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Food for Thought ... the First Ten Years

Exactly ten years ago, we started a regular series of Food for Thought … (FFT) articles in ALTEX (http://www.altex.ch/Food-for-Thought.104.html). On the scale of the journal, this series was an enormous success and contributed, together with the workshop reports and white papers by the transatlantic think tank for toxicology (t^4) (http://www.altex.ch/t^4-Workshop-Reports.105.html), to the boost in impact factor (from 1.3 in 2007 to 5.8 today). The regular reference to personal experiences and the use of personal pronouns in a scientific text is somewhat reminiscent of the blog style. The more formal appearance in a print medium moves them more toward editorials or commentaries. However, the FFTs also aim at being extremely thorough concerning the review of facts; they are typically also extensive review articles. Most importantly, they are – at least for me – fun to write and hopefully also fun to read. The series was named FFT, because I like food and hope that the reader will enjoy this intellectual food, which will necessarily be varied, some parts essential, others dull, hopefully including some unexpected flavors or even delicacies. It needs to be digested by the reader to stimulate thought, representing the analytical and creative processes the authors hope to stimulate.

In 2006, the journal ALTEX was reorganized and ALTEX Edition was formed to publish it. Joining its board, I presented the idea for the FFT series at a meeting to mark the 30th anniversary of FFVFF (now Animalfree Research, Berne, Switzerland) that year. I combined the offer with a request to change the journal from bilingual to English. The first FFT article was published in issue 2/2007. At first, we published both a German and an English version, then short German summaries until 2010.

In total, I have (co-)authored 33 FFT articles (plus the one in this issue) with 48 co-authors over ten years, 9 more by 46 authors were published without myself as an author, though often upon my invitation. They have become an attractive part of the journal, showing that there is a place for opinion in science: The articles received a total of 1046 citations (907 mine – 139 other FFT according to Google Scholar by March 6, 2017). The best cited are on the human toxome (86 citations), preclinical studies (79 citations) and integrated testing strategies (69 citations). As these articles are open access, they are now accessible via different repositories, including the ALTEX and AltWeb (http://altweb.jhsph.edu) websites but also ResearchGate (https://www.researchgate.net/profile/Thomas_Hartung). For the latter, I found recently to my surprise that of 40,000 reads my articles had received, 3,100 were for the FFT on economics of animal testing written with Antonella Bottini.

Over the years, the FFT series has covered large parts of safety sciences and related areas. Often, following the publication of an FFT on a given topic, additional materials, developments and ideas came up, but this traditional way of publishing does not allow one to go back and update easily. Often, like Denis Diderot, who from 1751 onwards published the Encyclopédie starting with letter A and very often covered materials under strange terms because the issue containing the entries with the first letter of the correct term already had been printed, I sneaked these points into later articles of the series or into other articles that were written in a similar style. In other cases, similar articles or workshops with their reports allowed us to pursue the discussion. In the following, a few major threads of articles shall be revisited.

Validation and Evidence-based Toxicology

At the time my bread and butter business at ECVAM, the first FFT on validation (doi:10.14573/altex.2007.2.67) summarized the intense discussion including some staff retreats on this topic. It shows how much this process is necessarily in flow. I owe my former team much credit for jointly shaping these ideas. Most of the questions formulated in the article are still open. It builds on our major revision of the validation process, the Modular Approach, which introduced ideas like retrospective validation, i.e., validation based on existing data, lean design of validation studies, applicability domains for *in vitro* tests, and performance standards for future variants of validated assays. An updated review was published in 2012 (doi:10.1039/c2tx20011b).

The emergence of mechanistic regulatory toxicology with the advent of the adverse outcome pathway (AOP) concept in 2011, a major implementation of the "Toxicology for the 21st century" concept (doi:10.1038/460208a), necessitated a new approach, which focuses more on the scientific validity of a test than its correlative performance to traditional methods. We coined the concept of mechanistic validation (doi:10.14573/altex.2013.2.119) to do exactly this, very much relying on the toolbox of Evidence-based Toxicology (doi:10.1093/toxsci/kfi099). The concept of systematic review of (diagnostic) methods and retrospective validation converge as laid out in another article in ALTEX, which very well could have been in the FFT series (doi:10.14573/altex.2010.4.253). The EBT Collaboration was also the starting point for another FFT-like paper on the validation of high-throughput assays (doi:10.14573/altex.2013.1.051).

The concept of Evidence-based Toxicology was prompted on our side very much around the need to improve the valida-

tion process. This met the needs of the regulatory community to carry out meta-analyses of the often-controversial evidence about a given substance. These different roots and their needs were the topic of another FFT (doi:10.14573/altex.2009.2.75). We also discussed the weighing of evidence (doi:10.14573/altex.1412231), which is very different to EBT but also must not be its opposite: it should be done where possible as a quantitative data integration using multicriteria decision analysis and Bayesian methods (www.ebtox.org). It is wonderful to see that the EBT Collaboration has established its own governance and is tackling important issues such as systematic reviews and study quality assessments in toxicology.

Shortcomings of in vivo AND in vitro approaches

It is my strong belief that we use more animals in research than necessary, because we do not openly speak about their shortcomings. Science is not good at self-criticism: Nobody writes an article or a grant application stressing the limitations of the chosen model. After we have used the model for a while, we might know better, but it is not in our apparent best interest to challenge our earlier work. It takes a lot of frustration for a researcher to turn against his tools. So, the fourth FFT actually aimed to summarize typical limitations of animal models (doi:10.14573/altex.2008.1.3). To my surprise, I could at the time not identify any such review in PubMed, at the time comprising 20 million articles. This has been a continuing theme in this series, especially in "*Look back in anger – what clinical studies tell us about preclinical work*" (doi:10.14573/altex.2013.3.275), "*Uncertainty of Testing Methods – What Do We (Want to) Know?*" (doi:10.14573/altex.2013.2.131) or the FFT in this issue (doi:10.14573/altex.1703291).

Only most recently, the creation of larger databases allows a thorough analysis for example of the reproducibility problem of animal tests. After making the database resulting from the European REACH registration program machine-readable (doi:10.14573/altex.1510052), we were able to analyze hundreds and thousands of chemicals, which were tested more than once in the same guideline tests (doi:10.14573/altex.1510053; 10.14573/altex.1510054; 10.14573/altex.1510055). I was blown away to see that two chemicals were tested more than 90 times in rabbit eyes and 69 were tested more than 45 times! This waste of animals, however, allows an objective assessment of reproducibility. And the results back what the smaller analyses of the past suggested, i.e., that the reproducibility of guideline studies (highly standardized with quality assurance!) is only 70-90%. This does not even mean that the tests are 70-90% correct in predicting human or environmental hazards, but they certainly cannot be better than this. This shows that too often the expectations of what an alternative method can achieve when validating against animal data, were unrealistic. We cannot design a test that performs better than the reference data we are forced to compare it to. Prompted by these results we organized a workshop on the topic in 2016 and are currently working toward a reference document that compiles what is known about the reproducibility of standard tests.

This objective criticism of animal tests is not animal welfare advocacy in disguise. In fact, it is only credible if we apply similar measures to the alternative approaches. Already the second FFT addressed the shortcomings of cell culture work (doi:10.14573/altex.2007.3.143). It was based on, at the time, ten years work toward Good Cell Culture Practice (GCCP) and the experiences of validating *in vitro* tests in ECVAM. These limitations have been regularly addressed in this series (doi:10.14573/altex.2012.3.251; 10.14573/altex.2013.3.275). We also reflected on the need for complexity in our cell-based assays (doi:10.14573/altex.2012.4.359). With the advent of more organotypic cultures, we felt the need to reactivate the GCCP Collaboration, now expanding it to consider stem cell-derived and organotypic models (doi:10.14573/altex.1607121).

Toxicology for the 21st century

While originally perceived as the concept of the National Research Council Report *Toxicity Testing for the 21st Century: a vision and a strategy*, "Toxicology for the 21st century" (Tox-21c) today comes in many flavors: Most prominently, this is represented by US EPA's ToxCast and the multi-agency Tox21c alliance, which pursue high-throughput screening (HTS). Astonishingly, this has never been the main topic of a dedicated FFT, but the pioneering program has been referenced consistently. The panorama of the (r)evolutionary change going on, was captured in an FFT with Marcel Leist on "*the evolution of toxicology and the phasing out of animal testing*" (doi:10.14573/altex.2008.2.91) and a companion article in the same issue including Pierluigi Nicotera (doi:10.14573/altex.2008.2.103).

A number of FFT articles were written in the context of Tox-21c. The invited "*Can case study approaches speed implementation of the NRC report: 'Toxicity Testing in the 21st Century: A Vision and a Strategy?*"" (doi:10.14573/altex.2011.3.175) included as lead authors Mel Andersen and Kim Boekelheide, two of the opinion leaders of the 2007 NRC report. Our own reflection "*Food for Thought ... on mapping the human toxome*" (doi:10.14573/altex.2011.2.083) laid the foundation for the Human Toxome Project and a workshop. We also developed a vision toward systems toxicology (doi:10.14573/altex.2012.2.119). To some extent, the FFT series, complemented with our workshop reports, reflects the conceptual discussion on the road to Tox-21c, an important addition to the technical developments. The most recent FFT, "*The need for strategic development of safety sciences*", which happened to be my 500th scientific article, aimed to bring this discussion to the next level, i.e., the systematic integration and completion of the puzzle pieces to actually impact on regulatory practice (doi:10.14573/altex.1701031).

Technologies and approaches

Certain technologies have matured in recent years and are impacting increasingly on the safety sciences, representing an important pillar of Tox-21c. This started off in 3'09 with an FFT with Sebastian Hoffmann on *in silico* approaches (doi:10.14573/altex.2009.3.155), taking a quite critical stand to (Q)SAR. More recently, we initiated a program on read-across, starting with an FFT article (doi:10.14573/altex.1410071), leading to two from my point of view important documents (doi:10.14573/altex.1601251; 10.14573/altex.1601252) toward Good Read-Across Practice and, together with our in-house work, changing my view on what *in silico* can contribute to risk assessments (doi:10.14573/altex.1603091).

Another approach, the frontloading of toxicity measurements, i.e., Green Toxicology, was prompted by Nick Anastas, US EPA, one of the founding fathers of Green Chemistry. The idea is simple: Alternative *in silico* and *in vitro* tools can be applied earlier in the product development process, in principle even before synthesizing a substance, to make *in silico* predictions that help to avoid toxic substances in products and difficult decisions to abandon a substance after a lot of investment. The FFT article (doi:10.14573/altex.1406181) was our way of "wrapping our mind around the topic". Since then, half a dozen conference symposia and information events were held in the US and Europe.

One key approach, often discussed over the last 15 years but rarely tackled systematically, is the integrated testing strategy, i.e., the integration of different information sources such as *in vitro* and *in silico* tools, but not excluding *in vivo*, epidemiological or existing data. Our FFT (doi:10.14573/altex.2013.1.003) summarized the state of the art and stimulated a workshop (doi:10.14573/altex.1506201) and several symposia. The topic has now been taken up by OECD, coining the new term IATA (Integrated Approaches to Testing and Assessment), combining it with their AOP activities, and leading to a recent guidance document (http://bit.ly/2iTaZcz).

Views on different industrial sectors and their challenges and opportunities

While many concepts generally apply to the safety sciences, different industrial sectors have their own challenges because of the individual product categories, development processes and regulations. The start was made for the cosmetic sector, which was dramatically impacted by the 7th amendment in 2002, banning animal testing for cosmetic products and their ingredients with some deadlines (doi:10.14573/altex.2008.3.147). I have often referred to it as my welcome present to ECVAM as the political agreement was reached two months after I started there. This urged an important industrial sector to ultimately become a central engine of progress. Given the very small number of animals used in this sector at the time, singling out this industry might not have been fair, but we are blessed to have it as a driving force. Sure, the alternative methods were not fully available in 2013 when the last deadline hit (doi:10.14573/altex.2010.4.253), but it also prompted a very important road-mapping exercise (doi:10.14573/altex.2012.1.003; 10.14573/altex.1406091), which showed that most requested change is within reach. The FFT on cosmetics was a summary of a type of business plan developed in ECVAM with my team on how to tackle the ban. It represents our rather optimistic view with a very full pipeline in 2007/2008. The enormous budget cuts (I estimate about 80% reduction), which led to my change in responsibility and continued after, did not allow to set many of these things into practice, but still the pipeline has delivered until today, including a number of new and revised OECD test guidelines (Tab. 1), mostly but not only for cosmetics and their ingredients.

This can be seen as a glass half full or a glass half empty. On the one hand, it is considerable output of a short phase of work and its continuation by my successors. Please note that the work on endocrine disruptors occurred not by validation studies, but mainly on an OECD level, within ReProTect or steered by ICCVAM, but on all levels with ECVAM's input and help. On the other hand, there is still very little to offer for the systemic toxicities and the mentioned roadmap exercise awaits implementation. With more animal testing bans for cosmetics world-wide, a revised FFT might be timely.

The second FFT on an industrial sector was on food (doi:10.14573/altex.2008.4.259), teaming up with Herman Koeter, at the time acting director of EFSA. The FFT series addressed over the years a number of further industrial sectors: Industrial chemicals (doi:10.14573/altex.2010.1.3), nanomaterials (doi:10.14573/altex.2010.2.87), pharmaceuticals (doi:10.14573/altex.1506201), including countermeasures to biological and chemical terrorism and warfare (doi:10.14573/altex.2012.3.251), and most recently e-cigarettes (doi:10.14573/altex.1606291). The latter, was a very interesting case study showing the dynamics of safety sciences: In only one decade a new product category formed with enormous turn-over but also challenges for safety assessments, especially the most probably now close to 10,000 flavoring substances employed that have never been properly tested for inhalation toxicology. This urges the development and use of alternative approaches.

The series also gave economical aspects in general quite a bit of consideration with two FFT with Antonella Bottini, the first also with Patrick Amcoff, at the time at OECD (doi:10.14573/altex.2007.4.255; 10.14573/altex.2009.1.3). Similarly, our analysis with Costanza Rovida of REACH as to costs and animal numbers had enormous resonance (doi:10.14573/altex.2009.3.187; 10.1038/4601080a; 10.14573/altex.2010.3.175). More recently, we analyzed the animal use for

Tab. 1: ECVAM validations initiated and contributed to from 2002-2008 and resulting OECD test guidelines until 2017

Area	Test	ECVAM validity statement	OECD TG
Kinetics	P450 induction	Ongoing	–
Eye Irritation	Bovine Corneal Opacity and Permeability (BCOP)	2007	TG 437, 2009, revised 2013
	Isolated Chicken Eye (ICE)	2007	TG 438, 2009, revised 2013
	Cytosensor Microphysiometer (CM)	2009	–
	Fluorescein Leakage (FL)	2009	TG 460, 2012
	Reconstructed human Tissue (RhT)-based test methods: "SkinEthic Human Corneal Epithelium" and "EpiOcular Eye Irritation Test"	2014	TG 492, 2015
	Low Volume Eye Test (LVET)	2009	TG 491, 2015
Skin Corrosion	SkinEthic™ EpiCS® (EST-1000) (me-too validations to the 1998 and 200 validations of EpiSkin and EpiDerm)	2006, 2009	TG 431, 2004, revised 2014
Skin Irritation	EpiSkin™, EpiDerm™SIT (EPI-200), Modified EpiDerm (EPI-200) and SkinEthic™ reconstructed human epidermis	2007, 2009, 2008, 2009	TG 439, 2010, revised 2013
Skin Sensitization	Reduced Local Lymph Node Assay and non-radioactive variant	2007, – (US only)	TG 442 a & b, 2010
	Direct Peptide Reactivity Assay (DPRA)	2012	TG 442 c, 2015
	KeratinoSens™	2014	TG 442 d, 2015
	Human Cell Line Activation Test (h-CLAT)	2015	TG 442 e, 2016
	U937 Skin Sensitization Test (U-SENS™)	2016	draft TG, 2016
Acute Toxicity	Colony-Forming Unit Granulocyte/Macrophage (CFU-GM) Test	2006	–
	3T3 Neutralred Uptake Cytotoxicity Test	2011	Guidance Document 129, 2010
Carcinogenicity	Cell Transformation Assay	2012	TG 214, 2015
Genotoxicity	Micronucleus Test	2006	TG 487, 2010
	Comet *in vivo* assay	– (Japan)	TG 489, 2014
(Sub-)Chronic Toxicity	One-Year Dog Study obsolete for pesticides	2006	n/a
Endocrine Disruptors	Stably Transfected Human Oestrogen Rezeptor-α Transcriptional Activation Assay for the Detection of Oestrogenic Agonist Activity of Chemicals	– (OECD, US EPA)	TG 455, 2009, updated 2015, 2016
	H295R Steroidogenesis Assay	– (OECD, US EPA)	TG 456, 2011
	BG1Luc Estrogen Receptor Transactivation *in vitro* Assay to Detect Estrogen Receptor Agonists and Antagonists	– (USA, 2012)	TG 457, 2012
	Transcriptional Assay for the Detection of Estrogenic and Anti-Estrogenic Compounds using MELN Cells	– (validation stopped 2009 after consultation with test submitter)	ongoing
Acute Fish Toxicity	Upper threshold concentration step-down approach	2006	TG 203, Guidance 126, 2010
	Zebrafish Embryotoxicity Test	2014	TG 236, 2013
Pyrogenicity	Monocyte Activation Tests	2006	n/a

science in Europe (doi:10.14573/altex.1509081). Given the enormous interest in these economic aspects, also discussed above, this type of consideration should certainly be updated and expanded. The most important –omics is economics!

The other topics – not less important but not easy to group

CAAT, under the leadership of Alan Goldberg, teamed up with me, at the time heading ECVAM, to address developmental neurotoxicity (DNT) in a series of workshops. An FFT article (doi:10.14573/altex.1403271) summarized the state of the art. A CAAT workshop in 2011 developed a list of DNT reference compounds. The most recent DNT workshop report was just published (doi:10.14573/altex.1604201). Earlier this year, another DNT workshop was organized by CAAT-Europe in Konstanz, Germany, in this case reconvening the International STakeholder NETwork (ISTNET), a group representing the regulatory-focused DNT community (doi:10.1007/s00204-015-1464-2). This process has formed a DNT *in vitro* community and systematically contributed to joint research and the prioritization of models, endpoints and reference chemicals for this field for which there is a strong need to develop fast, robust and predictive alternatives. A major step forward was made in November 2016, when the OECD hosted a meeting including major parts of this community and agreed on the need to develop a testing strategy for DNT. Current in-house work develops around the concept of cellular resilience, which we again developed in an FFT article (doi:10.14573/altex.1509271). This is a rare example of long-term joint cooperation between stakeholders developing a roadmap toward an alternative testing strategy.

Another topic, which has shaped parts of my career is pyrogenicity testing. An FFT (doi:10.14573/altex.1503241) summarized the lessons learned in 20 years, which were mainly how long it takes for obvious solutions to become implemented. The assay was described in 1996, validated in 2003, received validity statements in 2006 and formal acceptance by European Pharmacopoeia and FDA by 2009-2010, but is still hardly used. This is despite the enormous number of rabbits still used, i.e., 15 times more than rabbit use for chemical eye and skin testing in Europe over the last decade. It is quite incredible how long it takes to get such a test implemented, but now, after more than 20 years, there is some movement, with European Pharmacopoeia making such Monocyte Activation Tests mandatory, acceptance by US pharmacopoeia and ISO, and some prospects for validation for medical devices. A sideline of the whole blood pyrogen test over the years was its use as an immunotoxicity test, which prompted interest in the topic summarized as an FFT with Emanuela Corsini (doi:10.14573/altex.2013.4.411).

Last but not least, as an academic institution we addressed, together with Marcel Leist and Bas Blaauboer, representing the three endowed Doerenkamp-Zbinden professors with a toxicological focus, education in alternative methods in toxicology (doi:10.14573/altex.2009.4.255). This was also followed by a t[4] workshop (doi:10.14573/altex.2011.4.341). As a consequence, we made the existing CAAT online courses of Johns Hopkins available for free: CAAT's Academic Programs educate students and professionals in the research field about alternatives and humane science, helping them gain a better understanding of the 3Rs and their role in improving the quality of science. CAAT has established a certified program in humane sciences and toxicology policy in Johns Hopkins School of Public Health, and any individual who completes the curriculum can be awarded this certificate. The Humane Science and Toxicology Certificate Program is central to CAAT's academic mission, with a curriculum consisting of six courses, offered both in the classroom and online through the Johns Hopkins Bloomberg School of Public Health (http://caat.jhsph.edu/programs/academics.html). CAAT has brought the certificated curriculum online, and the full program is thus available worldwide for the new Master of Science and Public Health (MSPH) in Toxicology.

CAAT is contributing with two online lecture courses in "*Toxicology for the 21st Century: Scientific Applications*" and "*Evidence-based Toxicology*". These two new courses have been recorded and the Tox21c course started in January and the EBT course in March 2017. Both courses are conducted online and each consists of 13 90-minute lectures, two assignments, and two exams. It is planned also to make them available online (uncredited).

The FFT series is thriving. As always, a few future texts are already in the making, such as ones on the implementation of the 2016 US chemical legislation, micro-physiological systems, probabilistic risk assessment, etc. I am excited that the format is finding more interest. This is evidenced by the FFTs contributed by other authors but also by the numerous invitations to write further articles that are sparked by FFTs.

I had often hoped that the FFT format would prompt more discussions, but we have not really received many replies other than some personal emails, and these contain praise rather than counter-arguments. Apparently, we need other formats such as invited Pro/Con discussions to achieve that.

The problem of keeping the FFTs updated has not been solved. Publishing an addendum after a certain number of years could be an option, but perhaps we just have to take them for what

they are – a reflection of the state of thoughts at a moment in time. When too much has changed or other authors can bring in new views, it will be time to address the same topics again.

Finally, I would like to thank my teachers, colleagues, the ALTEX team, staff, co-authors, friends and my families – ten years of FFT would not have been possible without them.

Conflict of interest

TH is founder of Organome LLC, Baltimore, share-holder and chief scientific advisor of Atheralabs, Luxembourg, and consults AstraZeneca, Cambridge, UK, all in the field of organotypic cultures. He holds patents as inventor of the whole blood pyrogen test and the use of cryopreserved blood, which are licensed to Merck-Millipore; he receives royalties from Merck-Millipore from sales of the kit version. He is also a member of Apple's Green Chemistry Advisory Board and consults Underwriters Laboratories (UL) on computational toxicology, especially read-across, and has a share of their respective sales.

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