



Capturing the applicability of *in vitro-in silico* membrane transporter data in chemical risk assessment and biomedical research

Laure-Alix Clerbaux^{a,*}, Sandra Coecke^a, Annie Lumen^b, Tomas Kliment^c, Andrew P. Worth^a, Alicia Paini^a

^a European Commission, Joint Research Centre, Via E. Fermi 2749, 21027 Ispra, Italy

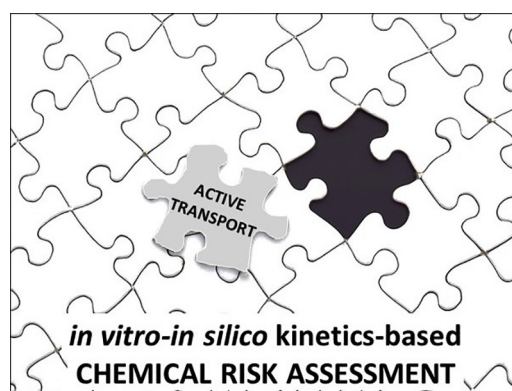
^b National Center for Toxicological Research, US Food and Drug Administration (FDA), Jefferson, AR, USA

^c Klimeto, Juzna 4, Roznava, Slovakia

HIGHLIGHTS

- Membrane transporters (MTP) are key determinants of drug and chemical kinetics.
- MTP studies are recommended during drug development.
- MTP data is an essential puzzle piece for kinetics-based chemical risk assessment.
- Integrated *in vitro-in silico* methods increase confidence in animal-free MTP data.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 24 April 2018

Received in revised form 9 July 2018

Accepted 10 July 2018

Available online 14 July 2018

Editor: Dr Wei Huang

Keywords:

Membrane transporters
Active transport
Toxicokinetics
Chemical risk assessment
Alternative methods
Survey

ABSTRACT

Costs, scientific and ethical concerns related to animal tests for regulatory decision-making have stimulated the development of alternative methods. When applying alternative approaches, kinetics have been identified as a key element to consider. Membrane transporters affect the kinetic processes of absorption, distribution, metabolism and excretion (ADME) of various compounds, such as drugs or environmental chemicals. Therefore, pharmaceutical scientists have intensively studied transporters impacting drug efficacy and safety. Besides pharmacokinetics, transporters are considered as major determinant of toxicokinetics, potentially representing an essential piece of information in chemical risk assessment. To capture the applicability of transporter data for kinetic-based risk assessment in non-pharmaceutical sectors, the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) created a survey with a view of identifying the improvements needed when using *in vitro* and *in silico* methods.

Seventy-three participants, from different sectors and with various kinds of expertise, completed the survey. The results revealed that transporters are investigated mainly during drug development, but also for risk assessment purposes of food and feed contaminants, industrial chemicals, cosmetics, nanomaterials and in the context of environmental toxicology, by applying both *in vitro* and *in silico* tools. However, to rely only on alternative methods for chemical risk assessment, it is critical that the data generated by *in vitro* and *in silico* methods are scientific integer, reproducible and of high quality so that they are trusted by decision makers and used by industry. In line, the respondents identified various challenges related to the interpretation and use of transporter data from non-animal methods. Overall, it was determined that a combined mechanistically-anchored *in vitro-in silico*

* Corresponding author at: European Commission, Directorate General Joint Research Centre, Health, Consumers and Reference Materials, Chemicals Safety and Alternative Methods, Via E. Fermi, 2749, I-21027 Ispra, VA, Italy.

E-mail address: laure.alix.clerbaux@gmail.com (L.-A. Clerbaux).

approach, validated against available human data, would gain confidence in using transporter data within an animal-free risk assessment paradigm. Finally, respondents involved primarily in fundamental research expressed lower confidence in non-animal studies to unravel complex transporter mechanisms.

© 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Humans and ecosystems are continuously exposed to various environmental chemicals, such as pesticides, manufactured chemicals, cosmetics ingredients or food contaminants. Their potential toxicity is of public concern. However, clinical trials are not conducted for pollutants, as compared to drugs, depriving the toxicologists of human *in vivo* data to rely on. Currently, the safety assessment of environmental chemicals for regulatory purposes mainly involves animal testing. However, costs, scientific and ethical concerns have created the need to develop reliable, relevant and economically feasible tools based on alternative (non-animal) approaches. In 2010 the EU adopted Directive 2010/63/EU which updated the 1986 Directive 86/609/EEC on the protection of animals used for scientific purposes. The aim of the new directive is to anchor in EU legislation the principle of the Three Rs: Replace, Reduce and Refine the use of animals for scientific purposes. Under this Directive, the EURL ECVAM was established to contribute to the development, validation, and international recognition of alternative methods. In 2015 EURL ECVAM published a toxicokinetic (TK) strategy proposing kinetics as the cornerstone in an integrative *in vitro-in silico* risk assessment (Bessems et al., 2015). Kinetics determine what amount of an external exposure dose of a compound reaches the systemic circulation and the target organ(s) by providing essential information on the ADME processes (Coecke et al., 2013; Tsaïoun et al., 2016). TK here defines kinetics of environmental toxicants in contrast to pharmacokinetics related to drugs. Despite the usefulness of TK information, there are only few legal requirements in EU chemicals legislation for the generation of TK data. However, the use of TK data to support the assessment of systemic toxicity is widely recommended in regulatory guidance and various scientific opinions (Bessems et al., 2015). TK data are proposed for use to evaluate cross-species differences, to waive specific *in vivo* studies when applicable, and to support the development of novel approaches in chemical safety assessment (Corvi et al., 2013; Prieto et al., 2014; Casati et al., 2013; Bessems et al., 2014; ECHA, 2011; EFSA, 2014). Moreover, TK data are valuable for the development of mathematical models, such as physiologically based kinetic (PBK) models and could increase the accuracy of *in vitro* fate models (Armitage et al., 2014; Fischer et al., 2017; Comenges et al., 2017).

Membrane transporters are well-recognized key determinants of kinetics, affecting the ADME processes of various endogenous and exogenous compounds (Klaassen and Aleksunes, 2010; “The Transporter Book”, 2017). The *in vivo* importance of transporters is demonstrated in several animal species, including knockout or mutated mice, as well as by genetic variants (polymorphisms) in humans (Klaassen and Hong, 2008). Furthermore, clinical data, -omics studies and non-invasive imaging on healthy volunteers or patients provide considerable information on the *in vivo* role of many transporters (Yee et al., 2010; Kusuvara, 2013). Besides *in vivo* studies, a plethora of *in vitro* methods exist to measure active transport. The most widely used are either membrane-based assays (including ATPase and vesicular transport assays) or cell-based systems such as cell lines, polarized and/or transfected with one or multiple transporters, providing information on specific transporter(s) interaction as well as animal or human primary cells representing rather holistic barrier models as their transportome profile is more similar to that found *in vivo* (reviewed in “The Transporter Book” 2017). Furthermore, hepatocytes, either suspended, plated or sandwich-cultured, are arguably one of the most valuable tools available to study drug metabolism and transport (Riley et al., 2016). Recently, more complex *in vitro* systems, such as organoids,

3D or co-culture are also exploited by transporter scientists (Zhang et al., 2017). In combination to experimental studies, several computational models are applied to gain deeper insight into transporter-substrate interaction or to integrate transport data at a systemic level. *In silico* models of transporters and transport processes range from quantitative structure-activity related relationship (QSAR), pharmacophore modelling and docking to PBK models and integrative platforms such as SimCyp, PKSim or GastroPlus, among others, as well as machine learning tools (Pajeva and Globisch, 2009; Ekins et al., 2015; You et al., 2015; Ekins, 2016; Kim et al., 2017). In recent decades, the pharmaceutical field has placed considerable effort to study transporters affecting drug disposition, therapeutic efficacy and adverse outcomes. Besides identification of substrates, inhibitors and inducers of transporters, drug-drug interaction (DDI) mediated by transporters are also intensively studied as they are a major cause of modulation of drug efficacy and toxicity. In 2010, an International Transporter Consortium (ITC) was formed (i) to identify transporters of clinical importance often called “drug transporters” and (ii) to discuss the appropriate methodologies to characterize drug-transporter interactions (Giacomini et al., 2010; Zamek-Gliszczynski et al., 2013; Brouwer et al., 2013). The ITC presented seven consensus transporters of clinical relevance, referred as the “ITC7”: MDR1, BCRP, OATP1B1-1B3, OAT1-3 and OCT2 (Giacomini et al., 2010). Then MATEs, Bsep and MRPs have been highlighted as additional transporters of emerging importance (Hillgren et al., 2013). The ITC recommendations have led several drug regulatory agencies to publish guidance documents on the evaluations of transporter implications in ADME processes and in DDI when developing new drug, in particular the European Medicines Agency (EMA) (EMA, 2012), the US Food and Drug Administration (FDA) (US FDA, 2012) and the Japanese Ministry of Health, Labour and Welfare (MHLW) (MHLW, 2014). Revised guidelines were released last year (2017) by the FDA including principally new *in vitro* guidance and adding MATE1 and MATE2-K transporters as well as time dependence of transporter inhibition studies in the requirements (US FDA, 2017). Not all transporters have to be investigated in all cases. The choice of experiments to be performed largely depends on the pharmacokinetic properties of the compound. To support scientists with the choice of relevant transporter studies, decision trees represent a central part of the regulatory recommendations.

With an increased focus on TK, it has been shown more recently that besides pharmaceutical compounds, membrane transporters also interact with various environmental contaminants, such as pesticides, manufactured chemicals, food contaminants and metals (Leslie et al., 2005; Tachampa et al., 2008; Van Herwaarden and Schinkel, 2006; Epel et al., 2008; Fardel et al., 2012; Wilks and Tsatsakis, 2014; Chedik et al., 2018a, 2018b). Expressed at key physiological barriers of the body, uptake and efflux transporters may modulate the systemic and intracellular concentrations of chemicals and hence directly impact their degree of toxicity (Leslie et al., 2005; Fardel et al., 2012). Furthermore, transporter-mediated interactions, polymorphisms or disease-related change in transporter function could cause a significant alteration in the intracellular concentration and consequent toxicity (Schuetz et al., 2014). These aspects have been discussed in several international workshops and conferences, that brought toxicologists, biologists and computational modelling experts together, and the role of active transport has been highlighted as a critical puzzle-piece of information for chemical risk assessment (Bessems et al., 2014; Paine et al., 2017a; Paine et al., 2017b). In this context, EURL ECVAM created and disseminated a survey entitled “Use of membrane transporter data and knowledge for chemical

safety assessment and biomedical research' to capture the applicability of using the important amount of available data and knowledge on transporters beyond the drug discovery field. The survey was designed with a specific interest to identify *in vitro* and *in silico* methods that are currently in use for transporter studies. The overall survey results, with a focus on the challenges and future areas of improvement pointed out by transporter experts to gain greater confidence in using transporter data for animal-free risk assessment, are reported and discussed here. Additionally, the methodologies to investigate membrane transporters in the context of fundamental sciences and the difficulties of moving away from animal studies to unravel transporter biology in basic research are also presented here.

2. Methodology

The survey was created by the European Commission – Joint Research Centre (JRC), EURL ECVAM (<https://eurl-ecvam.jrc.ec.europa.eu/>) as an online questionnaire using the public European tool EU survey (<https://ec.europa.eu/eusurvey/home/welcome>). The survey was available for the participants from the 24th of January until the 16th of March 2018.

2.1. Target group and dissemination

The survey was circulated to a target group of experts in membrane transporters from the academic, industrial, enterprises and regulatory communities, starting with EURL ECVAM collaborators, and selected scientists having publications on transporters (the survey was sent to around 250 contacts). The survey was also circulated to the members of the European Society of Toxicology *In Vitro* (ESTIV) (<http://www.estiv.org/>), the European Partnership for Alternative Approaches to Animal Testing (EPAA) (https://ec.europa.eu/growth/sectors/chemicals/epaa_en), the Virtual Physiological Human institute (VPHi) via their newsletter (<http://www.vph-institute.org/>), the EU-TOX-Risk consortium (<http://www.eu-toxrisk.eu/>). Furthermore, the recipients were encouraged to share the survey with their own network of experts. The goal was to reach as many scientists in the transporter field as possible. The exact number of recipients of the survey is therefore not known.

2.2. Structure of the survey

The survey contained a total of 20 questions including Yes/No, multiple choice, and free-text answers type questions. In most of the cases, the respondents could specify their answers in a free-text format. For multiple choice questions, participants could select one or multiple answers. The survey was divided into 4 sections: (1) respondent's profile (country, professional sector and expertise), (2) use of transporter data and knowledge (fields and applications of transporter studies as well as mechanisms, organs/barriers and individual transporters under study), (3) tools and methods used to study transporters (decision-support tools and experimental methods: *in vivo*, *in vitro* and *in silico*) and (4) opinions on the availability and applicability of the non-animal methods to study transporters, especially for chemical risk assessment and biomedical research. The full questionnaire is available in the Supplementary material. Responses were aggregated for statistical analysis and individual responses were kept anonymous.

2.3. Mapping and analysing the results

The answers collected by the EU Survey tool were exported to a Microsoft Excel file for analysis and interpretation. Data analysis and graphical representation was conducted using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and via <http://eulerr.co/> for the Venn diagrams. Geographical distribution of the survey responses was elaborated by means of an interactive infographic using

graphical mapping built on Google Chart API as a HTML5 and JavaScript web app by Klimeto (<http://klimeto.com/>). This visualisation approach was chosen as it allows for a simplified and clear illustration of the spread in demographics of the survey participants while keeping their identities and contact information anonymous.

3. Results

3.1. Country, professional sector and expertise of the participants

A total of 73 participants completed the online survey from 21 countries: Austria, Belgium, Canada, Croatia, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Netherlands, Norway, Portugal, Singapore, Spain, Sweden, Switzerland, United Kingdom, and United States of America (Fig. 1).

Survey participants comprised experts with representation from a variety of communities: academia (59%, $N = 43$), industry (18%, $N = 14$), governmental organisations (12%, $N = 9$), small medium enterprises (SME) (8%, $N = 6$), contract research laboratory (CRL) (8%, $N = 6$), and regulatory agencies (3%, $N = 2$) (Fig. 2A). The academia sector includes clinical research. Two academics reported to work in parallel for a non-profit organization. Three academics worked also in industry and one in a SME. Two respondents declared working in both SME and CRL. From the industrial sector, more than half of the participants were from the pharmaceutical industry. The others were spread evenly between the agrochemical, chemical and cosmetics sectors. Some recipients from regulatory agencies declared not having the expertise in-house thus could not fill in the survey and were not counted among the respondents.

Nearly half of the survey participants declared multiple kinds of expertise. The overall span of professional expertise of the respondents is summarized in Fig. 2B. Analysing the expertise by professional sectors, academics indicated expertise mainly in laboratory science (67%, $N = 29$ out of 43 academics) and research and development (R&D) (63%, $N = 27$), followed by risk assessment (14%, $N = 6$) and mathematical model development (5%, $N = 2$). Respondents from industry, SME and CRL all declared expertise in R&D (100%, $N = 24$), then in laboratory science, risk assessment and mathematical model development (29% each, $N = 7$), followed by expertise in products and services (21%, $N = 5$) and finally in statistical analysis (13%, $N = 3$). Experts from governmental organisations or regulatory agencies reported expertise in risk assessment, mathematical model development and research and development (55% each, $N = 6$ out of 11) and one in statistical analysis.

3.2. Use of transporters data and knowledge

3.2.1. Fields and applications of transporter studies

The results revealed that transporters are studied primarily for drug development of human and veterinary medicine (63%, $N = 46$), followed by risk assessment purposes on food and feed contaminants (such as pesticides) (32%, $N = 23$), industrial chemicals (28%, $N = 20$), cosmetics (21%, $N = 15$) and nanomaterials (10%, $N = 7$) (Fig. 3A). Comment from a respondent working in a CRL specialised in offering products and services to study transporters confirmed this dynamic: "Major clients are from the pharma but requests come also from food and chemical industry". Furthermore, 10 participants declared studying transporters in environmental species such as aquatic organisms or bees (Fig. 3A). Overall, the participants declared interest in internal exposure assessment (29%, $N = 21$), effects of combined exposures (mixtures) (22%, $N = 16$) or endocrine disruptors (20%, $N = 14$). When studying environmental species, most respondents declared investigating the impact of mixtures of pollutants on transporters ($N = 7$ out of 10). Finally, 40% of the total respondents declared studying transporters specifically for fundamental science purposes ($N = 30$) to improve the pharmacology and toxicology fields as well as to establish basic knowledge on transporter biology and on transporter-

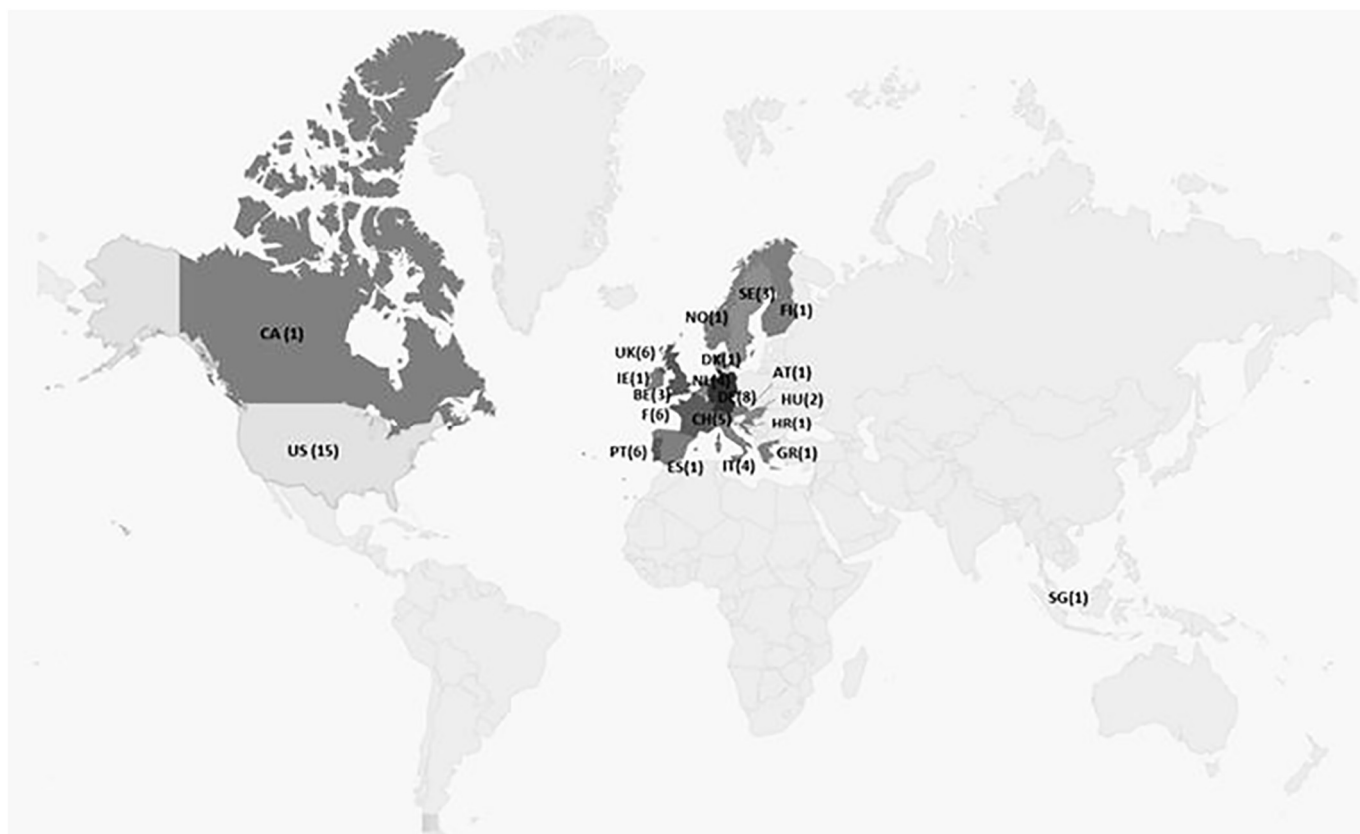


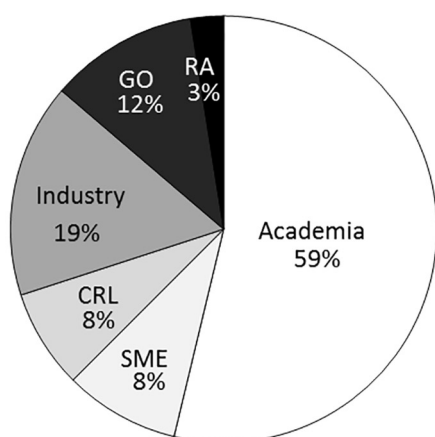
Fig. 1. Geographic distribution of the survey participants: 73 respondents from 21 countries. Each country is labelled with the ISO 3166 International Standard Country Code, two letters, followed by the number of responses received per country in brackets. The results by individual country can be accessed at <http://apps.klimeto.com/int/> by selecting a country. The colour scale represents the lowest number of replies (dark grey) to the highest number (light grey).

related diseases. These results allowed, (i) to compare the use of transporter data for drug development *versus* for chemical risk assessment purposes; (ii) to capture the specific methodologies currently used to study transporters in fundamental research.

When questioned about the applications of transporter studies, except for four academics interested in disease development and treatment only, all participants studied transporters for toxicity predictions of which 48% studied for risk assessment purposes ($N = 36$) and 40%

for screening and prioritising ($N = 29$). Furthermore, 37% studied transporters also for efficacy prediction ($N = 27$) and around 10% following customer requirements ($N = 7$) (Fig. 3B). When asked to specify the precise applications within toxicity/risk evaluations, participants mentioned studying transporters mainly to support *in vitro* to *in vivo* extrapolation (IVIVE) (70%, $N = 51$), to characterize and support design of *in vitro* assays (53%, $N = 39$), to evaluate species differences (49%, $N = 36$), to consider sensitive population (polymorphism, age, sex, diet,

A Professional sector



B Expertise

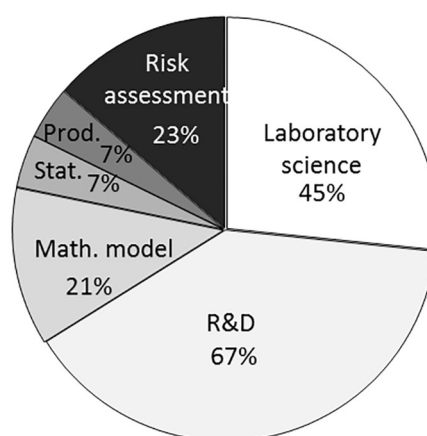


Fig. 2. Overall professional sector (A) and expertise (B) of the survey participants. Small medium enterprises (SME), contract research laboratories (CRL), governmental organisations (GO), regulatory agencies (RA), research and development (R&D), Mathematical model development (Math. model), Statistical analysis (Stat.), Products and Services (Prod.).

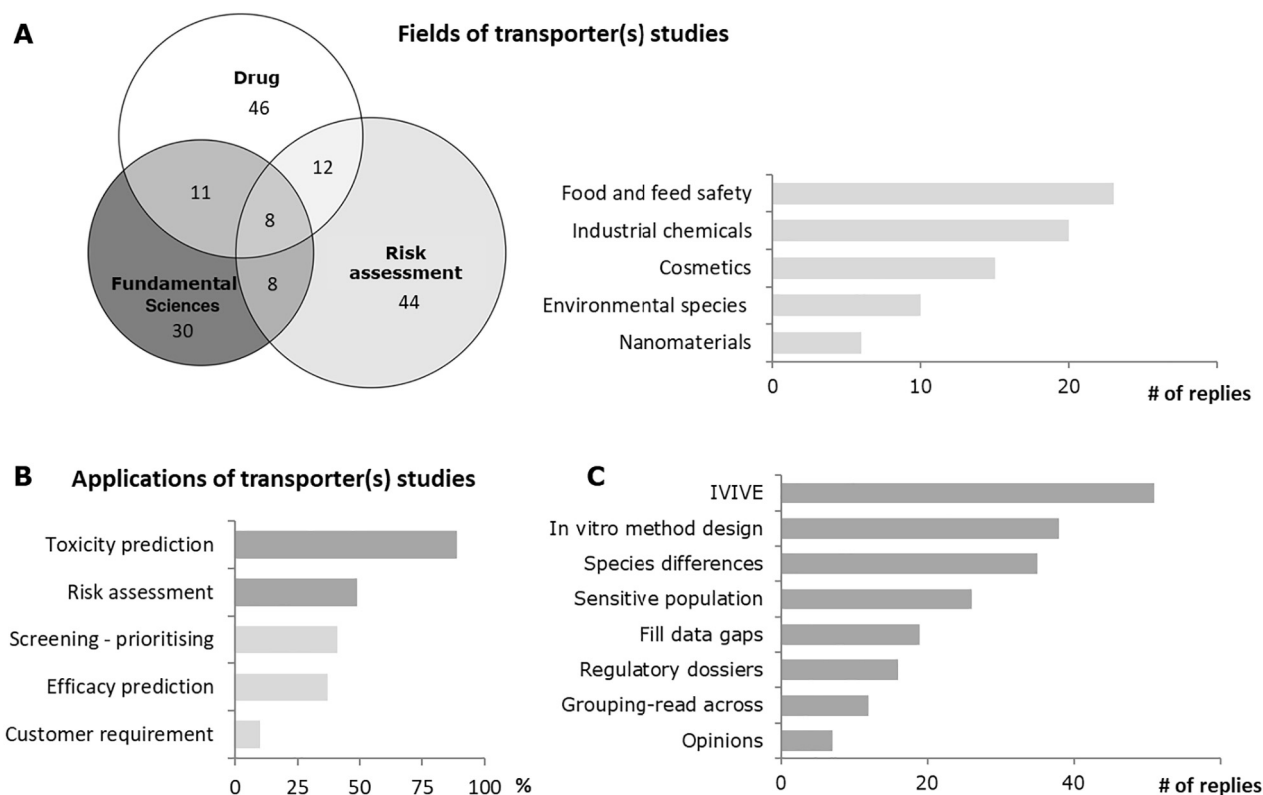


Fig. 3. Fields of transporter studies as number of replies, showing overlap of the answers as respondents could select more than one answer (A). The different fields of study encompassed in “Others” are detailed on the right side. Applications of transporter studies as percentage of total replies (B) and specific toxicity-related applications as number of replies (C). Drug: Drug development, IVIVE: *in vitro* to *in vivo* extrapolation.

diseases) (37%, $N = 27$), to fill data gaps (26%, $N = 19$) and to support substances grouping and read across (16%, $N = 12$) (Fig. 3C). Some respondents also mentioned investigating transporters for submission of regulatory dossiers or opinions ($N = 16$ and 7, respectively).

3.2.2. Mechanisms, physiological barriers and transporters currently studied

Participants were proposed to state whether they conduct studies to identify (i) substrates or (ii) inhibitors of transport proteins, (iii) transporter-mediated interactions or (iv) inducers of transporter expression. Almost all respondents declared investigating substrates (90%, $N = 66$), followed by inhibitors (75%, $N = 55$) and transporter-mediated interactions (56%, $N = 41$). Finally, induction studies were part of the work plan for less than half of the participants ($N = 36$). Several experts further specified that transporter induction is too poorly studied. Around 40% of participants declared investigating all mechanisms. The mechanisms were investigated in the same proportions whether for drug development or for chemical risk assessment or toxicity prediction purposes (Fig. 4A).

Transporters are expressed at the external barriers (intestinal, dermal and lungs) and major blood-tissue barriers (brain, placenta, testes) as well as at the hepatic and renal excretory organs. There, transporters affect the ADME processes of various compounds. Whether for drugs or for chemicals, intestinal, hepatic, renal and blood-brain barriers were predominantly considered by participants as shown in Fig. 4B. In this survey, brain and lungs were mentioned to be investigated more for drugs, while skin, placenta and testes more for chemicals (Fig. 4B). Some participants also mentioned working on the cardiovascular system, eyes, adipose tissues and on fish gills and embryos. Altogether, the participants rank-ordered the impact of transporters first on absorption, then on excretion, followed by distribution, and to a lesser extent on metabolism.

Related to transporters of interest, the participants could choose between the main “drug transporters” namely (*with gene name*): MDR1 also called P-gp (*ABCB1*), BCRP (*ABCG2*), OATPs (*SLCOs*), OATs (*SLC22A*), OCTs (*SLC22A*), MATEs (*SLC47*), Bsep (*ABCB11*) and MRPs (*ABCCs*). Their relative investigations among participants are represented in Fig. 5. Uptake and efflux transporters were equally considered. Besides, participants mentioned studying various supplementary ATP-binding cassette (*ABC*) and solute carrier (*SLC*) members; such as cholesterol efflux transporter (*ABCA1*), *ABCA7* transporter, excitatory amino acid transporter (*EAATs*, *SLC1A*), amino acid transporters (*SLC7*), sodium-taurocholate cotransporting polypeptide (*NTCP*, *SLC10A1*), monocarboxylate transporters (*MCTs*, *SLC16*), vesicular glutamate transporters (*VGLUTs*, *SLC17A*), vesicular monoamine transporter (*VMAT2*, *SLC18A2*), reduced folate carriers (*RFCs*, *SLC19A*) urate transporters (*URATs*, *SLC22A2*), concentrative nucleoside transporters (*CNTs*, *SLC28A*) and equilibrative nucleoside transporters (*ENTs*, *SLC29A*).

3.3. Tools and methods used to study transporters

3.3.1. Decision-support tools

Survey participants decided to investigate active transport based on literature (70%, $N = 51$), in house knowledge (61%, $N = 44$), *in vitro* to *in vivo* discrepancies (48%, $N = 35$), PBK models (42%, $N = 31$) or regulatory requirements (33%, $N = 24$), and also following biotech company guidelines/decision trees (11%, $N = 8$) or related to a specific research question to establish basic knowledge, as stated by some academics ($N = 5$). To identify which methodologies would be appropriate for their project, experts relied on in house experiences (70%, $N = 51$), literature (65%, $N = 47$), regulatory guidance and requirements (32%, $N = 23$) and biotech company advices/decision trees (10%, $N = 7$). For 22% of all respondents, financial considerations were also taken into consideration to select the method(s) ($N = 16$). For several academics,

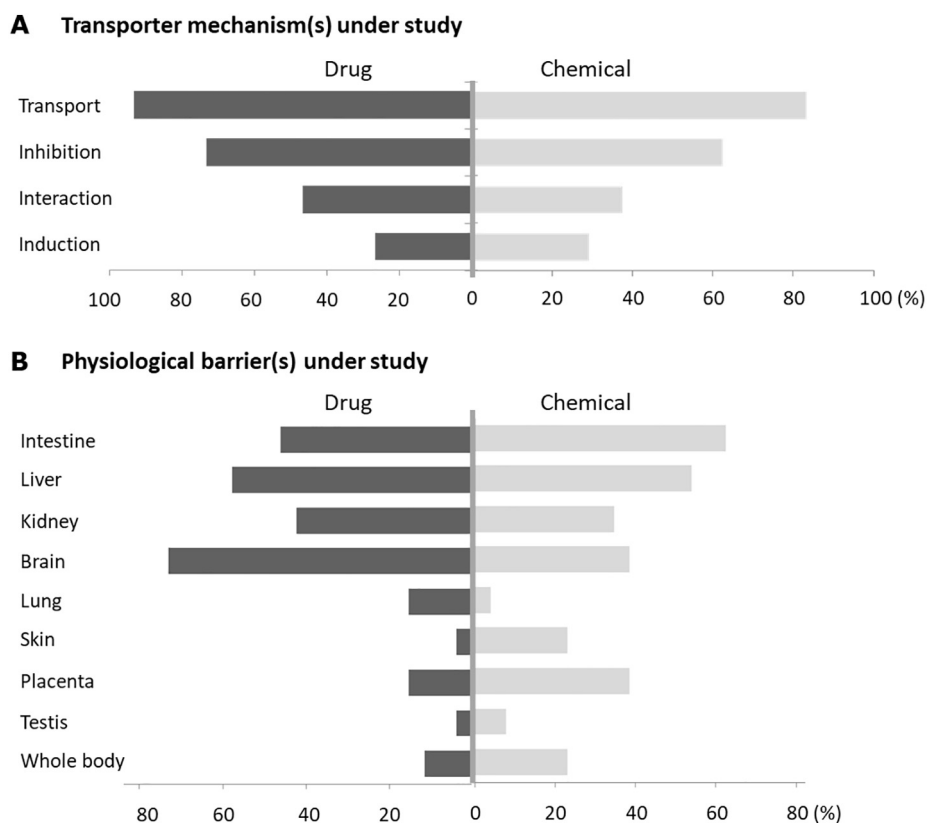


Fig. 4. Transporter mechanisms (A) and physiological barriers (B) currently investigated by survey participants. The bars represent the replies in percentage from respondents working either in the drug development field (Drug, $N = 26$) or carrying out risk assessment of food and feed contaminants, industrial chemicals, cosmetics, nanomaterials on both humans and environmental species (Chemical, $N = 24$). In the survey, the respondents could select more than one answer. Replies from respondents declaring working in both drug development and chemical risk assessment were not considered here, nor the replies from participants working in fundamental sciences only.

the methodology will depend on the research question. One respondent also based his choice on availability of the assays for screening. Finally, around 36% of the respondents followed guidelines or guidance documents to set up their transporter work plan, mainly the ones from FDA (92%, $N = 24$) and EMA (69%, $N = 18$), but also from MHLW (8%, $N = 2$) and others ($N = 4$) (not specified).

3.3.2. Experimental methods

Among survey participants, transporters were investigated primarily *via in vitro* (82%, $N = 60$), then *in silico* (41%, $N = 30$) and *in vivo*

(38%, $N = 28$) methods, either as stand-alone method or in combination as shown in Fig. 6A. The survey was designed so that the respondents replied only to the questions related to the methods they mentioned working with: *in vivo*, *in vitro* or *in silico*.

All the scientists working *in vivo* ($N = 28$) declared working also with *in vitro* and/or *in silico* methods (except for one participant who worked only with data from healthy volunteers) (Fig. 6A). Among the participants carrying out animal studies ($N = 23$ respondents), most of them performed those in the context of fundamental sciences ($N = 19$), followed by pharmaceutical drug development ($N = 6$). The

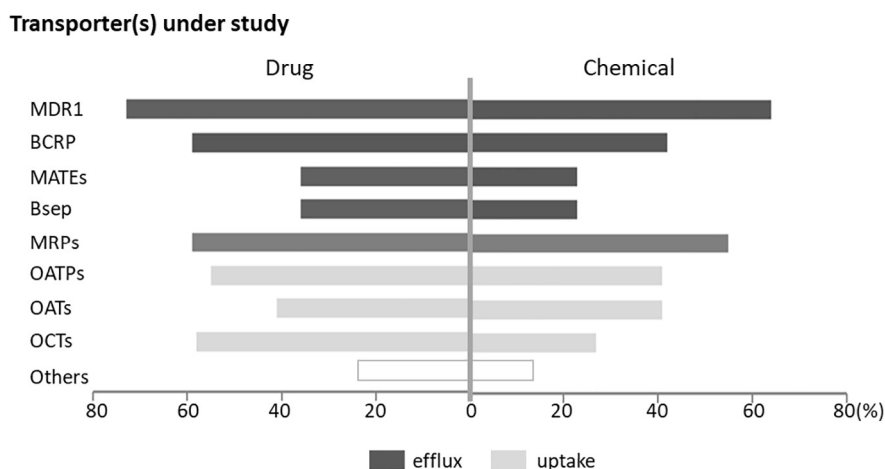


Fig. 5. Transporters currently studied by the survey participants. The bars represent the replies from respondents (number of replies presented in percentage) working either for drug development (Drug) or for risk assessment of food and feed contaminants industrial chemicals, cosmetics, nanomaterials on both humans and environmental species (Chemical). The respondents could select more than one answer. Replies from respondents declaring working in both drug development and chemical risk assessment were not considered here, nor the replies from participants working in fundamental sciences only.

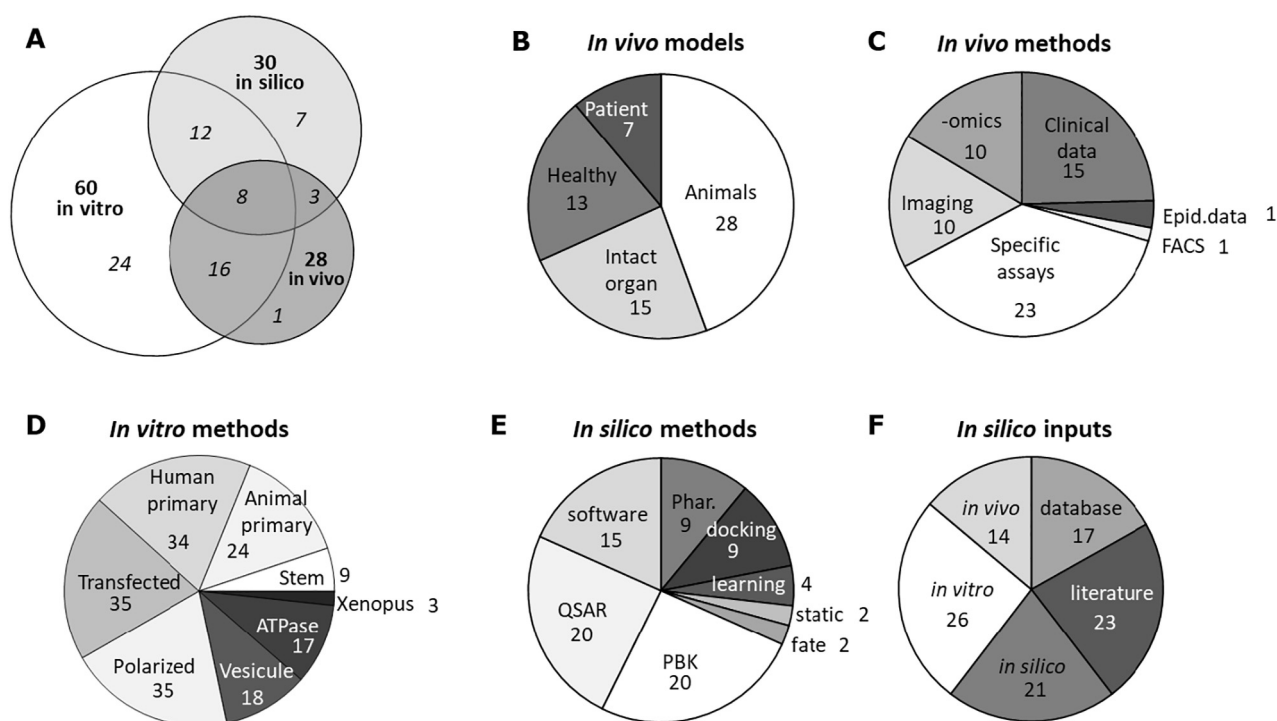


Fig. 6. Overview of the overall experimental methods used by survey participants as number of replies showing the overlaps in the answers (A). Number of replies describing the *in vivo* models and methods (B–C), *in vitro* methods (D), and computational methods and sources of input parameters for *in silico* model development (E–F) used by the survey participants. The respondents could select more than one answer. Epid. data: epidemiological data, FACS: fluorescent activated cell sorting, Phar.: pharmacophore modelling.

participants reported working with one or multiple *in vivo* models among those proposed in the questionnaire: knockout/mutated or “humanised” animal models ($N = 16$ and 8 replies respectively), intact organs ($N = 15$), healthy volunteers ($N = 13$) or patients (sick or presenting transporter polymorphisms) ($N = 7$) (Fig. 6B). The animals under study here were specified as mice, rats, dogs, lactating cattle's, zebrafish (adults and embryos) and sea urchins. Additionally, fungi were also mentioned, as well as meta-analysis of *in vivo* data from the literature. The *in vivo* analytical methods proposed as multiple choice answer and used among participants ranged from imaging (microscopy and positron emission tomography mainly) ($N = 10$ replies), -omics approaches ($N = 10$) and specific functional methods ($N = 23$) (Fig. 6C). Analysis of human clinical data ($N = 15$) were also reported. One additionally mentioned using fluorescent activated cell-sorting and another epidemiological data. Finally, among the respondents working *in vivo*, thirteen (13) of them declared being interested in sharing their *in vivo* data in public databases.

Participants who mentioned working *in vitro* ($N = 60$) could report the use of one or multiple experimental systems (questionnaire provided in Supplementary material). The methods reported were primarily cell-based ($N = 137$ out of 176 replies), followed by membrane-based ($N = 35$) and few cases of *Xenopus* oocytes ($N = 3$). The membrane-based methods referenced here included ATPase and vesicular transport methods, both reported to be used with equal prevalence (Fig. 6D). Cell systems proposed and reported were polarized and transporter-transfected cell lines ($N = 35$ each), animal and human primary cells ($N = 24$ and 34, respectively) as well as stem cells ($N = 9$) (Fig. 6D). Two participants also mentioned working with fish cell lines. Furthermore, participants could report whether working with plated ($N = 22$), suspension ($N = 18$) and/or sandwich-cultured hepatocytes ($N = 18$) (not shown). More complex systems were also reported to be used here such as co-culture ($N = 19$), 3D culture ($N = 17$) and organoids ($N = 9$) (not shown). Finally, more than half of the *in vitro* respondents expressed interest in sharing their experimental data into public databases ($N = 36$ out of 60). Furthermore, the same proportion of experts also agreed to share their protocols to explore

standardization of these methods to draft international guidance documents.

Then the computational models, proposed as a multiple choice answer, were reported by the *in silico* respondents ($N = 30$, accounting for 82 replies) as primarily physiologically-based kinetic (PBK) models and quantitative structure-activity related relationship (QSAR) ($N = 20$ replies each), then integrative platforms ($N = 15$), followed by pharmacophore modelling and docking ($N = 9$ each), machine/deep learning ($N = 4$) and finally static models and *in vitro* fate models ($N = 2$ each). Individual participants also mentioned biologically-based dose-response and network models. The sources of parameters for the *in silico* models related to transporters as reported in this survey (101 replies) were distributed between existing data, found in literature ($N = 23$) or in databases ($N = 17$), and data generated through new studies, including experimental *in vitro* data ($N = 26$), *in silico* predictions ($N = 21$) as well as *in vivo* data ($N = 14$). Finally, about half of the modellers showed interest in sharing their *in silico* data in public databases ($N = 13$).

3.4. Availability and applicability of *in vitro*-*in silico* methods to study transporters in the context of an animal-free chemical risk assessment strategy

3.4.1. Gap-analysis of available transporter *in vitro* methods and database information to support animal-free chemical risk assessment

In line with previous results section, the respondents identified some missing appropriate *in vitro* methods to generate transporters data without animal studies. Here are summarized the replies that were reported in a free-text format. First, transporter induction assessment is understudied and remains a big challenge as already stated. Further *in vitro* systems need to be developed to gain greater insight into mechanisms and signals inducing transporter expression. Then with regard to barrier models, it was mentioned by several respondents that holistic *in vitro* models are not fully developed yet and that improvement of current methods are needed. Particularly, it was mentioned a need for quantifiable and reproducible kidney and blood-brain barrier

models and additional methods to study placental transport. For individual transporter, methods for all species and polymorphisms were stated as lacking. Furthermore, it was mentioned that most of the data are on MDR1 (Pg-p), other transporter methods are available mainly as vesicles and this tool is not appropriate for permeable compound. It was also said that reliable high-throughput methods to assess transporter functionality are needed in association with a necessary understanding for data interpretation. Besides, some mentioned that non-animal methods to study transporters are highly costly and time consuming, suggesting that economically feasible methods are missing. Finally, it was reported that standardised protocols are lacking for these methods.

When questioned about the available information in transporter databases in the context of chemical risk assessment, around 80% of the respondents declared that important data are currently missing and specified which in free-text format. First, several respondents highlighted the need of databases based on environmental chemical datasets (pesticides, industrials, cosmetics, excipients...) and not only with respect to pharmaceuticals as it is mostly the case currently. Besides, the need of publicly accessible databases was also raised. Then, quantitative data on kinetics and on transporters abundance were particularly cited. This latter encompasses transporter expression and functionality in the various cell lines used compared to tissues, in different species and in specific diseases. It was stated by many that it would benefit the community to make available the raw *in vitro*, *in silico* and *in vivo* data and to report the negative results. Ideally supplemented with the methods or protocols used to ensure high quality of the data. Finally, non-mammalian data, *in vitro-in vivo* scaling factors, substrates specificity, mechanistic data, effects of co-exposure on the internal concentrations and toxicity were also mentioned.

3.4.2. Challenges and future needs in the applicability of *in vitro-in silico* transporter data to support animal-free chemical risk assessment

Respondents were proposed via multiple choice answers to identify and rank various challenges in the interpretation and use of transporter data generated by *in vitro* methods. Results highlighted complex interplay with metabolic enzymes and other transporters (70%, $N = 51$ replies), species differences (54%, $N = 39$), lack of specific transporter substrates and inhibitors (33–47%, $N = 24$ –34 respectively), loss of cell polarity (22%, $N = 16$), lack of negative controls (11%, $N = 8$). Finally more than half of the respondents clearly acknowledged problems of inter-laboratory variability (51%, $N = 37$) and lack of standardised protocols (54%, $N = 40$) (Fig. 7A). In regards to the last two points, several experimental parameters affecting transporter activity in the *in vitro* systems were brought up by experts (Fig. 7B). First, the culture medium is a concern for almost all of the survey participants. Moreover, 80% acknowledged and shared equally the concern of the differences in transporter expression and function depending on the origin/provider (for cell lines) or the inter-individual variability (for primary cells), concentration of the compound and culture timing, while 75% cited cell density. Matrices, cofactors, temperature and oxygen levels were also identified as affecting parameters. Furthermore, the contribution of passive diffusion and the *in vitro* artefacts such as plastic binding or evaporation was additionally mentioned by several respondents, as well as the issue of relevant IVIVE regarding transporter abundance differences in cell systems compared to tissues.

The challenges in the interpretation and use of *in silico* transporter data to support chemical risk assessment proposed in a multiple choice format and acknowledged by the participants were the lack of mechanistic understanding (77%, $N = 40$), the robustness of the models (58%, $N = 30$), the broad substrate specificity of transporters (54%, $N = 28$), the accuracy or size of the initial input data set (50%, $N = 26$), uncertainty (40%, $N = 21$), lack of metabolites libraries (21%, $N = 11$), divergent membrane topologies (9%, $N = 7$) and low levels of sequence identity (6%, $N = 3$) (Fig. 7C). Again the quantitative difference of transporters in cell lines *versus* tissues, that should be implemented as a

correction factor in computational models, was raised. Finally, respondents remarked that small changes in the inputs or parameters may have large effects on predictions and emphasized that it is essential to understand what models can be used for what purpose.

Finally, when questioned about the need for further guidelines and guidance documents to conduct studies on transporters, half of the participants said yes while 12% (all from academia) thought that it is not necessary for fundamental sciences (Fig. 7D). The others did not have an opinion on this question. When asked to specify in a free-text format about the content of these recommendations, experts replied: to know the right controls and standardised protocols, to inform on risk assessment and PBK model development for industrial chemicals, to provide an overview of the options (tools and methodologies) to study transporters and to guide on which transporters are relevant to study. Several respondents also mentioned the need for harmonization across geographical regions.

To the final question on how to gain greater confidence in describing transporter kinetics in the absence of *in vivo* data, the participants strongly acknowledged a better understanding of the mechanisms (80%), the development of new uncertainty assessment (21%) and a combined *in vitro-in silico* approach (65%) as key elements. However, with the aim of using transporter data for animal-free risk assessment, respondents from the regulatory sector specified that they would only gain confidence in the predictions if they are shown to match *in vivo* data, at least in some cases such as with known compounds or with literature data. Similarly, another respondent proposed to first obtain transporter TK data *via in vitro* and *in silico* tools to parametrise PBK models and then use available human data to evaluate the model performance and further refine the PBK parameters obtained from initial *in vitro/in silico* methods. This resounds to other comments stating that “because we do not know how to use the *in vitro* data for further use in kinetic models, it may limit the end value of the data and therefore, it will be difficult to gain confidence in describing transporter TK in the absence of *in vivo* data” and “that guidance as to how much *in vitro* data is good enough to parameterize a transporter function *in silico* would be important to characterize at an earlier stage so that the transporter-specific parameters do not end up becoming fudge-factors say in a PBK model”. Additionally, a quantitative understanding of inter-species and *in vitro-in vivo* differences in transporter expressions and activities is very important for the development of PBK models and for risk assessment as already stated in this paper.

Ultimately, the confidence in unravelling transporter (patho)-physiology without animal studies is low for basic researchers. It was recurrently mentioned that *in vitro* and *in silico* models have limited *in vivo* relevance in that context and cannot mimic complex pathways, such as enterohepatic circulation. Lack of funding of alternative-based projects was also expressed as a barrier to encourage scientists to move toward studies not requiring animals.

4. Discussion

Several international workshops and conferences identified active transport as representing essential TK information for chemical risk assessment (Bessems et al., 2014, 2015; Paine et al., 2017a; Paine et al., 2017b). As such, the EURL ECVAM released a survey entitled ‘Use of membrane transporter data and knowledge for chemical safety assessment and biomedical research’ to gather the current state of science in this matter. The survey was designed with a view of identifying the availability, applicability and future areas of improvement to generate and use transporter data using non-animal methods within a TK informed risk assessment strategy.

Thanks to the 73 respondents from different working sectors and with various kinds of expertise, the results reported that transporters are investigated during drug development first, but also for human and environmental risk assessment purposes and in fundamental research. With the exception of few working on basic science, all

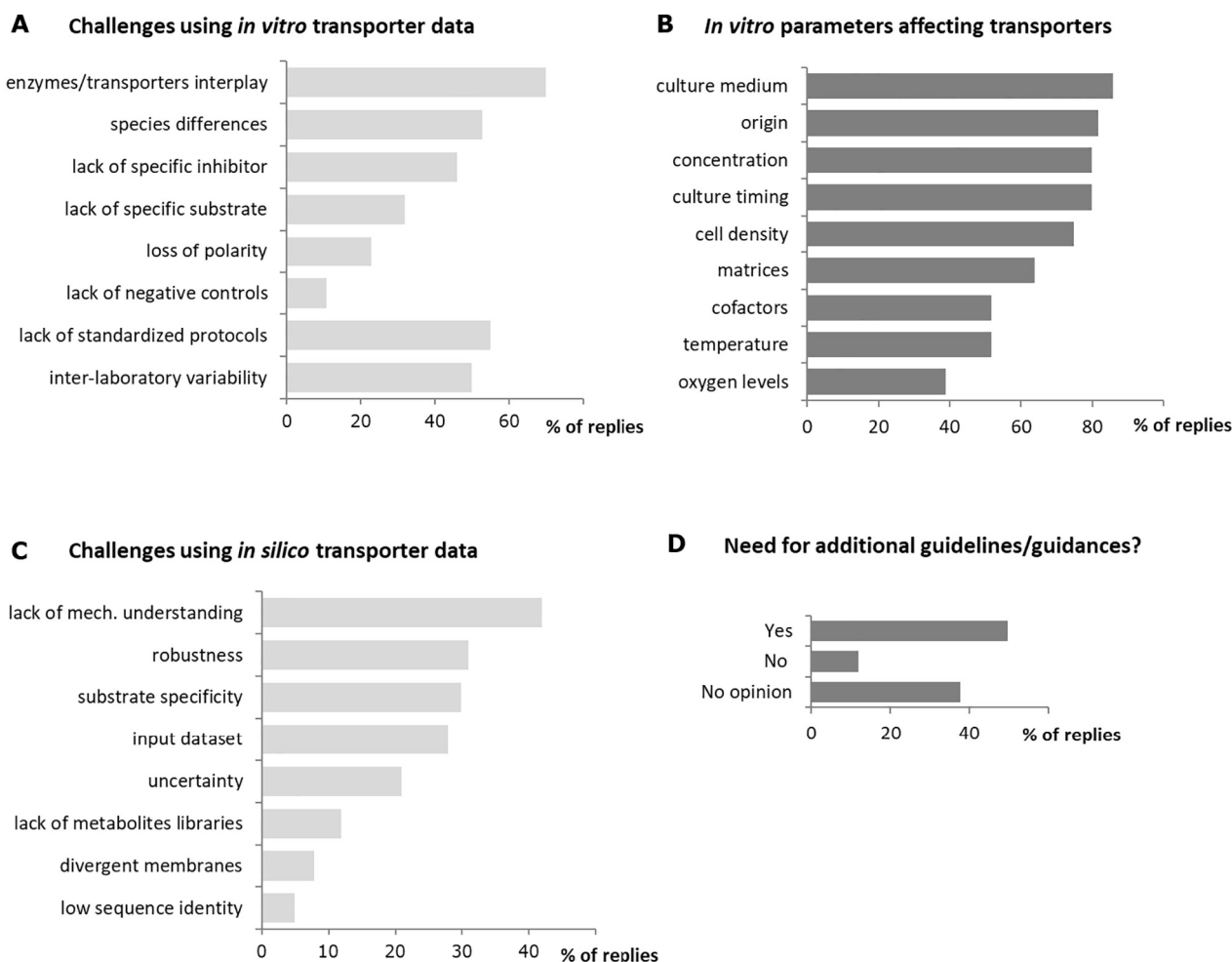


Fig. 7. Bars showing the challenges when using and interpreting *in vitro* transporter data as percentages of replies (A) and experimental elements affecting transporter expression and activity *in vitro* as percentages of replies (B). Bars representing the challenges when using and interpreting transporters *in silico* data as percentage of replies (C). The respondents could select more than one answer. Replies to the question of the need for further guidelines or guidance documents as percentage of the total answers (D). mech.: mechanistic.

participants studied transporters for toxicity predictions mainly to support IVIVE, considering species differences and sensitive population as well as real internal exposure, but also to characterize and design *in vitro* methods used in toxicology or pharmacology, to support screening and prioritising and to support substances grouping and read across. These results are in line with recent recommendations of how TK data can be used in regulatory decision making (Corvi et al., 2013; Prieto et al., 2014; Casati et al., 2013; Bessems et al., 2014; ECHA, 2011; EFSA, 2014). However, recipients from some regulatory agencies declared not having transporter expertise in house, supporting that transport TK data are not sufficiently considered so far within a regulatory context.

Whether a compound is a substrate or an inhibitor of transporters are the primary focus of active transport studies for drugs as for chemicals. Then, transporters are notably implicated in DDI resulting in adverse effects. Similarly, transporters could be involved in the deleterious effects of chemical-drug or chemical-chemical interactions (Fardel et al., 2012). Therefore impacts of transporters on TK of pollutants or on pharmacokinetics of drugs due to exposure to chemicals should be likewise carefully characterized in humans as well as in environmental species. In bees or aquatic organisms for example, efflux transporters represent protective mechanisms against toxins. The impact of interaction of several environmental chemicals on transporters could threaten the sustainability of a population (Hawthorne and Dively, 2011; Epel et al., 2008; Luckenbach et al., 2014). Finally, in both the toxicology and pharmacology fields, transporter induction is

understudied by lack of consideration and of adequate experimental systems. Though, systemic inducers may be absent *in vitro*; such as hormones resulting in loss of sex-specific expression of transporters in the experimental systems. Furthermore hormones, and more generally chemicals, can modulate transporter levels not only by inducing transcriptional activation but also by repressing it (Aleksunes et al., 2012; Jigorel et al., 2006; Bucher et al., 2013). Considering transporter induction or repression is therefore of particular importance for environmental chemicals as no human *in vivo* data are available to evaluate them in contrast to existing clinical data in pharmacology. Furthermore, studying transporter expression regulation could be particularly relevant for chronic exposure of environmental chemicals. So even if, so far, clinically relevant transporter-mediated DDI seem to be linked to transport activity inhibition and not to altered expression (König et al., 2013; Chedik et al., 2018a, 2018b), the mechanisms regulating transporter expression clearly deserve more attention and research for chemical risk assessment purposes.

During drug development, particular attention is paid on transporters expressed at the intestinal, hepatic, renal and blood-brain barriers considered as pivotal for pharmacokinetics (Giacomini et al., 2010). Similarly, these barriers are predominantly investigated for TK in the context of estimating the internal concentration that corresponds to the external exposure levels to environmental chemicals. In this survey, brain was particularly considered for drugs, probably because brain-to-blood efflux represent a major obstacle in drug penetration and efficacy (Löscher and Potschka, 2005; Kalvass et al., 2013). While

the prevalence of skin studies for chemicals compared to drugs may be related to the European cosmetics regulations that banned animal testing in the field of cosmetics in March 2013, promoting alternative methods. Finally, placental and testes barriers are of importance for reproductive and developmental toxicology and for the evaluation of window of sensitivity for exposure-based adverse effects in reproductive life-stages. The individual transporters studied for drugs as for chemicals reflect the recommendations of drug regulatory agencies (EMA, 2012; US FDA, 2017). Interestingly, while MRPs are not required but should only be eventually considered, more than half of the respondents declared studying them. It is important to note that the list of known drug transporters listed here is neither exhaustive nor complete as the transporter field is rapidly advancing. Literature on additional membrane transporters of clinical and toxicological relevance is continuously emerging. This is notably reflected by the additional transporters cited by the participants and detailed in the results section. Furthermore, there exists the possibility for some transporters to be more relevant for chemicals than for drugs. This should be kept in mind when assessing relevant transporters for chemical risk assessment. However, considering first the intensively studied transporters offer extensive data and knowledge to start our assessments with.

Regarding experimental methods, all respondents performing animal studies declared working also with *in vitro* and/or *in silico* tools. This already reflects a shift toward the use of alternative methods in the transporter field. However, several challenges and future needs have been raised in this survey to gain scientific and regulatory confidence in the use of transporter data to support animal-free risk assessment purposes. First, improvements of some experimental methods are needed including induction assessment, holistic barrier models, specific species- and polymorphic-transporter methods, while considering the economical, quantifiable, reproducible and high-throughput capacity aspects of the methods. Secondly, standardization of methods should be the next priority to ensure high-quality data that would be consistent across laboratories. In this regard, the various experimental parameters that should be taken into account when developing, validating or using a given transporter experimental method have been identified in this survey. And several respondents stated their willingness to share their protocols in support of development of new guidance documents. Thirdly, irrespective of the type of computational models used, their uncertainties have to be systematically characterized and documented. Earlier reports have highlighted the very need and have begun to address the issue of standardization in uncertainty characterization for computational models (Barton et al., 2007; Loizou et al., 2008; Project and No, 2010); however, such systematic evaluation and documentation is yet to become common practice. Finally, several large-scale efforts have been already implemented to systematically gather the important amount of transporter data into databases (summarized in Table 1). However, datasets were constituted by drugs in most of these databases and not by environmental chemicals. Going in this direction, Sedykh et al. assembled a large human intestinal transporter database constituted half by drugs and half by chemicals (Sedykh et al., 2013). This database was used to develop QSAR models predicting transport and inhibition of transporters with drug candidates but also with environmental chemicals (Sedykh et al., 2013).

Additionally, it was particularly emphasized here the need to report transporter abundance in cell lines versus tissues and between species in order to address a major gap in the ability to mathematically describe the fate of a chemical within the tissue. Besides, abundance data represents an essential characterization of the biological system used to generate *in vitro* data. The need for phenotypic characterization of toxicological test systems by *in vitro* method developers has been raised already in a guidance document on Good Cell Culture Practices by Coecke et al. (2005). Current work at OECD is ongoing to issue a Guidance Document on Good Method *In Vitro* Method Practices (GIVIMP) where this aspect is stressed (<http://www.oecd.org/chemicalsafety/testing/draft-guidance-review-documents-monographs.htm>). In line,

Table 1
Transporter databases.

Name	Link	References
TP-Search	www.tp-search.jp	(Ozawa et al., 2004)
TransporterDB (2.0)	www.membranetransport.org	(Ren et al., 2007; Elbourne et al., 2017)
UCSF pharmacogenetics database	www.pharmacogenetics.ucsf.edu	(Giacomini et al., 2010)
U Washington DDI	www.druginteractioninfo.org	(Hachad et al., 2010)
FDA Transporter Database	www.transportal.combio.ucsf.edu	(Morrissey et al., 2012)
TCDB	www.tcdb.org	(Saier et al., 2014; 2016)
TrSSP: The Transporter Substrate Specificity Prediction Server	www.bioinfo.noble.org/TrSSP	(Mishra et al., 2014)
SwissADME	www.swissadme.ch	(Daina et al., 2017)

several respondents acknowledged that it would be beneficial to share the raw data and many declared being in favour of sharing them.

Overall, to gain scientific and regulatory acceptance of non-animal transporter data, the approach should be integrative and iterative similarly to Integrated Approaches to Testing and Assessment (IATA) (Worth and Patlewicz, 2016). First, computational models combined with decision trees and/or public databases could be used to determine if a chemical would interact with a transporter and with which transporter(s). This first step would help to guide experimental work to focus on the appropriate *in vitro* methods related to the transporter of interest and it will also specify what confidence there may be in the predictions that lack any active transport kinetics. Then the data generated by specific human *in vitro* methods could be implemented into PBK models, built on mechanistic understanding from literature. The *in vivo* predictions obtained could then be evaluated with human biomonitoring or volunteer study based data when available. The PBK model equations and parameters as well as the initial *in vitro* or *in silico* generated data and models could then be adjusted accordingly to obtain matching with *in vivo* data. Through iterative calibration, the model predictions would gradually describe the human data better, increasing confidence in predictions from alternative methods. Importantly, the focus of the study here was active transport. However, passive as well as facilitated diffusion via membrane channels or via binding to human serum albumin, have to be taken into consideration (Cheng and Ng, 2017), especially for lipophilic contaminants, as most of the industrial chemicals are.

Finally animal studies were primarily performed in the context of fundamental research by the survey participants. Conversely, half of respondents declaring fundamental sciences as their field of interest, mentioned working with non-animal approaches. However, the transition away from animals seems to be less engaged in basic research than in the application-centric toxicological field. Considering the widely recognized inter-species differences in transporter expression and activity, human cell-based data implemented into computational model describing human physiology and validated with human biomonitoring data may be more relevant than animal studies. Several researchers pointed out that *in vitro* methods will never be able to replace *in vivo* studies, especially not to unravel complex pathways involving multi-transporters or transporter-metabolic enzyme interplay. In this context, the other Rs of the Directive 2010/63/EU could still be considered, namely the Reduction and Refinement of the use of animals for scientific purposes. *In vitro* and *in silico* TK data as well as imaging can allow to (i) reduce the number of animals used to obtain the same information or with the same number of animals expand the information collected, (ii) refine animal experimentation by moving from severe to mild suffering by favouring non-invasive approaches as an example (Ricketts et al., 2011; Kramer and Font, 2015). Besides, the most commonly used

in vitro models are often reproached to lose their tissue-specific physiology including their transportome profile. The recent fields of 3D cultures and organs-on-chips are proposed by some to have promising potential to improve these issues by offering more complex systems notably with co-cultures (van der Meer and van den Berg, 2012; van Der Helm et al., 2016). In addition to the role of transporters in TK, they can also play a role in toxicodynamics. This could be more extensively captured in the context of adverse outcome pathways (AOPs), which provide a simplified organizational construct linking molecular initiating events and early key events at lower levels of biological organization to disease outcomes. Transporters may be part of molecular initiating events or key events, such as in the AOP:27 describing cholestatic liver injury induced by inhibition of Bsep transporter (AOP Wiki <https://aopwiki.org>). As such, AOPs could facilitate the compilation of information to increase mechanistic understanding of human (patho)physiological pathways (Bal-Price and Meek, 2017) that involve the role of transporters. Ultimately, better funding of alternative-based projects would encourage basic researchers to replace, reduce and refine animal studies and applied researchers to adopt more standardised non-animal methods in toxicity testing and regulatory decision-making.

5. Conclusion

This paper reported the current state-of-play and challenges in the application of non-animal (*in vitro* and *in silico*) methods to study transporters for chemical risk assessment purposes and in biomedical research. The results provide insights into the future improvements needed to gain regulatory and scientific acceptance, including *in vitro* methods amelioration and standardization, missing database information as well as uncertainty characterization at the different steps. Future guidelines or guidance documents should as a priority contain standardised protocols preceded by an overview of *in vitro* and *in silico* tools available and relevant to evaluate active transport. Overall, the extensive existing knowledge on drug transporters from the historical interest of the pharmaceutical sector could provide essential pieces of information to support chemical risk assessment and to gain greater confidence in the predictive value of non-animal data.

Disclaimer

The views expressed in this paper are those of the authors and do not necessarily reflect the views of the author/s institutions. Authors declare no conflicts of interest.

Acknowledgements

The authors would like to sincerely thank the 73 scientists that replied to the survey. The authors would also like to make special thanks to the following JRC colleagues, Ivana Campia, Alfonso Lostia, Stephanie Bopp, Raffaella Corvi and Pilar Prieto-Peraita for providing valuable feedback on the questionnaire. Adelaide Dura for the graphical figure for disseminating the link of the survey together with Sofia Batista Leite and Madalena Cipriano. The authors would like to thank the following associations: ESTIV, EPAA, VPHi and EU-TOX-Risk consortium for circulating the survey to their members. The authors are also grateful to the preliminary discussion on the role of membrane transporters in science and regulatory with Dr. Catherine Mahony, Dr. Corie Ellison, and Dr. John Troutman from P&G as well as Dr. Barbara Brik, Dr. Eric Fabian and Dr. Caroline Gomes from BASF.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.07.122>.

References

- Aleksunes, Lauren M., Yeager, Ronnie L., Wen, Xia, Cui, Julia Yue, Klaassen, Curtis D., 2012. Repression of hepatobiliary transporters and differential regulation of classic and alternative bile acid pathways in mice during pregnancy. 130 (2):257–268. <https://doi.org/10.1093/toxsci/kfs248>.
- Armitage, James M., Wania, Frank, Arnot, Jon A., 2014. Application of mass balance models and the chemical activity concept to facilitate the use of *in vitro* toxicity data for risk assessment. Environ. Sci. Technol. 48 (16):9770–9779. <https://doi.org/10.1021/es501955g>.
- Bal-Price, Anna, and M. E. (Bette) Meek. 2017. "Adverse outcome pathways: application to enhance mechanistic understanding of neurotoxicity." *Pharmacol. Ther.* 179. The Authors: 84–95. doi:<https://doi.org/10.1016/j.pharmthera.2017.05.006>.
- Barton, Hugh A., Chiu, Weihsueh A., Woodrow Setzer, R., Andersen, Melvin E., John Bailer, A., Bois, Frédéric Y., Robert S. Dewoskin, Et Al., 2007. Characterizing uncertainty and variability in physiologically based pharmacokinetic models: state of the science and needs for research and implementation. Toxicol. Sci. 99 (2):395–402. <https://doi.org/10.1093/toxsci/kfm100>.
- Bessemers, Jos, George Loizou, Kannan Krishnan, Harvey Clewell, Camilla Bernasconi, Frederic Bois, Sandra Coecke, et al. 2014. "PBTK modelling platforms and parameter estimation tools to enable animal-free risk assessment. recommendations from a joint EPAA - EURL ECVAM ADME workshop." *Regul. Toxicol. Pharmacol.* 68 (1). Elsevier Inc.: 119–39. doi:<https://doi.org/10.1016/j.yrtph.2013.11.008>.
- Bessemers, Jos, Coecke, Sandra, Gouliarmou, Varvara, Whelan, Maurice, Worth, Andrew, 2015. EURL ECVAM strategy for achieving 3Rs impact in the assessment of toxicokinetics and systemic toxicity. 22. <https://doi.org/10.2788/197633>.
- Biotechnology, SOLVO, 2017. *The Transporter Book. 3rd Edition. SOLVO Biotechnology Produced and Published by.*
- Brouwer, K.L.R., Keppler, D., Hoffmaster, K.A., Bow, D.A.J., Cheng, Y., Lai, Y., Palm, J.E., Stieger, B., Evers, R., 2013. *In vitro* methods to support transporter evaluation in drug discovery and development. Clin. Pharmacol. Ther. 94 (1):95–112. <https://doi.org/10.1038/clpt.2013.81>.
- Bucher, Simon, Le Vee, Marc, Jouan, Elodie, Fardel, Olivier, 2013. Regulation of hepatic drug transporter activity and expression by organochlorine pesticides. J. Biochem. Mol. Toxicol. 28 (3).
- Casati, S., Worth, A., Amcoff, P., Whelan, M., 2013. EURL ECVAM strategy for replacement of animal testing for skin sensitisation hazard identification and classification. JRC Scientific and Policy Reports 23. <https://doi.org/10.2788/84214>.
- Chedik, Lisa, Arnaud Bruyere, Astrid Bacle, Sophie Potin, Marc Le Vée, Lisa Chedik, Arnaud Bruyere, Astrid Bacle, Sophie Potin, and Marc Le Vée. 2018a. "Interactions of pesticides with membrane drug transporters: implications for toxicokinetics and toxicity." Expert Opin. Drug Metab. Toxicol. Taylor & Francis. doi:<https://doi.org/10.1080/17425255.2018.1487398>.
- Chedik, Lisa, Arnaud Bruyere, and Olivier Fardel. 2018b. "Interactions of organophosphorus pesticides with solute carrier (SLC) drug transporters." *Xenobiotica* 0 (0). Informa UK Ltd.: 1–12. doi:<https://doi.org/10.1080/00498254.2018.1442030>.
- Cheng, Weixiao, Ng, Carla A., 2017. A permeability-limited physiologically based pharmacokinetic (PBPK) model for perfluorooctanoic acid (PFOA) in male rats. Environ. Sci. Technol. 51 (17), 9930–9939.
- Coecke, Sandra, Pelkonen, Olavi, Leite, Sofia Batista, Bernauer, Ulrike, Bessemers, Jos G.M., Bois, Frederic Y., Gundert-Remy, Ursula, Loizou, George, Testai, Emanuela, Manuel Zaldivar, José, 2013. Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. Toxicol. in Vitro 27 (5):1570–1577. <https://doi.org/10.1016/j.tiv.2012.06.012>.
- Coecke, S., Balls, M., Bowe, G., Davis, J., Gstraunthaler, G., Hartung, T., Hay, R., Merten, O.W., Price, A., Schechtman, L., Stacey, G., Stokes, W., Second ECVAM Task Force on Good Cell Culture Practice, 2005. Guidance on good cell culture practice. a report of the second ECVAM task force on good cell culture practice. Altern Lab Anim. 33 (3), 261–287.
- Comenges, J. M., Zaldivar, E., Joossens, J. V., Sala Benito, A., Worth, and A. Paini. 2017. "Theoretical and mathematical foundation of the virtual cell based assay – a review." Toxicol. in Vitro 45. The Author(s): 209–21. doi:<https://doi.org/10.1016/j.tiv.2016.07.013>.
- Corvi, Raffaella, Madia, Federica, Worth, Andrew, Whelan, Maurice, 2013. EURL ECVAM Strategy to Avoid and Reduce Animal Use in Genotoxicity Testing. <https://doi.org/10.2788/43865>.
- Daina, A., Michielin, O., Zoete, V., 2017. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep. 7, 42717. <https://doi.org/10.1038/srep42717>.
- ECHA, European Chemicals Agency, 2011. *The use of alternatives to testing on animals for the REACH regulation the use of alternatives to testing on animals for the REACH regulation.* Chemistry Vol. 117. <https://doi.org/10.2823/22471>.
- EFSA, European Food Safety Authority, 2014. Modern methodologies and tools for human hazard assessment of chemicals. EFSA J. 12 (4):3638. <https://doi.org/10.2903/j.efsa.2014.3638>.
- Ekins, Sean, 2016. The next era: deep learning in pharmaceutical research. Pharm. Res. 33 (11):2594–2603. <https://doi.org/10.1007/s11095-016-2029-7>.
- Ekins, Sean, Clark, Alex M., Wright, Stephen H., 2015. Short communication making transporter models for drug–drug interaction prediction mobile S. Drug Metab. Dispos. 43 (October), 1642–1645.
- Elbourne, L.D.H., Tetu, S., Hassan, K., Paulsen, I.T., 2017. TransportDB 2.0: a database for exploring membrane transporters in sequenced genomes from all domains of life. Nucleic Acids Res. <https://doi.org/10.1093/nar/gkw1068>.
- EMA, European Medicines Agency, 2012. *Guideline on the Investigation of Drug Interactions Discussion.*
- Epel, David, Luckenbach, Till, Stevenson, Charlotte N., Macmanus-Spencer, Laura A., Hamdoun, Amro, Smital, Tvrtko, 2008. Efflux transporters: newly appreciated roles in protection against pollutants. Environ. Sci. Technol. 42 (11), 3914–3920.

- Fardel, Olivier, Kolasa, Elise, Le Vee, Marc, 2012. Environmental chemicals as substrates, inhibitors or inducers of drug transporters: implication for toxicokinetics, toxicity and pharmacokinetics. *Expert Opin. Drug Metab. Toxicol.* 8 (1):29–46. <https://doi.org/10.1517/17425255.2012.637918>.
- FDA, U.S. Food and Drug Administration, 2012. *Guidance for industry guidance for industry drug interaction studies – study design, data analysis*.
- Fischer, Fabian C., Henneberger, Luise, König, Maria, Bittermann, Kai, Linden, Lukas, Goss, Kai Uwe, Escher, Beate I., 2017. Modeling exposure in the Tox21 in vitro bioassays. *Chem. Res. Toxicol.* 30 (5):1197–1208. <https://doi.org/10.1021/acs.chemrestox.7b00023>.
- Giacomini, Kathleen M., Huang, Shiew-Mei, Tweedie, Donald J., Benet, Leslie Z., Brouwer, Kim L.R., Chu, Xiaoyan, Dahlin, Amber, et al., 2010. Membrane transporters in drug development. *Nat. Rev. Drug Discov.* 9 (3):215–236. <https://doi.org/10.1038/nrd3028>.
- Hachad, H., Ragueneau-Majlessi, I., Levy, R.H., 2010. A useful tool for drug interaction evaluation: the University of Washington Metabolism and Transport Drug Interaction Database. *Hum. Genomics* 5 (1), 61–72.
- Hawthorne, David J., Dively, Galen P., 2011. Killing them with kindness? In-hive medications may inhibit xenobiotic efflux transporters and endanger honey bees. *PLoS One* 6 (11). <https://doi.org/10.1371/journal.pone.0026796>.
- Hillgren, Kathleen M., Keppler, Dietrich, Anik, Zur, Giacomini, Kathleen M., Stieger, Bruno, Cass Carol, E., Lei, Zhang, 2013. Emerging transporters of clinical importance: an update from the international transporter consortium. *Clin. Pharmacol. Ther.* 94 (1).
- Jigorel, Emilie, Marc L E Vee, Claire Boursier-neyret, Yannick Parmentier, and Olivier Fardel. 2006 "Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes." doi:<https://doi.org/10.1124/dmd.106.010033>.
- Kalvass, J.C., Polli, J.W., Bourdet, D.L., Feng, B., Huang, S.M., Liu, X., Smith, Q.R., Zhang, L.K., Zamek-Gliszczynski, M.J., 2013. Why clinical modulation of efflux transport at the human blood-brain barrier is unlikely: the ITC evidence-based position. *Clin. Pharmacol. Ther.* 94 (1):80–94. <https://doi.org/10.1038/clpt.2013.34>.
- Kim, Soo-Jin, Toshimoto, Kota, Yao, Yoshiaki, Yoshikado, Takashi, Sugiyama, Yuichi, 2017. Quantitative analysis of complex drug–drug interactions between repaglinide and cyclosporin A/gemfibrozil using physiologically based pharmacokinetic models with in vitro transporter/enzyme inhibition data. *J. Pharm. Sci.* 106 (9), 2715–2726.
- Klaassen, Curtis D., Aleksunes, Lauren M., 2010. Xenobiotic, bile acid, and cholesterol transporters. *Pharmacol. Rev.* 62 (1):1–96. <https://doi.org/10.1124/pr.109.002014.1>.
- Klaassen, Curtis D., Hong, Lu, 2008. Xenobiotic transporters: ascribing function from gene knockout and mutation studies. *Toxicol. Sci.* 101 (2):186–196. <https://doi.org/10.1093/toxsci/kfm214>.
- König, Jörg, Müller, Fabian, Fromm, Martin F., 2013. Transporters and drug-drug interactions : important determinants of drug disposition and effects. *Pharm. Rev.* 944–966.
- Kramer, Matthew, and Enrique Font. 2015. "Reducing sample size in experiments with animals: historical controls and related strategies" doi:<https://doi.org/10.1111/bv.12237>.
- Kusuhara, Hiroyuki, 2013. Imaging in the study of membrane transporters. *Clin. Pharmacol. Ther.* 94, 33–36.
- Leslie, Elaine M., Deeley, Roger G., Cole, Susan P.C., 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* 204 (3):216–237. <https://doi.org/10.1016/j.taap.2004.10.012>.
- Loizou, George, Spendif, Martin, Barton, Hugh A., Bessems, Jos, Bois, Frédéric Y., D'Yvoire, Michel Bouvier, Buist, Harrie, et al., 2008. Development of good modelling practice for physiologically based pharmacokinetic models for use in risk assessment: the first steps. *Regul. Toxicol. Pharmacol.* 50 (3):400–411. <https://doi.org/10.1016/j.yrtph.2008.01.011>.
- Löscher, Wolfgang, Potschka, Heidrun, 2005. Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Prog. Neurobiol.* 76 (1):22–76. <https://doi.org/10.1016/j.pneurobio.2005.04.006>.
- Luckenbach, Till, Stephan Fischer, and Armin Sturm. 2014. "Current advances on ABC drug transporters in fish." *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* 165. The Authors: 28–52. doi:<https://doi.org/10.1016/j.cbpc.2014.05.002>.
- MHLW, The Ministry of Health Labour and Welfare, 2014. *Drug Interaction Guideline for Drug Development and Labeling Recommendations* (Tentative English Version of the Draft for Public Comment). p. 450.
- Mishra, N.K., Chang, J., Zhao, P.X., 2014. Prediction of membrane transport proteins and their substrate specificities using primary sequence information. *PLoS One* 9 (6), e100278.
- Morrissey, K.M., Wen, C.C., Johns, S.J., Zhang, L., Huang, S.M., Giacomini, K.M., 2012. The UCSF-FDA TransPortal: a public drug transporter database. *Clin. Pharmacol. Ther.* 92 (5), 545–546.
- Ozawa, N., Shimizu, T., Morita, R., Yokono, Y., Ochiai, T., Munesada, K., Ohashi, A., Aida, Y., Hama, Y., Taki, K., Maeda, K., Kusuhara, H., Sugiyama, Y., 2004. Transporter database, TP-Search: a web-accessible comprehensive database for research in pharmacokinetics of drugs. *Pharm Res.* 21 (11), 2133–2134.
- Paini, Alicia, Joossens, Elisabeth, Bessems, Jos, Desalegn, A., Dorne, Jean-Lou, Gosling, J., Heringa, M., et al., 2017a. EURL ECVAM Workshop on New Generation of Physiologically-Based Kinetic Models in Risk Assessment. <https://doi.org/10.2760/035371>.
- Paini, Alicia, Jeremy A. Leonard, Tomas Kliment, Yu Mei Tan, and Andrew Worth. 2017b. "Investigating the state of physiologically based kinetic modelling practices and challenges associated with gaining regulatory acceptance of model applications." *Regul. Toxicol. Pharmacol.* 90. Elsevier Ltd.: 104–15. doi:<https://doi.org/10.1016/j.yrtph.2017.08.019>.
- Pajeva, Ilza K., Globisch, Michael Wiese Christoph, 2009. Combined pharmacophore modeling, docking, and 3D QSAR studies of ABCB1 and ABCG1 transporter inhibitors. *ChemMedChem* 4 (AA), 1883–1896.
- Prieto, Pilar, Burton, Julien, Graepel, Rabea, Price, Anna, Whelan, Maurice, Worth, Andrew, 2014. EURL ECVAM Strategy to Replace, Reduce and Refine the Use of Animals in the Assessment of Acute Mammalian Systemic Toxicity. <https://doi.org/10.2788/084219>.
- Project, Harmonization, No, Document, 2010. Characterization and Application of Physiologically Based Pharmacokinetic Models (Ipc - Who).
- Ren, Q., Chen, K., Paulsen, I.T., 2007. TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Res.* 35, D274–D279.
- Ricketts, Sally-ann, Hockings, Paul D., Waterton, John C., 2011. Non-invasive imaging in the pharmaceutical industry. *Small Anim. Imaging* <https://doi.org/10.1007/978-3-642-12945-2>.
- Riley, R. J., S. A. Foley, P. Barton, M. G. Soars, and B. Williamson. 2016. "Hepatic drug transporters: the journey so far." *Expert Opin. Drug Metab. Toxicol.* 12 (2). Taylor & Francis: 201–16. doi:<https://doi.org/10.1517/17425255.2016.1132308>.
- Saier, M.H., Reddy, V.S., Tamang, D.G., Vastermark, A., 2014. The transporter classification database. *Nucl. Acids Res.* 42, D251–D258.
- Saier, M.H., Reddy, V.S., Tsu, B.V., Ahmed, M.S., Li, C., Moreno-Hagelsieb, G., 2016. The Transporter Classification Database (TCDB): recent advances. *Nucleic Acids Res.* 44, D372–D379.
- Schuetz, John D., Swaan, Peter W., Tweedie, Donald J., 2014. Special Section on Transporters in Toxicity and Disease – The Role of Transporters in Toxicity and Disease. April. 42 pp. 541–545 doi:[dmd.113.055806](https://doi.org/10.1130.055806).
- Sedykh, Alexander, Fourches, Denis, Duan, Jianmin, Huckle, Oliver, Garneau, Michel, Zhu, Hao, Bonneau, Pierre, Tropsha, Alexander, 2013. Human intestinal transporter database: QSAR modeling and virtual profiling of drug uptake, efflux and interactions. *Pharm. Res.* 30 (4):996–1007 doi:[https://doi.org/10.1016/S0140-6736\(02\)11602-3](https://doi.org/10.1016/S0140-6736(02)11602-3).
- Association.
- Tachampa, Kittipong, Takeda, Michio, Khamdang, Suparat, Noshiro-Kofuji, Rie, Tsuda, Minoru, Jariyawat, Surawat, Endou, Hitoshi, 2008. Interactions of organic anion transporters and organic cation transporters with mycotoxins. *J. Pharm. Sci.* 106:435–443. <https://doi.org/10.1254/jphs.FP0070911>.
- Tsaioun, Katya, Blaauboer, Bas J., Hartung, Thomas, 2016. Evidence-based absorption, distribution, metabolism, excretion (ADME) and its interplay with alternative toxicity methods. *ALTEX* 33 (4):343–358. <https://doi.org/10.14573/altex.1610101>.
- US FDA, U.S. Food and Drug Administration, 2017. *In Vitro Metabolism- and Transporter-Mediated Drug-Drug Interaction Studies* (Draft) no. October.
- van Der Helm, Marinke W., Van Der Meer, Andries D., Eijkel, Jan C.T., Van, Albert, Berg, Den, Segerink, Loes I., et al., 2016. Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers* 8370 (August):1–13. <https://doi.org/10.1080/21688370.2016.1142493>.
- van der Meer, Andries, van den Berg, Albert, 2012. Organs-on-chips: breaking the in vitro impasse. *Integr. Biol.* 4 (5), 461–470.
- van Herwaarden, Antonius E., Schinkel, Alfred H., 2006. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. *Trends Pharmacol. Sci.* 27 (1):10–16. <https://doi.org/10.1016/j.tips.2005.11.007>.
- Wilks, Martin F., Tsatsakis, Aristidis M., 2014. Environmental contaminants and target organ toxicities - new insights into old problems. *Toxicol. Lett.* 230 (2):81–84. <https://doi.org/10.1016/j.toxlet.2014.08.003>.
- Worth, Andrew, Patlewicz, Grace, 2016. Integrated approaches to testing and assessment. In: Eskes, C., Whelan, M. (Eds.), *Validation of Alternative Methods for Toxicity Testing. Advances in Experimental Medicine and Biology*, pp. 317–342 no. Chapter 13.
- Yee, Sook Wah, Chen, Ligong, Giacomini, Kathleen M., 2010. Pharmacogenomics of membrane transporters: past, present and future. *Pharmacogenomics* 11 (4):475–479. <https://doi.org/10.2217/pgs.10.22>.
- You, Hwan, Lee, Kyungro, Lee, Sangwon, Hwang, Sung Bo, Kim, Kwang-Yon, Cho, Kwang-Hwi, No, Kyoung Tai, 2015. Computational classification models for predicting the interaction of compounds with hepatic organic ion importers. *Drug Metab. Pharmacokinet.* 30 (5), 347–351.
- Zamek-Gliszczynski, M.J., Ca Lee, A Poirier, J Bentz, X Chu, H Ellens, T Ishikawa, et al. 2013. "ITC recommendations for transporter kinetic parameter estimation and translational modeling of transport-mediated PK and DDIs in humans." *Clin. Pharmacol. Ther.* 94 (1): 64–79. (doi:<https://doi.org/10.1007/978-1-62703-673-3>).
- Zhang, Lei, Junfang Zhao, Chenmeizi Liang, Mingyao Liu, Feng Xu, and Xin Wang. 2017. "A novel biosensor based on intestinal 3D organoids for detecting the function of BCRP." *Drug Deliv.* 24 (1). Informa Healthcare USA, Inc: 1453–59. doi:<https://doi.org/10.1080/10717544.2017.1381199>.