Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Federal Department of Home Affairs FDHA
**Federal Food Safety and Veterinary Office FSVO**
Research Management

**Section** **Fields (of activity)**

**Style sheet (Bitte die Vorlage direkt mit Ihrem Text überschreiben / veuillez substituer le texte du modèle par votre texte)**

# Integration von komplexen toxikologischen Daten: in vitro Antwortsprofile und in silico Modellierung zur Vorhersage von Lebertoxizität im Menschen verursacht durch Chemikalien in Lebensmitteln

*Shana J. Sturla, ETH Zurich, Laboratory of Toxicology, Institute of Food, Nutrition and Health*

**Key words**

NAFLD, Chemical Food Safety, Toxicology in vitro, IVIVE (In vitro to in vivo extrapolation)

**Aim of the study**

The objective of this research is to improve food safety by enabling the integration of in vitro data concerning mechanisms of toxicity to humans in chemical risk assessment. In this context, the aim of this study was to establish a systems-toxicology platform to predict liver injury from food-relevant chemicals. Using non-alcoholic fatty liver disease (NAFLD) as an adverse outcome of significant human relevance, our central hypothesis is that quantification and integration of profiles of multiple cellular and molecular responses in human liver cells exposed to low doses of chemicals that induce NAFLD through complementary mechanisms can be used to identify response patterns relevant to NAFLD. The testing strategies developed are candidate new approach methods to include in regulatory testing batteries for hepatotoxicity risk prediction in food safety assessment.

**Material and methods**

The project involved a combination of in vitro toxicological and computational modeling strategies:
***Cell culture and chemical exposure***-Undifferentiated human liver cells (HepaRG) cells were used to establish a novel NAFLD assay. Their differentiation was induced by a multi-week culturing protocol to generate a metabolically competent co-culture system. Chemical cytotoxicity was evaluated by standard approaches for at least 3 independent biological experiments with 6 technical replicates per condition.
***High-content imaging assay***-A key experimental aspect was establishing a new high-content imaging assay and data analysis pipelines to measure sentinel cellular alterations involved in the NAFLD adverse outcome pathway. After exposures to concentrations of chemicals below their $EC_{50}$ values, live cells were exposed to fluorescent indicators of lipid accumulation, mitochondrial stress, oxidative stress and altered nuclear morphology. Automated quantitative image analysis pipelines were established to quantify key events on a cellular population level, as well as single cell level.
***Dose response data and model fitting.*** Data was normalized to untreated cells to derive fold change values of the four endpoints. To determine a point of departure from concentration-response data, benchmark concentration (BMC) was modelled using the benchmark dose software (BMDS) version 3.1.2.
***In vitro to in vivo extrapolation***. IVIVE was performed using the high-throughput toxicokinetic (httk) R package (version 1.10.1) developed by the US-EPA. The httk package contains four toxicokinetic models which can be parameterized using high-throughput derived in vitro data on plasma protein binding and hepatic clearance. Moreover, it has a monte carlo sampler to simulate population variability and includes tools for

reverse dosimetry with functions for the analysis of concentration versus time simulations. In our study, a three compartment steady state pharmacokinetic (PK) model was parameterized and used for simulations.
***Testing of food-related chemicals and pesticides***. After we could induce and quantify chemically induced key events relevant to NAFLD using reference compounds, we applied the workflow to food-related chemicals selected based on their in silico nuclear binding prediction. For the selection, smiles were extracted from the regulated food-related use/occurrence chemical database from the Swiss federal food safety and veterinary office and from the toxcast database (US EPA). Subsequently, a structure-based workflow was used to predict binding to nuclear receptors, and chemicals predicted to bind one or more nuclear receptor were further filtered by available NAFLD-related and toxicokinetic data.

**Results and significance**

Systematic assessments of chemicals as NAFLD risk factors are limited in part due to a lack of in vitro and in silico toxicological testing strategies. In this study we developed a rapid, quantitative and predictive approach for prioritizing chemicals for NAFLD hazard assessment. The approach integrates in vitro high-content phenotypic response data including lipid accumulation, mitochondrial membrane potential, oxidative stress and nuclear morphology from chemical-exposed liver cells with PBPK modeling to derive relevant human dose predictions of potential concern. The model was trained using FDA-approved drugs and experimental chemicals with known mechanism of action for NAFLD pathogenesis. The four key events were analyzed after 24 h and were quantified on a population and single cell level. Dose modeling showed a strong predictive performance in terms of stimulating NAFLD key events at exposure concentrations that were closely to human dose equivalents for reported in vivo NAFLD development from drug use.

A limitation of the strategy relates to the immune component that impacts NAFLD etiology. Preliminary studies were conducted to include a pro-inflammatory cellular milieu and we evaluated excreted cytokines as a potential strategy to integrate this aspect of disease etiology in future refinement of the method. Further, we envisioned using proteomic analysis to refine predictions from the high-content imaging assay, however, these were not pursued due to the good performance of the high-content imaging assay, and the high cost and effort without a clear benefit to the immediate goal. Finally, studies were also performed to measure intracellular concentrations in order to better understand dose-response relationships for inducing NAFLD-associated cellular events. These studies involved HPLC analysis of the test chemical amiodarone. Data showed efficient uptake into liver cells that was rate-limited at high doses. The analysis used is chemical-specific, so application of this approach to all of the chemicals tested would require developing an analysis method for each, which is recommended therefore only as a follow-up for chemicals showing positive responses.

Having established a novel and reliable in vitro NAFLD test strategy, we evaluated food-related chemicals prioritized on the basis of structure-based receptor binding interactions relevant to NAFLD. Of the 14 chemicals tested, we found that lipid accumulation was dose-dependently increased after exposure to uric acid, tartrazine, bisphenol A, atrazine, metazachlor and vinclozolin. Mitochondrial membrane potential increased in a dose-dependent manner in cells exposed to orotic acid, tartrazine, fructose and carbofuran where it exceeded the threshold for all except fructose. A dose-dependent decrease in mitochondrial membrane potential was observed for mepanipyrim, but it did not exceed the threshold. On the basis of comparison of the exposure levels eliciting cellular responses, we identified orotic acid, which is biosynthesized in mammals, present in milk, and added to some dietary supplements, as having the potential to induce NAFLD in humans.

**Publications, posters and presentations**

In vitro and in silico testing strategies for predicting human liver toxicity. A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH (Dr. sc. ETH Zurich) by FABRICE ALAIN MÜLLER

Müller, F. A. & Sturla, S. J. Human in vitro models of nonalcoholic fatty liver disease. Curr. Opin. Toxicol. 16, 9–16 (2019).

Müller, F.A., Stamou, M., Diedrich, S., Wambaugh, J.F., Sturla, S.J. Multiparametric analysis of chemically induced nonalcoholic fatty liver disease in vitro combined with in silico dose predictions. Manuscript in preparation

American Chemical Society Fall 2020 Virtual Meeting and Expo: Fabrice A. Müller, Marianna Stamou and Shana Sturla.; “In vitro and in silico testing strategies for predicting human liver toxicity from foodborne chemicals”. Virtual meeting, 17-20 Aug, 2020 (Poster)

Annual Meeting of Swiss Society of Toxicology: Fabrice A. Müller, Marianna Stamou and Shana Sturla.; “In vitro and in silico testing strategies for predicting human liver toxicity from foodborne chemicals”. Basel, Switzerland, 28-29 Nov, 2019 (Poster + selected for talk)

Swiss 3RS Day 2019: Fabrice A. Müller, Marianna Stamou, Sabine Diedrich, Shana J. Sturla: “In vitro and in silico testing strategies for predicting human liver toxicity from foodborne chemicals”. Bern, Switzerland, September 2nd, 2019 (Talk)

Toxcon 2019: Fabrice A. Müller, Marianna Stamou, Sabine Diedrich, Shana J. Sturla: “In vitro testing strategies for investigating key cellular events involved in chemically induced nonalcoholic fatty liver disease”. Vyhne, Slovakia, 26-28 June, 2019 (Talk)

D-HEST Research Day 2018: Fabrice Müller, Marianna Stamou and Shana Sturla.; “In vitro and in silico modeling for predicting human liver toxicity from chemicals in food”. Zürich, Switzerland, Dec 4, 2018. (Talk)

Annual Meeting of Swiss Society of Toxicology: Fabrice Müller, Marianna Stamou and Shana Sturla.; “High content in vitro assessment of chemically induced liver injury: Lipid accumulation and mitochondrial dysfunction”. Basel, Switzerland, 29-30 Nov, 2018. (Poster)

Wissenstransfer at the Swiss Federal Food Safety and Veterinary Office: Fabrice Müller, Marianna Stamou and Shana Sturla.; “In vitro and in silico modeling for predicting human liver toxicity from chemicals in food”. Bern, Switzerland, Sept 25, 2018 (Talk)

Annual Retreat MTB PhD program 2018: Fabrice Müller, Marianna Stamou and Shana Sturla.; “High content in vitro assessment of chemically induced liver injury: Lipid accumulation and mitochondrial dysfunction”. Zürich, Switzerland, Sept 12, 2018 (Poster)

Eurotox 2018: Fabrice Müller, Marianna Stamou and Shana Sturla.; “High content in vitro assessment of chemically induced liver injury: Lipid accumulation and mitochondrial dysfunction”. Brüssel, Belgium, 2-5 Sept, 2018 (Poster)

Annual Meeting of Swiss Society of Toxicology: Fabrice Müller, Marianna Stamou and Shana Sturla.; “Quantification of time- and concentration-dependent accumulation of lipid droplets in human liver cells exposed to steatosis-inducing chemicals”. Basel, Switzerland, 30. Nov – 01. Dec, 2017. (Poster)

Annual Retreat MTB PhD program 2017: Fabrice Müller, Marianna Stamou and Shana Sturla.; “Quantification of time- and concentration-dependent accumulation of lipid droplets in human liver cells exposed to steatosis-inducing chemicals.” Zürich, Switzerland, Nov 02, 2017 (Poster)

***Project (Formatvorlage Überschrift 3)*** *Text (Formatvorlage Überschrift 4)*

***Project duration*** *01.03.2017-30.06.2021*