, and validation of alternative methods for regulatory use. The Alternative Methods Work Group (AMWG), which Dr Fitzpatrick chairs under the Office of the Commissioner, helps qualify a method for regulatory use. FDA developed the concept of "qualification" as a refinement of "validation." Qualification indicates that the results of an assay can be relied upon to have a specific interpretation and application in product development and regulatory decision-making. In comparison, validation lacked the definition of a purpose and use of the test method, which would allow an incremental transition, and it was conceptualized as a one-for-one full replacement, which was not effective. Inextricable from qualification is the concept of "context of use," which is a clearly articulated description that delineates the manner and purpose of use of a particular approach. It is necessary to clarify the questions that need to be answered and their purposes to determine how much validation or qualification is required. To move toward regulatory use, assays require stable platforms/cells, reproducibility, and clarifications on applicability domain, sensitivity, and specificity.

4 Status of technology for safety and efficacy

A report on the latest technological progress in AATs in Japan, the EU, the US, and Asia was presented and moderated by **Dr Yasuhiro Tanaka** (ILSI Japan, JP) and **Dr Jan van der Valk** (Utrecht University, NL).

Prof. Akira Hosono (Nihon University, JP) focused on research on the function of foods in the gastrointestinal tract and the role of the intestinal flora in his talk on the "Characteristics of *in vitro* and *in vivo* tests for food functionality research." In 1991, the Japanese Ministry of Health established a new Foods for Specified Health Uses (FOSHU) system to respond to increasing consumer awareness on maintaining and promoting health. This is the world's first social system to focus on the health effects of functional foods. The FOSHU application requires the submission of medical and nutritional evidence to ensure efficacy and safety. After confirming the effectiveness of these foods in animal experiments, it is necessary to assess their effects on human health and to establish a safe intake level by conducting human studies. In recent years, especially in the food industry, there have been considerations that animal experiments should be conducted under very restricted conditions and should be avoided where possible to meet societal demands. However, it is difficult to establish appropriate *in vitro* evaluation methods for the function of food ingredients. For example, when studying the functions of the gastrointestinal tract, the evaluation system may not fully consider the environmental factors, e.g., the contribution of symbiotic bacteria in the gut and the interaction between the intestinal and systemic immune systems. Therefore, assessing food function in the intestinal tract requires a combination of *in vivo* and *in vitro* studies.

Prof. Hiroshi Yamazaki (Showa Pharmaceutical University, JP) presented on the topic "Prediction of metabolic fates of food chemicals for risk assessment." The presented research aimed to predict the pharmacokinetic profiles of food components based on estimated key parameters derived from disparate chemicals in rat or human physiologically based pharmacokinetic (PBPK) (Miura et al., 2018) models. A simple PBPK model with three compartments (gut, liver, and central) and three important parameters (absorption constant, ka; volume of systemic circulation, V1; and hepatic intrinsic clearance, CLh, int) was developed. The intestinal permeability (Fa) was determined in Caco-2 *in vitro* systems. A PBPK simulation of plasma concentrations in humans after oral doses of astaxanthin and epigallocatechin gallate was presented. The simulated plasma concentration of astaxanthin was very close to the reported figure. Plasma concentrations of epigallocatechin gallate also were simulated well, whereas the predicted Fa value was higher than expected. He exhibited the results of *in silico* prediction of PBPK parameters for 200 chemicals, where *in vivo*-derived LogV1 was well correlated with the *in silico* derivation. Other parameters like ka and CLh, int, provided similar correlations, whereas the estimation of Fa and the fraction gut metabolism needed to be improved. Finally, he presented two case studies on neopetasitenine, a pyrrolizidine alkaloid found in fuki in Japan, and coumarin, a hepatic toxicant contained in Japanese food like sakura-mochi.

Dr Satoshi Kojima (ILSI Japan, JP) presented on the topic "*In silico* prediction of food chemical toxicity: Strategy and recent activity of ILSI Japan." Systemic toxicity is a significant concern in food safety evaluations. Since complex mechanisms cause systemic toxicity, it is necessary to make comprehensive judgments integrating various types of information (physicochemical properties, and *in silico*, *in vitro*, and *in vivo* studies). *In silico* methods, such as QSAR and read-across, are important for predicting toxicity. The hazard evaluation support system integrated platform (HESS), developed mainly by the National Institute of Technology and Evaluation, Japan, is a pioneering system that is useful in supporting repeated-dose toxicity prediction of chemical substances. However, the HESS contains little toxicity data for food ingredients. Therefore, a toxicity test

[2] https://www.fda.gov/media/109634/download
[3] https://www.epa.gov/sites/default/files/2018-06/documents/epa_alt_strat_plan_6-20-18_clean_final.pdf
[4] https://ntp.niehs.nih.gov/go/iccvam-rdmp

database for food ingredients is under development. He introduced repeated-dose toxicity data of 50 food ingredients collected from published literature into HESS and examined their effects on toxicity predictions for 41 trial food ingredients. The addition of data affected the prediction results for 13 components. For caffeic acid, the reliability of read-across was improved owing to increased reference to similar structures. For chlorogenic acid, for which toxicity prediction was unavailable before the addition of the additional data, read-across based on the estimated metabolite became an option. The above results suggest that toxicity prediction by HESS may be extended to food ingredients by including further data. Plans were announced to further expand the database.

Dr Jan van der Valk introduced methods to improve the reproducibility of research in his presentation on "Ingredients for improving *in vitro* methods: A piece of cake!" Irreproducible research results may hinder the validation of new methods and decision-making. Multiple reproducibility issues originate from the cells such as genetic drift and cell line contamination/misidentification. To avoid genetic drift, which is widely prevalent in many of the cell lines used today, a cell line should not be passaged for more than two months. Cell line contamination is another common problem, particularly when cell lines are not obtained from established cell banks. In both cases, the cell lines may not exhibit the expected key functions or characteristics. To avoid such cases, cell lines should be authenticated regularly. Another source of irreproducible results is fetal bovine serum (FBS). FBS is a product of biological origin and thus it inherently varies in composition from batch to batch. For issues in reproducibility and animal welfare and safety, the European Centre for the Validation of Alternative Methods Scientific Advisory Committee, as well as other organizations including Organization for Economic Co-operation and Development (OECD), European Medicines Agency, and FDA discourage the use of FBS. A chemically defined medium, which enables complete control over composition, decreases variability, and avoids animal use is an alternative. As different media compositions are required for different cell types, the FCS-free database[5] can help researchers find or develop optimal serum-free media. Considering these factors may lead to better reproducibility of results.

Prof. Bob van de Water (Leiden University, NL) presented on the topic "Application of NAMs for safety testing: Learning from Europe's flagship non-animal toxicology project EU-ToxRisk and beyond." The EU-ToxRisk[6] project aimed to unite all relevant disciplines and stakeholders to establish: i) pragmatic, solid read-across procedures incorporating mechanistic and toxicokinetic knowledge, and ii) *ab initio* hazard and risk assessment strategies for chemicals with little background information. The project focused on repeated-dose systemic toxicity (liver, kidney, lung, and nervous system) and developmental and reproductive toxicity. Different human tiered test systems were integrated to balance speed, cost, and biological complexity. The project combined *in silico* tools and *in vitro* assays using computational modelling approaches to provide quantitative data on the activation of key AOP events. This information, together with detailed toxicokinetic data and *in vitro-in vivo* extrapolation algorithms, forms the basis for improved hazard and risk assessment. 150 test method descriptions, novel *in silico* NAMs, an advisory document on regulatory requirements for acceptance of NAM-assisted read-across, a BioStudies database for case studies, the SaferWorldbyDesign webinars, and newsletters were outcomes of the project. The validation of NAMs for regulatory use is an issue that remains to be resolved. As the EU-ToxRisk project ended in 2021, the new RISK-HUNT*3R*[7] project, which will run until 2026, aims to establish a chemical risk assessment based on non-animal approaches via *ab initio* safety evaluation, from hazard identification to full risk assessment.

Dr Xudong Jia (China National Center for Food Safety Risk Assessment (CFSA), CN) presented on the topic "Alternative methods in food toxicology in China." In China, the procedures and methods for the safety assessment of chemical substances in food are described in the National Food Safety Standard GB15193. Of the 23 toxicity test methods listed, 17 are animal tests and six are *in vitro* methods. Animal testing remains mandatory in food safety regulations, however, there has been an increase in investment and financial support from NGOs, companies, and governments toward non-animal testing. The National Natural Sciences Foundation Committee supported several projects on non-animal toxicity testing, and assisted with the key project, "Toxicity pathway-based new technology for toxicological assessment." In addition, the Ministry of Science and Technology also provided special financing to promote the development and application of non-animal alternatives in food safety and supported a project titled "Toxicity pathway and mode-of-action (MOA)–based toxicological assessment of food chemicals." The Society of Food Toxicology and Society of Toxicological Alternatives and Translational Toxicology were established under the Chinese Society of Toxicology. The objectives and responsibilities of these societies are, 1) to promote the development, validation, administrative acceptance, international cooperation, and application of non-animal alternatives in toxicology research and risk assessment; 2) to provide a platform for national and international scientific communications on toxicological alternatives and translational toxicology, and for strong collaboration between different groups; 3) to facilitate professional education and dissemination of non-animal alternatives and translational toxicology. The CFSA was established in 2011 as a public health laboratory under the National Health Commission, with the primary mission of food safety risk assessment, risk monitoring, risk communication, and standards development, and it contains laboratories in chemistry, microbiology, and toxicology. The toxicology laboratory performs research on alternative methods to animal testing focusing on zebrafish models, embryonic stem cell models, threshold of toxicological concern (TTC), and toxicological pathways and adverse outcome pathways (AOP). As an example, the TTC developed by CFSA was adopted as a national standard and published as "Guidelines for the application of TTC to food safety risk assessment." In summary, alternative methods for toxicity testing in the food industry have not been accepted or approved by the Food Safety Authority of China; however, scientific research on alternative methods has been

[5] https://fcs-free.org/
[6] https://www.eu-toxrisk.eu/
[7] https://www.risk-hunt3r.eu/

supported both academically and financially. In terms of information, workshops and other information from relevant societies helped promote and disseminate new ideas on alternatives.

Dr Lim Hui Kheng (Agency of Science, Technology, and Research (A*STAR), SG) introduced a new genotoxicity testing protocol for the small intestine in her presentation on the topic "All disease begins in the gut: 3D reconstituted human intestinal models for food safety testing." The small intestine is an essential site for digestion and nutrient absorption, and it is constantly exposed to various orally ingested, potentially toxic substances. The tissue structure of commercially available 3D reconstructed human small intestine microtissues constructed from cells derived from the human ileum resembles native small intestine tissue. Micrographs showing that the structure and various differentiation markers are relatively similar, were presented. Permeability and transepithelial electrical resistance values that demonstrate the barrier function were also presented. The model was used in a micronucleus test protocol called the reconstructed intestine micronuclei cytome assay. Consistent results were obtained for mitomycin C, vinblastine, and benzo(a)pyrene. In addition, positive data for various genotoxins and negative data for non-genotoxic substances were obtained, suggesting that this model can clearly distinguish between genotoxic and non-genotoxic substances. Subsequently, a detailed comparison between the 2D and 3D models was presented. Evaluation of mitomycin C and vinblastine showed that micronucleus induction was observed only at higher doses in the 3D model and that this difference could be attributed to differences in 3D tissue structure and barrier function. Evaluation of nano-sized food additives showed a difference between the 2D and 3D models, as dose-dependent micronucleus induction of TiO_2 nanoparticles was observed in the 3D model but not in the 2D model. In summary, the new model could clearly identify genotoxic material.

Dr A. B. Pant (CSIR-Indian Institute of Toxicology Research, IN) presented on the topic "Implementation of the 3R's in regulatory studies: The Indian perspective." He explained the status of alternative methods to animal experiments in India. India became a signatory to the agreement on the mutual acceptance of data developed by the OECD in 2011, ushering in alternatives to animal testing. In 2019, the Society for Alternatives to Animal Experiments was established, which is working to promote and lead the way for alternative methods in India. Alternative techniques used in India include the *in vitro* cytotoxicity test according to ISO 10993-5, the *in vitro* eye irritation test (OECD TG 492), and acute toxicity tests using *in vitro* fish cell lines (OECD TG 249). Other drug screening methods have also been established, such as an experimental system using cultured cell membranes as a substitute for rat brains and the *Drosophila* method for the safety assessment of chemical substances. Recently, researchers have begun to develop a 3D organoid model for electrophysiological experiments, are investigating methods to extrapolate data generated from *in vitro* experiments to humans by pharmacokinetic modelling, and and developing human-specific bioprinted 3D tissues using 3D printers. A fully functional 3D miniaturized form of the US chip has been developed.

Prof. Thomas Hartung (Johns Hopkins University, US) presented on the topic "The new alternatives from microphysiological to micropathophysiological systems and computational approaches toward AI technologies." It is critical to change the communication approach on alternative methods to focus on the scientific quality, economic benefits, and opportunities of NAMs. The creation of large toxicological databases ("big data") and data-mining technologies ("AI") allows predictive computational approaches on a new scale, for example, a new automated read-across (RASAR, i.e., read-across-based structure-activity relationships). The development of alternatives to traditional approaches for product development and safety assessment is benefiting from the increasing pace and integration of technological developments in modern cell culture. Meanwhile, the combination of cell culture with bioengineering has resulted in several technologies that make cell cultures more closely resemble organs, such as 3D culture, human stem cell-derived systems, perfusion, co-cultures, and combinations with scaffolds and sensors. These technologies lead to organ-on-chip or even multi-organ human-on-chip solutions that reflect physiological conditions by reproducing organ architecture, cellular environment homeostasis, and organ function. An example is a human induced pluripotent stem cell-derived mini-brain used to examine neurotoxicity. The market introduction of organoid platforms helped to improve standardization. In addition, the combination of the increased mechanistic base of reasoning (i.e., AOP concepts), integrated testing strategies, and evidence-based methods of data evaluation and integration has set in motion revolutionary changes in how we assess the biological effect of substances.

5 Panel discussion

In the last session, evaluation strategies using NAMs in the food sector were discussed followed by a panel discussion on sharing awareness of the current state of NAMs in the food sector and the construction of technologies that enhance human predictability within the applicable range as activity proposals for the future, with **Dr Hajime Kojima** (National Institute of Health Sciences, JP) and **Dr Jan van der Valk** as moderators. Finally, the workshop statement was compiled.

Dr Kazutoshi Saito (ILSI Japan, JP) presented on the topic "Alternatives to animal testing in food safety: Evaluation strategy by ILSI Japan." First, he introduced how to assess the safety of functional food compounds in Japan, which often requires *in vivo* tests on systemic toxicity. He pointed out three major issues for applying NAMs to the risk assessment of functional food compounds: 1) the development of a non-animal pharmacokinetics (PK) prediction system applicable to functional food compounds, 2) the preparation of a database to estimate the systemic toxicity of functional food compounds, and 3) the integration of NAMs to achieve the non-animal risk assessment of food-related compounds. He explained that ILSI Japan has organized three parallel working groups (WGs) to address the aforementioned issues and briefly introduced the activities of WG1: Internal exposure prediction applicable to functional food compounds and WG3: Making case reports. Regarding WG1, he mentioned the collaboration with Prof. Hiroshi Yamazaki, who developed an *in silico* PK estimation system based on general chemicals and pharmaceuticals. This prediction system estimated PK parameters of vitamin A in rats within a three-fold difference. As for WG3, Dr Saito demonstrated how the case reports should be created. He mentioned that pyrrolizidine alkaloids, which can induce

hepatotoxicity, would be the first target compounds for WG3. Finally, he presented ILSI Japan's NAM-based evaluation strategy for the risk assessment of food-related compounds. The strategy consists of four steps: 1) Decision on the target food compound(s), 2) utilization of existing safety data, 3) determination of *in vitro* points of departure, and 4) risk assessment. He explained that WG activities in ILSI will contribute to each step.

Dr Sandra Coecke (European Commission Joint Research Centre, EU) presented on the topic "Good *in vitro* method practices and the pursuit of innovative *in vitro* mechanistic methods for food safety testing." NAMs are key drivers that can impact food safety, and many innovative *in vitro* test methods are being developed to improve hazard and risk assessment. Although these NAMs provide insights into the mechanisms by which food functions, quality assurance of such methods is critical. The OECD guidance document on GIVIMP (OECD, 2018) covers quality assurance principles, including validating and ensuring reproducibility and accuracy of the prediction of NAMs, and aims to harmonize at all levels, including obtaining and reporting data. She warned that not implementing GIVIMP may lead to a waste of effort, time, and other resources put into research. Thus, it is crucial for researchers involved in data generation to comply with GIVIMP concepts for quality outputs. Method reproducibility is essential when data are to be used for regulatory purposes. As an example for validation capacity building, the activities of the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) related to the assessment of endocrine disruptors, especially thyroid hormone system–disrupting chemicals were presented. In this effort, 15 EU-NETVAL laboratories and the European Commission Joint Research Centre's European Union Reference Laboratory for Alternatives to Animal Testing launched a series of validations of over 18 mechanistic methods to detect potential thyroid hormone system disruptors. Validation is being done in two stages: Part 1 validation consists of producing related standard operating procedures, further developing the method, and assessing robustness and reliability using a few reference and control chemicals in five valid runs. Part 2 validation aims to confirm the relevance of the method based on the underlying mechanism.

Dr Suzanne C. Fitzpatrick presented on the topic "New approach methodologies in the FDA Foods Program." She stated that it is no longer acceptable in the global market to rely on animals when looking at food supply and expressed FDA recognition of the need for NAMs. Several NAMs currently being developed by the FDA were highlighted. She emphasized that intra- and interlaboratory reproducibility give us more confidence for regulatory decision-making. The FDA has established a 'closer to zero' program to reduce exposure to toxic elements from foods eaten by babies and young children. Although this program should assess arsenic in infant rice cereals, developmental neurotoxicity models in animals are inadequate. A development and activity test in *Caenorhabditis elegans* was developed where certain combinations of metals lead to a shift in maturation and motor activity. Next, she explained the expanded decision tree to categorize chemicals into toxicity classes. Improvements include that the number of classes has been increased from three to six, functional modalities have been increased, and modes of action and species differences have been incorporated. The decision tree can be used to improve the prioritization of a higher number of low exposure chemicals. Other areas where NAMs could be applied include tests for food colorants and additives, and skin absorption tests for cosmetics could be applied to bio-printed skin. The Alternative Methods Working Group (AMWG) will discuss NAM FDA-wide and work with US federal partners and other global stakeholders to discuss and develop draft performance standards. For example, they have developed a public-private partnership to provide reliable, science-based food toxicology information, including industry, academics, and animal welfare associations. Dr Fitzpatrick is also working with the European Food Safety Authority to develop a NAMs program in the Global Harmonization of Risk Assessment initiative. She concluded by declaring the importance of global collaboration of all attendees at the meeting for the development and implementation of NAMs.

The panel discussion involved seven speakers (Dr Kazutoshi Saito, Prof. Akira Hosono, Dr Sandra Coecke, Dr Suzanne C. Fitzpatrick, Prof. Thomas Hartung, Dr Xudong Jia, Dr B Sesikeran); two moderators; and two panelists (Prof. Masaaki Akita, Kamakura Women's University, JP and Dr Benjamin Smith, Agency of Science, Technology and Research (A*STAR), SG).

The organizers prepared a draft workshop statement to facilitate discussion. In addition, the definition of NAMs and the scope of food to be evaluated were supplemented as explanatory information based on the results of the discussion. In addition, the following five questions were prepared in advance, and statements were created after discussions between panelists and consensus building: 1. Are there any regions within the range of scope (e.g., food additives)? 2. The pharmaceutical and chemical industries are ahead in the development of NAMs. What types of NAMs are being developed? Which of these can be recommended (especially for the food industry)? 3. Are there opinions on validating and ensuring the quality (e.g., reproducibility and accuracy of prediction) of NAMs? 4. Case reports are important for the promotion of non-animal risk assessments. However, the priorities of the target compounds and strategies differ in each region. Is it possible for international coordination and cooperation to produce a case report and promote non-animal risk assessment? 5. Is it possible to establish guidelines or standards for new methods to assess the efficacy of functional foods? Panelists discussed the issues in the five questions. After the workshop, ILSI Japan and ILSI EU collaborated to summarize the discussions and prepare the workshop statement and explanatory information.

Workshop statement

✓ By focusing on developing and implementing NAMs* for safety and efficacy assessment of food-relevant substances in humans, we may improve predictability within the applicable scope***.**

* NAMs – new approach methodologies include *in silico* approaches, *in chemico* and *in vitro* assays, physiologically based pharmacokinetics (PBPK) modelling and simulation, and a variety of new testing tools such as high-throughput screening and high-content methods, e.g., genomics, proteomics, metabolomics, that can be used to provide information in the context of chemical hazard and risk assessment. These new approaches include integrated

approaches to testing and assessment (IATA), defined approaches for data interpretation, and performance-based evaluation of test methods.
** food-relevant substances: Food test items include pure substances, mixtures and multi-constituent substances, and other types of food test items. Each region may have region-specific terminologies.
*** applicable scope: Food-relevant substances which require safety evaluation focusing on systemic toxicity and efficacy considering exposure with dynamics and kinetics.
✓ To establish comprehensive assessment strategies that involve NAMs, we create and disseminate case reports in accordance with the strategies.

Explanatory information related to workshop statement:

Selection of applicable scope of NAMs
Since the focus of this international workshop was on new technologies and evaluation strategies without using animals for evaluating systemic toxicity for substances taken orally by humans as foods or as parts of foods, the evaluation targets were set in fields where *in vivo* systemic toxicity tests are currently required for risk evaluation.
Requirements to improve predictability of NAMs
Maintenance and utilization of a highly reliable database, refining internal exposure prediction considering *in vitro-in vivo* extrapolation (IVIVE) (Bell et al., 2018), and validation of individual methods and comprehensive evaluation strategies are necessary to improve predictability and reliability.
Requirements for test reliability assurance of NAMs
To maximize the reproducibility, reliability, acceptance, and proper application of test results, *in vitro* methods should be conducted according to good practice such as Good *In vitro* Method Practices (GIVIMP) (OECD, 2018) and Good Cell Culture Practice (GCCP) (Pamies et al., 2022).
Requirements of the database
To ensure the usefulness and quality of assessments using NAMs, it is useful to compare results to legacy data from animal experiments compiled in a highly reliable database. Such database also could be used for *in silico* toxicology assessments of newly developed chemicals. The quality and coverage of the database should be carefully considered.
Requirements for implementation of case reports (also related to public administrative acceptability):
It is necessary to acquire a common understanding by all stakeholders by preparing case reports based on highly predictable and reliable methods and evaluation strategies. Ultimately, it is desirable to develop international guidelines/guidance and standards.

The ILSI Japan AAT project will contribute to maintaining biodiversity, improving animal welfare, and achieving sustainable development goals based on the outcome of this workshop with international ILSI members.

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Acknowledgments

This workshop was co-sponsored by ILSI Japan and ILSI EU, and both equally contributed to the formulation and issuance of the workshop statement. The authors would like to thank Dr Hajime Kojima and Prof. Dai Nakae for their invaluable advice in organizing this workshop and editing this paper.

Satoshi Ohta, Shigeki Kimura, Daisuke Maejima, Kazutoshi Saito, Yasuhiro Tanaka, Satoshi Kojima, Tomohiro Kawaguchi, Takuya Kikuchi, Takuya Seki, Hsuan Chung, Risa Funaki, Tetsuya Kuhara, Hisako Hori and Yuichi Ito

International Life Sciences Institute (ILSI) Japan, Tokyo, Japan
(webmaster@ilsijapan.org)