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# Innovative replacement methods at the Istituto Superiore di Sanità in the spirit of the 3Rs principle

Edited by
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FORMAZIONE
E INFORMAZIONE

# ISTITUTO SUPERIORE DI SANITÀ

# Innovative replacement methods at the Istituto Superiore di Sanità in the spirit of the 3Rs principle

Edited by
Isabella De Angelis (a), Laura Ricceri (b), Augusto Vitale (b)

*(a) Department of Environment and Health*
*(b) Reference Centre for Behavioural Sciences and Mental Health*

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2022, 82 p. Rapporti ISTISAN 22/18

The 3Rs principle (Replacement, Reduction, Refinement) was proposed by the end of the 1950s by two British academics, William Russell and Rex Burch. The principle focuses on the idea of a necessary but aware use of animal models. This idea must be put into practice by development of experimental procedures open to technological innovation, with the aim of constantly promoting a balance between scientific progress and animal welfare. Replacement proposes use of non-animal experimental models, or use of animals considered as non-sentient (in line with current legislation), or again use of *in silico* models. The present volume is a sort of a snapshot of research activities carried out at the Istituto Superiore di Sanità (ISS, the National Institute of Health in Italy), which imply, with different aims, replacement methods. Research described here illustrates the efforts by ISS personnel to devise innovative methodological approaches and experimental models, especially in biomedical research and regulatory studies, in tune with the principle of the 3Rs.

*Key words*: Non-animal experimental models; *In vitro* models; *In silico* models; New advanced methodologies

Istituto Superiore di Sanità
**Metodi innovativi di sostituzione applicati in Istituto Superiore di Sanità in accordo con il principio delle 3R.**
A cura di Isabella De Angelis, Laura Ricceri, Augusto Vitale
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Il principio delle 3R (*Replacement, Reduction, Refinement)* fu formulato alla fine degli anni ’50 del secolo scorso da due accademici britannici William Russel e Rex Burch. Esso contestualizza l’idea di un utilizzo consapevole e necessario del modello animale attraverso la messa a punto di prospettive sperimentali aperte all’innovazione metodologica e attente a stabilire un equilibrio tra conoscenza scientifica e rispetto per gli animali. La R di *Replacement* (Sostituzione) prevede l’utilizzo di modelli sperimentali non animali o di modelli animali considerati non senzienti secondo la normativa vigente o, infine, di modelli computazionali. Il rapporto rappresenta una fotografia, sia pur parziale, delle attività presso l’Istituto Superiore di Sanità (ISS) che promuovono e realizzano metodi di Sostituzione con finalità diverse. Nel complesso le attività descritte testimoniano l’interesse dei ricercatori ISS alla messa a punto di metodi e modelli innovativi, soprattutto nella ricerca biomedica e negli studi di tipo regolatorio, nel rispetto del principio delle 3R.

*Parole chiave*: Modelli sperimentali non animali; Sistemi *in vitro*; Sistemi *in silico*; Metodologie innovative

Per informazioni su questo documento scrivere a: isabella.deangelis@iss.it; laura.ricceri@iss.it; augusto.vitale@iss.it

Il rapporto è accessibile online dal sito di questo Istituto: www.iss.it.

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viale Regina Elena, 299 – 00161 Roma

*All models are wrong, but some are useful*

George Box, 1976

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# REPLACEMENT IN THE SPIRIT OF THE 3RS PRINCIPLE

Augusto Vitale
*Reference Centre for Behavioural Sciences and Mental Health, Istituto Superiore di Sanità, Rome, Italy*

In 1959 two British academics, Rex Burch e William Russell, published a book destined to become a milestone in the field animal experimentation: "The principles of humane experimental technique" (Russell & Burch, 1959). Burch and Russell were inspired and guided by Charles Hume's vision. Hume was at the time the President of the University Federation of Animal Welfare (UFAW) (a very active association in the field of animal welfare to this day), and was aware of a gap existing between scientific laboratory techniques and humanist values. Those humanist values were going to be instrumental in changing the attitudes towards experimental animals, considering these something more than just mere laboratory objects. Therefore, to bridge the gap, Hume thought it was necessary to introduce a new understanding of the experimental subjects, away from a strictly instrumental framework. The animals had to be intended as able to experience negative mental states, and a new methodological approach to laboratory techniques should have aimed at turning these negative states in at least neutral, if not positive ones.

As we all know now, this new methodological approach resulted in the introduction of the "3Rs principle", that is, "Replacement, "Reduction" and "Refinement". Therefore, when a researcher approaches a study utilising an animal model, he/she has to enquire firstly whether an alternative model to the use of a sentient animals can be used, then to consider whether to reduce the number of individuals utilised in his/her protocol, and finally to adopt all of the possible measures to avoid or minimise sufferance and pain. As said before, the final aim would be to alleviate, if not eliminate the negative mental states experienced by animals in research laboratories.

Replacement is the topic of the present report. In this contribution I will focus on some theoretical considerations concerning this particular concept.

## Replacement as the first "R"

How Russell and Burch defined Replacement? They offered the following definition:

> "We shall use the term 'replacement technique' for any scientific method employing non-sentient material which may in the history of experimentation replace methods which use conscious living vertebrates. Among this non-sentient material, we include higher plants, microorganisms, and the more degenerate metazoan endoparasites, in which nervous and sensory systems are almost atrophied" (Russell & Burch, 1959 p. 69).

Replacement is the first "R" to be introduced by the two authors. As a matter of fact, nor Reduction, neither Refinement would be necessary where full Replacement is in place. It is also the most popular "R", because it is close to the point of view of animal rights movements, and is the easiest to communicate: as Olsson and colleagues pointed out, "not tested on animals" purveys a more convincing message than "tested on fewer aninals, but treated well" (Olsson *et al.*, 2012).

The "R" of Replacement is also the most controversial one, and can be potentially exploited by opposite camps in the debate on animal experiments. On one hand, the "3Rs principle" is sometimes considered as not working and being obsolete, because animals are still used in

research laboratories, and therefore no effective Replacement has been achieved; on the other, some researchers still consider the principle impossible to put into practice because "I still need my animal model". In both cases it is worth to cite Russell and Burch in their book:

> "Desirable as replacement is, it would be a mistake to put all our humanitarian eggs in this basket alone. The progress of replacement is gradual, not is it ever likely to absorb the whole of experimental biology" (p. 105).

This sentence could have been written today and it is a further proof of the current relevance of the principle. The message here is not to consider Replacement as detached from the other two "Rs", and to understand the principle as a unitary concept. However, Replacement generally has a prominent profile in EU Commission funding politics: although applicants are required to take the principle of the 3Rs in full consideration, is the "R" of Replacement that receives more specific funding.

The three "Rs" could give the impression to focus on two different ethical perspectives: Replacement focuses on no use, whereas Reduction and Refinement say that it is admissible to use animals, but under certain conditions. It is my opinion that this is not the way to correctly interpret the Russell and Burch's principle. The two authors did not want to write a book on animal ethics, but a methodological one. The question whether the use of animals in research laboratory is morally legitimate, and therefore if they be used or not, belongs to the "animal rights" line of arguments. The principle fully belongs to the "animal welfare" line of arguments. It is within this last perspective that the principle has to be taken into consideration and discussed and, eventually, challenged.

# Partial Replacement?

What is "Partial Replacement"? Russell and Burch distinguish between "Relative Replacement" and "Absolute Replacement". For them "Relative Replacement" is when animals are still used, but not subjected to sufferance or distress, whereas in "Absolute Replacement" animals are not used at all. It is important to notice that, in its original meaning, Replacement is not indicated by the two authors as the elimination of the use of animals in experiments, but by the elimination of distress. In the case of "Absolute Replacement", no inhumanity is implied, simply because no animals are used. However, the use, for example, of complete anesthetized animals is indicated as a case of "Relative Replacement", provided the anesthesia is deep and running parallel to the course of the experiment.

In more recent times, Replacement has been defined in different ways: not using animals, or not using vertebrates, or using animals intended to be not sentient, or less sentient. For example, the "Guidelines for the care and use of mammals in neuroscience and behavioral research" of the Institute of Laboratory Animal Research defines Replacement as "use of non-animal systems or less-sentient animal species to partially or fully replace animals" (p. 10) (ILAR, 2003). One of the problems of such definition is the use of the term "less sentient". It could mean that the members of a particular species could be less capable to experience pain and/or distress than other species. However, the problem of how the define "sentience" in relation to, for example, behavioural biology is far from being solved (Vallortigara, 2017; *see also* Pollo & Vitale, 2019).

The NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research), based in London, defines Partial Replacement (equivalent to the original Relative Replacement used by Russell and Burch) as the use of some animals that, based on current scientific thinking, are not considered capable of experiencing suffering. This includes

invertebrates such as *Drosophila*, nematode worms and social amoebae, and immature forms of vertebrates. Partial Replacement also includes the use of primary cells (and tissues) taken from animals killed solely for this purpose (i.e., not having been used in a scientific procedure that causes suffering (https://www.nc3rs.org.uk/the-3rs).

This means that the use of animals who are "less sentient" than the original ones is not considered in this case a form of Partial Replacement (e.g., a mouse instead of a monkey, or a fish instead of a mouse). The comparison of level of potential sufferance experienced by different species is also problematic in interpretative terms. We are still away from really understanding what sufferance means for different species (Dawkins, 2008; Borgi *et al.*, 2021).

The assumption is that more "complex" animals should be able to experience level of sufferance similar to humans. That would be in connection to their level of "sentience", which would be linked with a greater ability to remember and anticipate pain and distress, among other things. Is this really so? Do we really have robust data to support this assumption? This point of view is more in tune with what has been defined the "socio-zoological scale" (Arluke & Sanders 1996; see also Olsson *et al.*, 2012). It refers to what most of us, humans, perceive about the sufference of other species, rather than biological perspective. On top of the scale we put great apes, at the bottom invertebrates. This scale surely overlaps in some points with data collected by animal welfare scientists and cognitive scientists (see, for example, Mendl & Paul, 2004), but more research is still needed to better focus this scale on biological and evolutionary reality.

# Replacement in the legislation

What are the references to Replacement and Partial Replacement in the European Directive 2010/63/EU, on the protection of animals used in scientific procedures? The principle is explicitly referred to in the normative text. For example, article 4 states:

> "Principle of replacement, reduction and refinement 1. Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure. 2. Member States shall ensure that the number of animals used in projects is reduced to a minimum without compromising the objectives of the project. 3. Member States shall ensure refinement of breeding, accommodation and care, and of methods used in procedures, eliminating or reducing to the minimum".

Furthermore, it is required from scientists, when deciding upon which procedure to use, to choose those that "involve animals with the lowest capacity to experience pain, suffering, and distress or lasting harm" (article 13.2) (Europe, 2010).

The Italian normative text on this regard, which is the application of the EU Directive into a national normative context, confirms this legal requirement (see, for example, article 13) (Italia, 2014). For what concerns Replacement, the article recommends to use procedures which implies the use of animals that show less capacity to experience pain, sufferance, distress or prolonged damage. Therefore, also here the normative wording does not seem to be in the spirit of the original idea by Russell and Burch, and points to the use of animals with less capacity to experience sufferance, a concept we saw before encountering some conceptual difficulties.

However, having said that, it is also expected that the understanding and application of the principle would change with the passing of time, from its original formulation, allowing for some new interpretations. The principle must also be understood as reflecting the state-of-the-art of animal welfare science.

# Conclusions

The Istituto Superiore di Sanità has embraced the spirit of the "3Rs principle" when it comes to Replacement techniques, and the contributions presented in this volume are a demonstration of this attitude. There are two ways in which Replacement techniques can be thought and implemented: one-way is to pursue studies to improve *in vitro* and/or *in silico* methodologies, without reference to a particular experimental protocol; the other strategy is to device an alternative method with reference to a specific protocol or specific scientific question. The term "innovation" is crucial here. It represents a dynamic approach to experimental procedures, where old ways are revisited in the spirit of Replacement, or new methodologies that do not entail the use of animals are devised anew. However, most importantly, innovation in experimental procedures reflects the contemporary flavour that the "3Rs principle" continues to have since the year of its publication, 60 years ago (*see* also De Angelis *et al.*, 2019).

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# NON-TESTING METHODS TOWARDS REPLACEMENT WITHIN 3RS PRINCIPLES

Chiara Laura Battistelli, Cecilia Bossa, Olga Tcheremenskaia*
*Department of Environment and Health, Istituto Superiore di Sanità, Rome, Italy*

## Introduction

In compliance with 3Rs principles (Replacement, Reduction, Refinement), alternatives to animal testing have achieved high interest in prevention strategies and regulatory frameworks.

Recently, the term New Approach Methodologies (NAM) has been adopted, which collectively refers to alternative test methods and strategies that can be used to provide information on chemical hazard and risk assessment while avoiding the use of intact animals. Although there is no harmonized definition of NAMs, these new approaches broadly include any technology, methodology, approach, including computational/*in silico* models – i.e., (Quantitative) Structure–Activity Relationships, (Q)SAR –, or combination thereof to reduce, refine or replace vertebrate animals (OECD, 2020).

Among NAMs, computational approaches, referred as *in silico* methods, are useful tools that can significantly contribute to reducing the use of laboratory animals and make fast predictions for a large set of compounds in the assessment of chemicals. *In silico* approaches include the use of (Q)SAR, which provide a correlation between the chemical structure and the biological activity. In the last years, *in silico* methods have been subjected to extensive refinements and fine-tuning, including continued curation of training set data by the developers (Amberg *et al.*, 2016; Benigni *et al.*, 2019; Honma *et al.*, 2019).

Modern approaches to toxicity testing rely on NAMs, incorporating batteries of assays, *in silico* approaches, and computational models, rather than a single alternative method. Non testing methods, together with *in chemico*, *in vitro*, *ex vivo*, *in vivo* or omics technologies, are integrated into a structured form, thorough the formalization of Integrated Approaches to Testing and Assessment (IATA) and defined approaches for data interpretation.

Progress has been made in capturing the toxic mode of action information in Adverse Outcome Pathways (AOP) frameworks. Further collection and organisation of the current knowledge on mechanisms leading to toxicity open future perspectives for creation of AOP-informed IATA schemes (Sakuratani *et al.*, 2018). Other areas of research and progress are efforts on collection and integration of the existing toxicity protocols, best practices for toxicity assessment and guidelines providing a practical advices on the uncertainties assessment (Benford *et al.*, 2018) and on weight of evidence approach (Hardy *et al.*, 2017).

This paper presents a comprehensive review of selected alternative test methods and strategies – such as grouping, Read-Across, (Q)SAR, AOP, IATA and Defined approaches (DA) –, which can be used to assess the potential toxicity of chemical substances. The need of high-quality datasets, the integration of evidence, and evaluation of uncertainties are also addressed. Finally, aspects related to the regulatory acceptance of non-testing methods, with a special focus on genotoxicity and carcinogenicity endpoints and areas of possible improvements in computational toxicology, are pointed out.

* The authors equally contributed to the manuscript

The computational toxicology group at the Istituto Superiore di Sanità (ISS, the National Instiute of Health in Italy) has more than twenty years of experience in development and evaluation of non-testing methods as replacement of animal testing. Our most relevant activities include development of curated toxicological databases and QSAR expert systems for prediction of various genotoxicity and carcinogenicity endpoints, participation in different international projects on standardisation of chemical safety data, including nanomaterials, coordination of recent (Q)SAR assessment framework project of the OECD (Organization for Economic Co-operation and Development), participation in numerous OECD expert groups (related to AOP, IATA, (Q)SAR, good computational data practices), participation in evaluation of *in silico* methods in regulatory contests – European Chemicals Agency (ECHA) and European Food Safety Authority (EFSA) – (Benigni *et al.*, 2019; Benigni *et al.*, 2013; Jeliazkova *et al.*, 2021).

# Selected non-testing methods: relevant concepts

## Grouping and Read-Across

The chemical grouping is an approach "considering more than one chemical at the same time", for filling data gap for (eco)toxicity endpoints, and includes analogue and category approaches. The analogue approach provides the prediction of experimental data of one chemical, from one (or few) similar chemicals. In the category approach, chemicals – whose physicochemical, (eco)toxicological and/or environmental fate properties are likely to be similar or follow a regular pattern, usually owing to structural similarity – are grouped together in a category. In this approach, the data gaps can be filled for different category members and different endpoints.

Within the grouping approach, data gap filling can be carried out by Read-Across, trend analysis, or (Q)SAR. In the Read-Across method, relevant information from one or more analogous (source) chemicals is used to predict the properties of a query compound(s) (target). The similarity between target and analogue(s) has to be intended in a broad sense, such as presence of common functional group, structural similarity, common chemical class, similar values of physical chemical parameters, common precursors and/or breakdown products. A quantitative trend in the experimental data for a given endpoint across chemicals in a category can also allow for quantitative interpolation or extrapolation (trend analysis).

Although the literature is rich in proposals for general workflows and criteria, Read-Across strategies can vary very much on a case-by-case basis, to reflect differences in data availability, types of chemicals, mechanisms of toxic action, as well as regulatory requirements.

## (Q)SAR

(Q)SARs are mathematical models providing a correlation between compounds chemical structure and a property or activity (such as physicochemical, biological, or environmental fate). These models can be used to predict the qualitative or quantitative properties of untested chemicals, based on structurally related compounds with known activity.

There are two main types of (Q)SAR models: rule-based and statistics-based. The rule-based models are approaches defining characteristic substructures in chemicals with positive results (called Structural Alerts, SA), for already known data. Toxicity tests results are predicted qualitatively, using the empirical rules supported by mechanistic information. If a chemical does not include any SA, this does not always indicate an absence of concern, instead it can point to a lack of knowledge on the structural determinants of toxicity. Statistics-based (Q)SARs are models

based on the correlation of the physicochemical properties expressed in terms of molecular descriptors such as geometric, electronic, physicochemical parameters, with positive test results. The test results are predicted without a mechanistic knowledge or external rule, but using algorithms derived from the training set of chemicals; negative predictions are generally more accurate.

Various tools and platforms, both in the public domain and commercial software, can be used to predict different (eco)toxicological endpoints. A non-exhaustive list of the most popular systems with their characteristics can be found in literature (Bossa *et al.*, 2018; Tcheremenskaia *et al.*, 2019). Among the tools freely available in the public domain, one of the most used software for regulatory purpose is the OECD (Q)SAR Toolbox (https://QSARtoolbox.org/), developed and maintained by Laboratory of Mathematical Chemistry (Bourgas, Bulgaria), under the coordination of the OECD and the ECHA. It is intended to be used by governments, chemical industry, and other stakeholders in filling gaps of (eco)toxicity data for assessing the hazards of chemicals. Designed to facilitate application of (Q)SAR approaches within regulatory frameworks, the software incorporates information and tools from many different sources into a logical workflow, which are crucial for Read-Across and grouping of chemicals.

### OECD (Q)SAR Assessment Framework Project

Scientific validity of the (Q)SAR models and the generated predictions should be evaluated, e.g., by applying the OECD principles of validation of (Q)SARs for regulatory purposes (OECD, 2007). An informed decision on the adequacy of the model and its applicability in the context considered is one of the most important issues in judging the acceptability of the prediction.

In March 2021 the (Q)SAR Assessment Framework Project, led by Italy-ISS, namely by the authors of this review, has been launched by the OECD. The expert group includes more than 30 experts from different OECD Member countries. The aim of the project is to develop a systematic and harmonised assessment framework for (Q)SAR, by providing a consistent means of addressing the uncertainty/confidence in a (Q)SAR predictions. The assessment framework will be applicable irrespective of the modelling technique used to build the model (battery), and irrespective of the endpoint and intended regulatory application, recognising the principle that the required level of confidence is context-dependent and should be established by the regulatory assessor. The expected duration of the project is 24 months.

## AOP and IATA

The AOP framework was originally developed to support the use of NAMs in chemical safety evaluation and to facilitate their regulatory acceptance.

AOPs are mechanistic-based tools in toxicology with broad potential, which provide a clear representation of critical toxicological effects over different layers of biological organization. AOPs describe a sequence of events starting from an initial interaction of a stressor and a biomolecule within an organism. This event causes some biological perturbation in its biology and calls Molecular Initiating Event (MIE). The rest of a AOP progresses through a casual sequence of intermediate Key Events (KEs) linked by Key Event Relationships (KER) and finishes in an Adverse Outcome (AO); this represents an apical effect relevant to risk assessment or regulatory decision-making. The AOP-Wiki software (www.aopwiki.org) facilitates collaborative AOP development by collecting and linking expert-curated AOP information through a controlled vocabulary available on AOP-Wiki website.

In the case of Read-Across or trend analysis, AOPs can allow to group chemicals according to their biological activity and mechanisms of actions at different levels of biological organisation that can increase the robustness of prediction for the final AO.

An AOP allows for the mapping, organization and integration of various types of information such as *in silico, in chemico, in vitro* and *in vivo* data, which is essential for supporting IATA (Sakuratani *et al.*, 2018). IATA have been defined as pragmatic, evidence-based approaches for chemical risk assessment in regulatory context (Sakuratani *et al.*, 2018). IATA approach consists in integrated analysis of all available existing information, and, if necessary, in the generation of new data using mostly *in silico* and *in vitro* strategies. IATAs represent an iterative methodology aimed to answer a defined question of regulatory relevance. The levels of uncertainty associated with each source of information in the decision context should be evaluated and reported. While IATA always include a conclusion based on an expert knowledge, DA to testing and assessment consists of a fixed Data Interpretation Procedure (DIP) applied to data generated with a defined set of information sources.

Both IATA and DA could benefit significantly from incorporating mechanistic AOP related evidence to be used with other existing data. For example, an AOP-informed IATA on skin sensitization has been implemented in a DA published under OECD guideline (OECD, 2021). This is an excellent example of a scientifically valid and sustainable application of AOPs in regulatory toxicology, which pave the way to their future application to other toxicological endpoints.

### Importance of data curation

All alternative approaches share the need of high-quality experimental data as a starting point. Therefore, good data management is the key point leading to an effective knowledge integration and reuse (Mahony *et al.*, 2018). Good data management practices are embodied by the FAIR data principles, defined as Findability, Accessibility, Interoperability, and Reusability (https://www.go-fair.org/fair-principles/). Strategies to promote greater data sharing and interoperability in support of public health research goals have become increasingly important (Jeliazkova *et al.*, 2021). For data standardisation, OECD has already designed and published several OECD Harmonised Templates (OHTs). Data interoperability in toxicology requires balancing the need for domain-specific details with the need to reduce complexity to enable broader use of the data.

The ISSTOX databases cluster (Benigni *et al.*, 2013) has been developed by our group at the ISS. The structure of these databases is inspired by that of the Distributed Structure-Searchable Toxicity (DSSTox) Network of the US Environmental Protection Agency (EPA), in order to contribute to the free diffusion of scientific data in a standardized, easy to read format. A wide range of toxicological databases is also available in the OECD (Q)SAR Toolbox software.

## Uncertainties associated with non-testing methods

To improve regulatory acceptance of non-testing methods, it is crucial to identify potential uncertainties as an integral part of their scientific assessment (Benford *et al.*, 2018).
For instance, uncertainty in Read-Across could be related to: a) shortcomings in the Read-Across hypothesis; b) deficiencies (incompleteness/inconsistencies) in the supporting data; c) insufficient analysis of similarities/differences of the chemicals under consideration; d) lack of data on mechanisms of toxicity and ADME properties (Benigni *et al.*, 2019).

Recently, the EFSA guidance on weight of evidence approaches (Hardy *et al.*, 2017) underlined the crucial role of the rational integration and weighting of similar types of evidence. The information should be combined by applying a formalized WoE method able to predict the outcome and estimate uncertainties. For example a decision approach based on Dempster-Shafer Theory (DST) to combine multiple sources of information and obtain the WoE final outcome has been used in an EFSA project for prediction of the genotoxicity of pesticides metabolites (Benigni *et al.*, 2019).

Similar considerations could be valid for (Q)SARs, when they are used within an AOP-informed IATA. For evaluation of uncertainties, the limitations related to the (Q)SAR models and their predictivity as well as the limitation related to the assays, on which they are based, need to be considered.

AOPs can support the regulatory decision and WoE based evaluations for IATA but there are still challenges in determining how the WoE supporting the KERs, and the causal support for the entire AOP, should be applied within an IATA context. It is crucial that during the construction and reporting of IATA, the combined considerations of biological plausibility and empirical support related the whole AOP, are performed. Rigorous validation allows not only for assessing the predictivity of the overall scheme, but also for identifying its strong and weak points suggesting refinements of the tests or developing of (Q)SARs for individual events in the AOPs.

# Regulatory applications: relevant frameworks

This section summarizes information on the most important regulatory frameworks explicitly considering the use of non-testing methods, such as (Q)SARs and Read-Across methodologies with special focus for genotoxicity endpoints.

## REACH Regulation

The Regulation (EC) 1907/2006 includes the Registration, Evaluation, Authorization, and Restriction (REACH) of chemicals. One of the fundamental principles of EU REACH Regulation is that information shall be generated whenever possible by means alternative to animal tests methods, for example, *in vitro* methods, (Q)SAR modelling or from structurally related substances (grouping or Read-Across). The aspects related to (Q)SARs, grouping and Read-Across approaches have been further elaborated in Chapter R.6 QSARs and grouping of chemicals of the "Guidance on information requirements and chemical safety assessment" (ECHA, 2011), in Read-Across Assessment Framework (RAAF) (ECHA, 2017) and in the fourth report under Article 117(3) of the REACH regulation on the use of alternative testing on animals (ECHA, 2020).

The main critical points explaining still low regulatory acceptance of *in silico* models are often related to a poorly documented justification and not proper discussion of results for Read-Across hypothesis or aspects related to the validity of the model (defined endpoint, unambiguous algorithm, defined domain of applicability, measures of performance and mechanistic interpretation where appropriate), and address the requirements that are specific for the validity of the prediction (e.g., aspects related to prediction domain and adequacy of the prediction) (OECD, 2007). Thus, the regulatory acceptance of *in silico* methods requires clear and transparent documentation for the model and for the way it has been used, sufficient to enable the regulator to conduct an independent review of the evidence.

### EFSA Panel on Plant Protection Products and their Residues

Since for metabolites and degradation products of pesticides the existing data are often limited or completely missing, alternative non-testing methods have been proposed for their toxicological assessment.

The Guidance (EFSA, 2016) on the establishment of the residue definition for dietary risk assessment, published by the EFSA in 2016, describes a tiered schema for the toxicological evaluation of residues of pesticides active substances. Genotoxicity assessment for pesticides metabolites and in absence of experimental data, non-testing methods such of (Q)SAR predictions and Read-Across can be used. EFSA promotes actively alternative methods based on mechanistical knowledge of mode of actions, as well as weight of evidence approach and uncertainties estimation (Benford *et al.*, 2018, Hardy *et al.*, 2017).

### ICH M7 guideline

The ICH M7 guideline for the "Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals" (EMA, 2017) is a framework for assessing and controlling DNA reactive impurities in a pharmaceutical products to limit potential carcinogenic risk. In the absence of sufficient experimental mutagenicity and/or carcinogenicity data for a specific impurity, the ICH M7 guideline recommends the use of (Q)SAR models for the Ames test for evaluating the mutagenic potential. The main requirements are to follow the general OECD principles (OECD, 2007) for model validity, and to use two complementary (Q)SAR methodologies: *expert rule-based and statistical-based.* If both predictions are negative, this is considered sufficient to demonstrate lack of mutagenicity or carcinogenicity.

In general, this guidance has acted as a catalyst for the scientific activity and represents an important step forward in expanding the regulatory use and acceptance of (Q)SAR predictions.

### SCCS Guidance for the testing of cosmetic ingredients and their safety evaluation

The complete ban of animal studies for cosmetic products applies since 11 March 2013, irrespective of the availability of alternative non-animal methods. The "Guidance for the testing of cosmetic ingredients and their safety evaluation" (SCCS, 2018) clearly recommends, among other data sources, the use of non-testing methods, such as (Q)SAR modelling, chemical categories, grouping, Read-Across. A memorandum (SCCS/1578/16) on the use of *in silico* methods for assessment of chemical hazard has been prepared by the Scientific Committee on Consumer Safety (SCCS) (European Commission, 2017). The document specifies and explains several limitations and problems related to the use of *in silico* methodologies combined with test *in vitro*, as part of weight of evidence approach.

## Conclusions

At the current stage of development, the integration of NAMs, such as *in silico* predictions, with all other available information using expert knowledge and careful weight of evidence assessment is crucial. The relationship between different types of evidence reflecting the current state of the art of non-testing methods for toxicology reviewed here, is graphically shown in Figure 1.

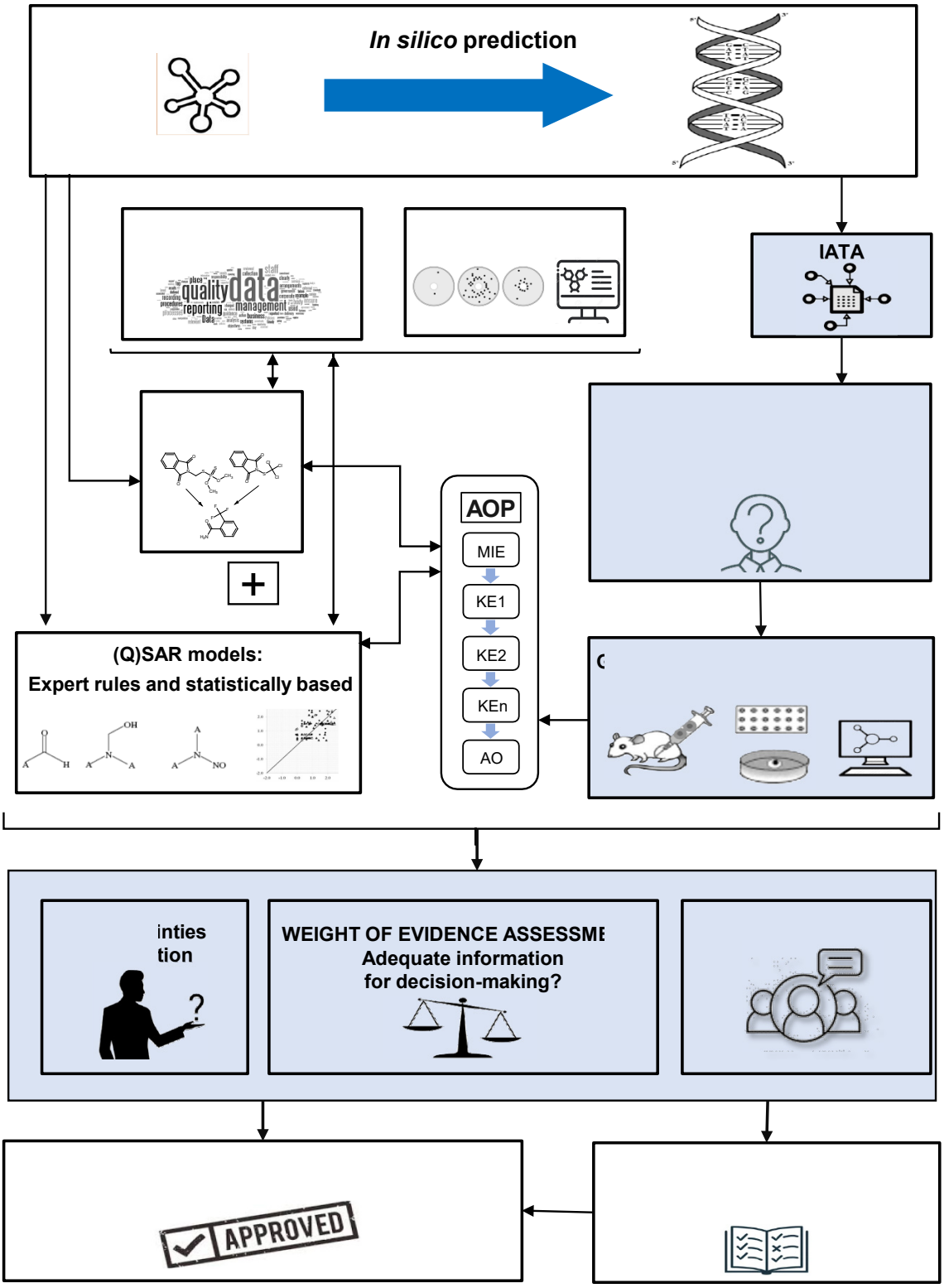


**Figure 1. Current state of the art of non-testing methods for toxicity prediction**

At present, existing (Q)SAR models and Read-Across methodologies for some endpoint (e.g., for some genotoxicity endpoint) have already sufficient reliability to be realistically used in prioritization processes and for regulatory decisions (e.g., for industrial chemicals, pesticides, and their metabolites, cosmetics ingredients, and pharmaceutical impurities).

The first key area for improvement is the generation of new experimental data and the creation of curated high-quality databases, in order to expand the chemical space of the data collections and consequently of the models.

Another important future direction should include the improvement of the understanding of intermediate effects of toxic mode of action. Development of AOPs for different endpoints, covering the entire mechanism at molecular, subcellular, cellular, organ, system and organism levels could increase the predictivity of *in silico* methods and could allow for the development and validation of custom predictive models. AOP informed IATA could play an important role in improving the acceptability of NAMs in regulatory contexts, by bringing more transparent mechanistically based integration of all available sources of information together with expert evaluation of uncertainty and weight of evidence assessment. In addition, progress towards standardization and validation of Defined Approaches and Guidelines for integrated assessment of toxicity can be expected in the next ten years.

Overall, the current state of the art of alternative approaches conveys optimism on meeting the increased expectations for trustable hazard identification and related regulatory application.

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# OMICS APPROACHES AND INTEGRATED BIOINFORMATIC ANALYSIS FOR THE IDENTIFICATION OF BIOMARKERS

Sabrina Tait
*Reference Centre for Gender Medicine, Istituto Superiore di Sanità, Rome, Italy*

## Introduction

In the last decades, the advent of omics technologies dramatically increased the understanding of signaling pathways and networks of co-regulated genes, proteins and metabolites, thanks to the availability of several omics applications, including genomics, transcriptomics, epigenomics, proteomics, metabolomics and, more recently, miRNAomics, which increased the possibility to cover all the aspects of cellular biology. Indeed, each omics technology provides a piece of information contributing to the understanding of the cellular processes in physiological conditions but also in different diseases or under the influence of exogenous stimuli like chemicals, physical agents, microorganisms or life style.

## Biomarker discovery

One of the main contributions that omics approaches can provide is the identification of relevant biomarkers. According to the World Health Organization definition, a biomarker is "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence or outcome of the disease" (UNEP, 2001). Different biomarkers could be discovered by the analysis of clinical or experimental samples, e.g., diagnostic, prognostic or predictive biomarkers that are differently able to discriminate, respectively, specific pathologies, the progress of a disease, the response to a drug. Other biomarkers may be also identified in response to environmental stimuli (biomarkers of effect) or as critical stepping points during development or in particular life stages.

Different omics are used to investigate physiological states, diseases, exposure effects, infections, and all affections of clinical relevance for human health.

Genome-Wide association studies (GWAS) are applied to the discovery of genetic risk factors related to a particular disease by comparing the genetic variants in large population groups such as Single Nucleotide Polymorphisms (SNPs) or Copy Number Variants (CNVs), thus helping in the identification of susceptible subpopulation as well as of relevant markers of disease onset/progression (Tam *et al.*, 2019).

Epigenomics is used to identify sites of gene expression regulation, such as DNA methylation sites and histone modifications, which may be altered by pathological conditions or environmental factors (Stricker *et al.*, 2016). Since these alterations are heritable when occurring in germ cells, the identification of altered epigenomic sites may not only elucidate mechanisms underlying disease regulation but also be relevant for the generations to come (Bošković & Rando, 2018).

Transcriptomics has been the first approach that provided fundamental insights on how signaling pathways proceed into cells and to which extent minimal derangements from normal functionality may provoke great imbalances in some pathways and limited in others. As an example, we demonstrated that the exposure to low and high doses of the same chemical, i.e. the plasticizer bisphenol A, exerted completely different effects on the angiogenesis of mouse placenta by triggering two distinct signaling pathways, respectively promoting and inhibiting the process (Tait *et al*., 2015).

Being started with microarray technology, transcriptomics has now moved to Next Generation Sequencing (NGS) approaches which provide different levels of information with a greater detail, such as profiles of mutation in a specific disease condition, profiles of the transcriptome or, particularly, of the exome. With Whole-Exome Sequencing (WES) the costs may be reduced due to the selective capture of only those part of the genome encoding for functional proteins (about the 3%), where genetic variants are more probably located; for this reason, WES is increasingly implied in diagnostics and prenatal screenings (Suwinski *et al.*, 2019; Best *et al.*, 2018). Otherwise, with the Whole-Genome Sequencing (WGS), RNA-Seq in particular, all the transcripts are analysed permitting to compare the gene expression levels in different conditions as well as to discover new disease-related transcripts, among which long non coding RNAs are receiving great attention due to their regulatory role in several pathologies (Stark *et al.*, 2019). In addition, by short sequencing approaches, also micro RNAs may be identified. The discovery that the so-called "junk DNA" contains small and long non coding RNA (lncRNA) sequences which play a variety of different roles in cell machinery, strongly improved the application of NGS for the identification of novel transcripts and their interaction with other coding and non-coding RNAs, as well as with proteins under both normal and pathological conditions (Qian *et al.*, 2019). We recently reported the expression of different miRNA and lncRNA patterns (including novel transcripts) in visceral adipose tissue of lean and obese patients affected by colorectal cancer (Tait *et al.*, 2020). Our results highlighted that, in each condition, different regulatory interaction networks occur between non coding and coding genes, supporting the relevance of obesity comorbidity in colorectal cancer.

By the proteomic approach, the abundances of proteins actually functioning in the cellular machinery and their post-translational modifications are identified by mass spectrometry, allowing to discover new functions as well as new interactions among co-regulated proteins (Monti *et al.*, 2019). The end products of cellular metabolism, including sugars, amino acids, lipids, etc., are identified by metabolomics using chromatographic approaches (gas or liquid) coupled with mass spectrometry or by Nuclear Magnetic Resonance (NMR) (Schrimpe-Rutledge *et al.*, 2016). Importantly, metabolites do not represent the end of the story since they trigger cellular processes then affecting signaling pathways, transcription, translation, etc. (Rinschen *et al.*, 2019). Conversely to nucleic acids, proteins and metabolites are not amplifiable, so less abundant entities cannot be quantified. However, in the last years, these two approaches rapidly increased in sensitivity due to instrument implementations, thus their ability to effectively quantify the number of peptides and metabolites improved dramatically.

# Challenges

For every omics approach, a series of critical issues should be considered in order to safely and confidently use the identified biomarkers. The greatest challenge is that omics approaches are costly, although less expensive than in the past, and time consuming, both at instrumental and data analysis level. Thus, the risk is to include limited numbers of samples, reducing the statistical

power of the analysis and finally compromising the robustness of the obtained results. This ultimately limits a biomarker from being validated and adopted into clinical routine.

Other common critical steps include appropriate sample collection and processing, identification of sequences (genes or proteins) against a reference genome, annotation and quantification. The steps involving sample manipulation relies on availability of high-quality starting material, coming from tissues of patients or other human matrices, tissues of animal models from *in vivo* studies, primary cells or cell lines cultured *in vitro*. The next steps are more related to instrument performance and its supporting software.

Bioinformatic analyses have then to be performed to manage the huge amount of data obtained and to uncover the biological significance behind the disease/condition/status under study. The pipelines to obtain such results may be quite different among computational groups and the scientific community has not reached yet a consensus on general criteria to consider in a data analysis workflow, so the results may differ. This poses the question on standardization of data format, filtering and cleaning, statistical methods and software used. First of all, to maximize transparency, each step of the analysis should be documented.

In this regard, the Organization for Economic Co-operation and Development (OECD) recently launched the Omics Reporting Project to produce guidance documents and reporting templates for omics approaches in chemical testing, to be used also in a regulatory context. The aim of these guidance documents is mainly to provide researchers a platform to report omics data in a harmonized framework, but they also help regulators and stakeholders to assess the quality of omics data with practical tools. So far, both the Transcriptomics and the Metabolomic Reporting Frameworks have been drafted and made available (OECD, 2021a; OECD, 2021b).

At least for genomic, transcriptomic and proteomic data, for which public repositories are available, each dataset produced should be deposited to guarantee transparency and permit other researchers to re-analyse the data or to integrate that dataset with others for a meta-analysis. Whatever the workflow used, the aim of bioinformatics analysis of omics data is to extract relevant information from complexity identifying main affected pathways and interaction networks. The elaboration would ultimately lead to the definition of one or more candidate biomarkers which could be further validated by molecular and/or clinical assessment.

Despite with each single omics we obtain a picture on derangements occurring at a certain level of cellular organization, this may be somewhat limiting when it is necessary to obtain a complete comprehension of the biological system, especially if the final goal is precision medicine. Moreover, several regulating processes occur in cells affecting the level of expression of genes, proteins and metabolites, thus it is not assured that information obtained at gene expression level is completely "translated" at protein or metabolite levels, and vice versa. Responses are also cell-type and tissue-specific, and relative abundances of biological molecules may be affected also by inter-individual variability. Therefore, different omics should be used in combination to improve the knowledge, also because each level of cellular organization may be differently affected when considering a disease condition or when studying alterations induced by external factors. The integration of different omics data may provide a holistic overview of the underlying mechanisms that a single omics approach may fail to identify. However, this represents a seriously challenging problem which computational biologists, bioinformaticians and biostatistics are facing. So far, an increasing number of studies integrated two omics approaches, mainly transcriptomics and proteomics or proteomics and metabolomics, whereas not many implemented the integration of data from three or more omics (Misra *et al.*, 2018). Our group is increasingly involved in the implementation of multi-omics approaches and data analysis. A work is in progress with the support of the Core Facility at the Istituto Superiore di Sanità (ISS, the National Institute of Health in Italy), in which we performed transcriptomic and proteomic analyses to investigate exposure effects of some chemical contaminants on a human liver cellular

model. By our integrated bioinformatics analysis, we are identifying the affected pathways also discriminating which genes and proteins are more relevant in the regulatory network.

# Data gaps

Overall, the biological relevance of omics approaches, especially if human *in vitro* models or human matrices are analysed, relies on the ability to provide relevant information on human health in a mechanism-based context, rather than being based on apical adverse effects as observed in animal studies. In particular, they give the possibility to identify which is the flow of the signal within cells, as well as the temporal profile, if time-course experiments are performed. In this frame, increasing efforts should be undertaken to cover the investigation of all possible adverse effects, avoiding underestimation of some biological systems since, at the moment, not all the organs/tissues are equally considered. Table 1 provides an illustrative example, showing a simple search in PubMed with few keywords just to compare the number of publications in this field, not filtering for review or other type of articles.

**Table 1. Number of publications retrieved in PubMed (date of search 07/2021) by using as keywords the name of the organs, the Boolean operator "AND" and, alternatively, the column names**

| Key words | Omics | Multi-omics | Omics AND biomarker | Multi-omics AND biomarker |
|---|---|---|---|---|
| **"AND"** | | | | |
| **Blood** | 1757 | 410 | 749 | 157 |
| **Brain** | 1036 | 275 | 275 | 80 |
| **Liver** | 920 | 253 | 263 | 59 |
| **Lung** | 746 | 219 | 248 | 69 |
| **Breast** | 704 | 236 | 246 | 77 |
| **Kidney** | 439 | 117 | 184 | 40 |
| **Skin** | 250 | 64 | 61 | 15 |
| **Colon** | 181 | 70 | 53 | 20 |
| **Bone** | 227 | 49 | 57 | 16 |
| **Eye** | 217 | 36 | 37 | 5 |
| **Prostate** | 191 | 52 | 71 | 20 |
| **Ovaries** | 93 | 18 | 15 | 2 |
| **Bladder** | 88 | 34 | 43 | 12 |
| **Bone marrow** | 81 | 16 | 17 | 3 |
| **Thyroid** | 70 | 12 | 24 | 3 |
| **Pancreas** | 63 | 14 | 20 | 7 |
| **Stomach** | 61 | 19 | 28 | 7 |
| **Testis** | 42 | 5 | 8 | 1 |
| **Placenta** | 49 | 8 | 16 | 3 |
| **Adrenal** | 40 | 10 | 12 | 3 |
| **Lymph nodes** | 37 | 13 | 12 | 6 |
| **Uterus** | 28 | 2 | 11 | 0 |
| **Esophagus** | 15 | 8 | 9 | 6 |
| **Thymus** | 10 | 2 | 1 | 0 |

It is noteworthy that, by querying blood AND omics, 1757 publications were found, whereas for thymus AND omics only 10 are available. Moreover, only in a limited number of these publications the word "biomarker" matched the query. By searching for multi-omics studies, the numbers dramatically decrease and, among them, studies including "biomarker" are really scarce. This mere speculative exercise easily demonstrates how some organs are poorly investigated through omics approaches.

As a further consideration, a relevant issue often neglected in experimental studies, including omics, is the sex/gender. As stated above, in a context of a precision medicine era, gender-specific effects should be always taken into account since signaling pathways, organ functionalities, metabolism, aging, response to stimuli, immunity, etc. are different between the two genders. Repeating the same PubMed Search as shown above, but including the keywords "gender OR sex" in the query, we can see in Table 2 that in a very limited portion of studies a match with the keywords was found, indicating a scarce consideration of this fundamental aspect.

**Table 2. Number of publications retrieved in PubMed (date of search 07/2021) by using as keywords the name of the organs, the Boolean operator "AND" and, alternatively, the column names**

| Key words | Omics AND (gender or sex) | Multi-omics AND (gender or sex) | Omics AND biomarker AND (gender or sex) | Multi-omics AND biomarker AND (gender or sex) |
|---|---|---|---|---|
| **"AND"** | | | | |
| **Blood** | 69 | 15 | 30 | 5 |
| **Brain** | 43 | 13 | 10 | 0 |
| **Liver** | 24 | 9 | 6 | 4 |
| **Lung** | 26 | 9 | 11 | 4 |
| **Breast** | 6 | 3 | 3 | 2 |
| **Kidney** | 13 | 6 | 6 | 1 |
| **Skin** | 7 | 2 | 1 | 1 |
| **Colon** | 4 | 3 | 2 | 1 |
| **Bone** | 3 | 0 | 1 | 0 |
| **Eye** | 5 | 2 | 0 | 0 |
| **Prostate** | 3 | 1 | 1 | 1 |
| **Ovaries** | 5 | 0 | 1 | 0 |
| **Bladder** | 0 | 0 | 0 | 0 |
| **Bone marrow** | 0 | 0 | 0 | 0 |
| **Thyroid** | 2 | 0 | 0 | 0 |
| **Pancreas** | 0 | 0 | 0 | 0 |
| **Stomach** | 1 | 0 | 0 | 0 |
| **Testis** | 7 | 0 | 1 | 0 |
| **Placenta** | 1 | 0 | 0 | 0 |
| **Adrenal** | 40 | 10 | 12 | 3 |
| **Lymph nodes** | 37 | 13 | 12 | 6 |
| **Uterus** | 28 | 2 | 11 | 0 |
| **Esophagus** | 15 | 8 | 9 | 6 |
| **Thymus** | 10 | 2 | 1 | 0 |

# Conclusions

In conclusion, the use of omics approaches, especially multi-omics, is crucial for the identification of novel adverse effects and biomarkers, providing valuable information on system biology in general and pathological mechanisms in particular.

Harmonization of data analysis workflow and use of reporting templates are highly encouraged to allow data comparison and meta-analysis, especially in an open science perspective.

Data gaps on some tissues/organs as well as on sex/gender differences are highlighted, suggesting that more efforts should be undertaken to systematically apply omics approaches and fully exploit their potential.

As an added value, omics allow to prioritize and consequently reduce the number of studies using animal models, limiting the research to studies substantiating observed early molecular events and modes of action and to link them to an apical adverse outcome. This approach is compliant with the 3Rs framework and, if largely applied, it will progressively increase the knowledge based on mechanisms and on the discovery of new biomarkers to be used in clinical, prevention and risk assessment applications without the use of animals.

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# ADVANCED *IN VITRO* SYSTEMS AS A VALID ALTERNATIVE TO ANIMAL MODELS FOR ONCOIMMUNOLOGY AND AIRWAY ALLERGY STUDIES

Fabrizio Mattei (a), Luca Businaro (b), Adele De Ninno (b), Claudia Afferni (c), Giovanna Schiavoni (a)
*(a) Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy*
*(b) Institute for Photonics and Nanotechnologies, Consiglio Nazionale delle Ricerche, Rome, Italy*
*(c) National Centre for Drug Research and Evaluation, Istituto Superiore di Sanità, Rome, Italy*

## Introduction

Immunology investigates the response of the human body to pathogens and other external and internal insults. The two arms of immune responses, the innate and the acquired immunity, elicited by infections, allergens or cancer can be properly monitored through a variety of methods. Hitherto, these have included classical *in vitro* experiments principally aimed at revealing the activation of specific immune cell populations upon specific stimulation and therapeutic settings, as well as *in vivo* studies using animal models. The use of animals allows designing more complex experimental strategies, which mimic with higher affordability and reliability the *in vivo* scenario of human immune system, and have been successfully employed to dissect immune-associated events in diverse pathological settings. In this context, mouse models have markedly boosted the scientific literature on the mechanisms at the basis of immunity to cancer and allergy, especially with the development of genetically modified organisms in which relevant defects allowed researchers to dissect the role of specific pathways of immune responses.

Nowadays, biotechnologies have greatly improved *in vitro* systems applied to investigate multifaceted aspects of immunity in life sciences. The rise of replacement tools has further elicited the generation and use of alternative solutions to animal models. Typical examples of currently adopted alternative models include organoids and microfluidic platforms, which can provide reliable, cost-effective and affordable tools to recapitulate *in vivo*-like environments in health and disease (Boussommier-Calleja *et al.*, 2016; Mattei *et al.*, 2021).

In this contribution, we focus on the state of the art of selected alternative methods to animal experimentation, with emphasis on microfluidic chips and other new *in vitro* approaches to investigate human immune responses in cancer and allergy.

## Microfluidic chips as a valid platform to study the tumor immune microenvironment

In cancer immunology, a field of research investigating the role of the immune system in the progression and development of cancer as well as the interactions of immune cells with cancer within its microenvironment, several advanced tools can be adopted to accompany or replace mouse models. As an example, organoids represent a suitable advanced *in vitro* model to study the tumor microenvironment. Tumor organoids are three-dimensional *ex vivo* cultures obtained from either patient or mouse tumor explants that grow *in vitro* and form a complex multicellular mass resembling the original tumor environment (Kim *et al.*, 2020). A peculiarity of such tumor

organoids is the presence of infiltrating immune cells that allow the characterization of immune *vs* tumor cell interactions, such as following immunotherapy or chemotherapy, which both activate the immune system to fight cancer. The use of mouse tumor organoids implies that mice will not be fully replaced. However, due to the improvement of related experimental procedures, according to Reduction and Refinement concepts, use of animals may be certainly optimized (). As an alternative, a complete replacement of mice is accomplished by the use of human tumor spheroids generated by tumor cell lines, although this solution does not exactly recapitulate the human tumor microenvironment due to the intrinsic absence of immune cells in tumor spheroids. This limitation can be circumvented by the use of patient-derived human xenografts (PDX) mice to generate human tumor organoids (Xu *et al.*, 2021).

Among other types of advanced alternative *in vitro* methods recently described, microfluidic devices, represent an innovative system to replace the use of mice, studying in detail the interactions between different cells types. Microfluidic platforms for cell or organ-on-a-chip cultures are an emergent technology aimed to reproduce specific biological environments, recapitulating the *in vivo* scenario with superior reliability with respect to classical *in vitro* techniques. These devices hold several key advantages, such as fully customized structural internal units, fabricated *ad hoc* to investigate specific scientific questions using a minimal quantity of living cells or tissues. In the field of cancer immunology, microfluidic chips proved as useful tools to study the interactions between immune cells and tumor cells (Mattei *et al.*, 2021; Mencattini *et al.*, 2020). Depending on the specific case study, the use of microfluidic chips can either fully replace the use of the living animal, or contribute to its partial replacement.

Our laboratory has been the first to describe a microfluidic chip to study the interactions between immune cells and cancer cells in the context of immunodeficiency. In this pioneering study, B16.F10 metastatic melanoma cell lines and spleen cells isolated from mice deficient for the transcription factor IRF-8 (IRF-8 KO) were used (Businaro *et al.*, 2013). These mice are characterized by a severe deficiency of key immune cell populations, such as dendritic cells and macrophages, and defective immune response to pathogens and cancer (Mattei *et al.*, 2012). The *ad hoc* fabricated chip employed was composed by three main chambers interconnected by two arrays of microfluidic channels (mimicking the blood vessels). The chambers were equipped with inlet wells for tumor cells and immune cells loading in the two opposite sides of the device (Figure 1).

The time-lapse video and fluorescence microscopy analyses demonstrated that while immunocompetent (wild type) immune cells retain the ability to migrate towards melanoma cells interacting with them and contrasting their spread within the device chambers, IRF-8 KO immune cells fail to do so. These findings mirrored previous *in vivo* observations indicating marked melanoma growth and impaired immune cell infiltration at the tumor site in IRF-8 KO mice compared to immunocompetent counterparts (Mattei *et al.*, 2014; Mattei *et al.*, 2012). This experimental setting was subsequently employed to demonstrate the requirement for Formyl Peptide Receptor 1 (FPR1) in host immune cells, particularly dendritic cells, for therapeutic efficacy of anthracyclines in anticancer immune responses (Vacchelli *et al.*, 2015). Here, experiments in the microfluidic device revealed that FPR1 and its ligand annexin-1 promote stable interactions between dying cancer cells and human or murine leukocytes following chemotherapy. A similar approach employing both KO mouse models and microfluidic chips has been employed in a study investigating the serous tubal intraepithelial carcinoma (STIC) in a canine model. Here, de Almeida Monteiro Melo Ferraz and co-workers used an oviduct-on-a-chip model and the CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats of Associated Protein 9) system to target the TP53 gene. Together, these three tools provided an *in vitro* biomimetics of STIC in canine models (de Almeida Monteiro Melo Ferraz *et al.*, 2020). These on chip-based experimental strategies for cancer immunology studies paved the way to novel approaches exploiting microfluidic platforms to evaluate chemo- or immunotherapeutic agents by using kinematic datasets.

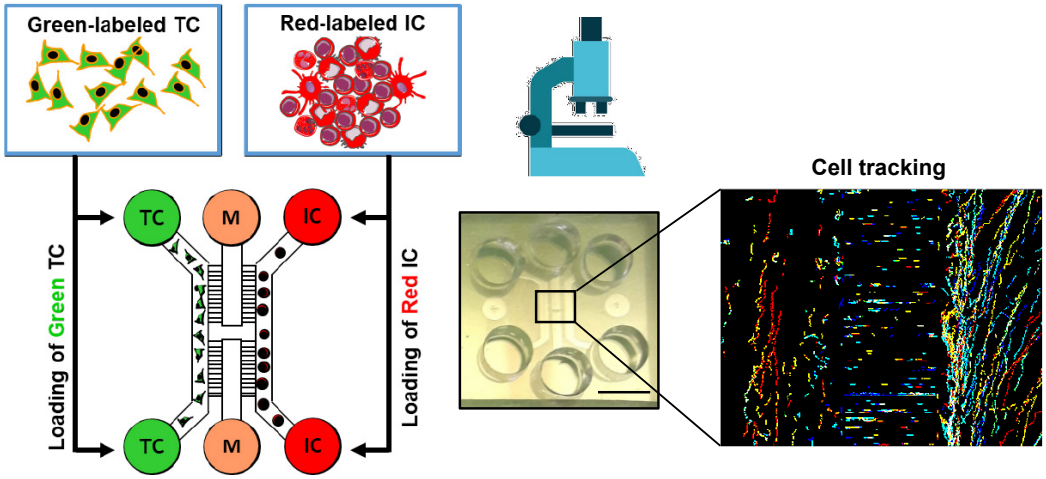


**Figure 1. Microfluidic device for cancer immunology studies. Fluorescent labeled tumor cells (TC) and immune cells (IC), from either immunocompetent or immune-deficient mice, are loaded into the chambers through appropriate inlets. Migration of immune cells to tumor cell chambers is monitored by time-lapse fluorescence microscopy and cell tracking analysis**

Other studies based on microfluidic devices are focused at investigating more complex issues, such as to recapitulate the immunotherapy responses of tumor biomimetic to some drugs or drug combinations (Mattei *et al.*, 2021). Our group has been among the first ones to propose an *ad hoc* fabricated competition-based chip looking at the synergic role of drug combinations in cancer. These devices are composed by a central chamber in which fluorescent-labelled immune cells, i.e., healthy donor human Peripheral Blood Mononuclear Cells (PBMC), are suspended in culture medium and loaded (Figure 2).

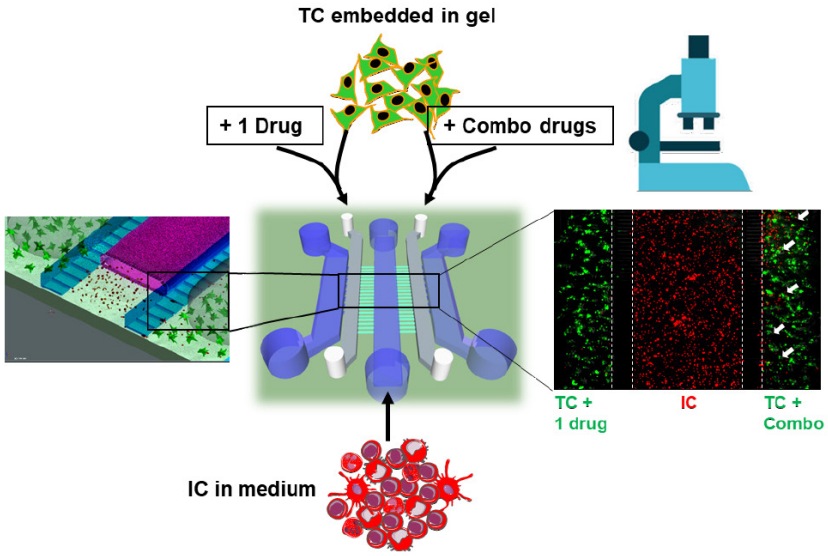


**Figure 2. Competitive microfluidic device for drug testing in cancer immunology. Fluorescent labeled tumor cells (TC) and immune cells (IC), or selected immune cell populations, are loaded into the chambers through appropriate inlets. Single and combined drugs (either chemotherapeutic or immunotherapeutic) are administered to the tumor cells as indicated. Migration of immune cells (red) to tumor cell (green) chambers containing either single and combo drugs is monitored by time-lapse fluorescence microscopy**

The device is equipped with two side chambers where tumor cells (i.e., melanoma cells) suspended in Matrigel are loaded. Matrigel can be viewed as a mix of extracellular proteins mimicking the stroma of the tumor microenvironment. Prior to loading onto chips, melanoma cells were pre-exposed to type I interferons (IFN) and 5-Aza-2'-Deoxycitidine (DAC) alone or in combination. By properly combining the two drugs in the chip (allowing to test two conditions simultaneously), we demonstrated that PBMCs preferentially migrated to and infiltrated the melanoma gel chamber exposed to both DAC and IFN. These studies were also paralleled by *in vivo* studies in melanoma-bearing mice exposed to single vs combined treatments, which confirmed the data obtained by on-chip experiments (Lucarini *et al.*, 2017).

A similar device platform was exploited to assess the function of specific immune cell populations against cancer. Parlato *et al.* analyzed IFN-α-conditioned dendritic cells (IFN-DC) within 3D tumor spaces for their ability to infiltrate and engulf collagen-embedded SW620 colorectal cancer cells treated with a combination of drugs (Romidepsin and IFN-α) with respect to untreated cells (Parlato *et al.*, 2017). We studied the ability of the cytokine IL-33 to attract eosinophils within melanoma tissue and showed that IL-33 triggered the infiltration of eosinophils into the gel chamber only when tumor cells were present, indicating an indirect effect (Andreone *et al.*, 2019). Overall, these applications constitute an added value to study the tumor microenvironment biomimetic by minimally resorting to the use of living animal models in agreement with the 3Rs principle.

The final ambitious objective of microfluidic chip research is to recapitulate the overall complex physiology of the human body, which may be accomplished by adopting a on chip based network of multi-organ modules (Mattei *et al.*, 2021). In this respect, many laboratories are conducting complex experiments based on modular chip units interconnected to other similar units through microfluidic channel systems, to form an advanced platform based on modular chip systems. The aim of this multi-modular microfluidic platform will be to faithfully recapitulate the superior complexity of human body, including physiological connections between organs in health and disease condition (Zhang *et al.*, 2009). Despite their complexity and high costs to face with, these advanced multi-organ chip platforms will have a significant positive impact in the optimization and reduction of animal experimentation in the medium or long-term future.

Advanced *in vitro* models for airway allergy studies

Respiratory allergies include a group of inflammatory syndromes affecting the upper and lower respiratory tract. They are caused by immunological hypersensitivity reactions to airborne allergens derived from dust mites, insects, animal epithelium or hair, pollen and mould. Clinical manifestations of respiratory allergies are rhinitis, rhino-conjunctivitis, sinusitis and asthma. The World Health Organization (WHO) estimates that 235 million people currently suffer from asthma (WHO, 2022). Although recognised as a result of an interaction between multiple genetic and environmental factors, allergies are mainly considered as an environmental disease (Renz *et al.*, 2017). Hypersensitivity reactions involved in respiratory allergies are defined as type I. They are mediated by class E immunoglobulins (IgE) and by T lymphocytes called type 2 helpers (Th2). Mechanistically, it has been observed that very similar symptoms, induced by the same allergenic sources, are often the result of different cellular and molecular interactions.

Murine models of allergic asthma have successfully reproduced many features of human allergic asthma such as the presence of high levels of both total and allergen-specific serum IgE, airway inflammation, Airway Hyper-Reactivity (AHR), and the presence of Th2-type cytokines in Broncho-Alveolar Lavage (BAL) (Epstein, 2004). These models are also used to assess the efficacy of allergen-specific immune therapy in pre-clinical testing.

By contrast, most *in vitro* methods have been primarily implemented for diagnostic purposes (using human biological samples) and to assess the effectiveness and safety of therapies by measuring significant endpoints. They include: 1) IgE binding tests, performed as

Immunoblotting and Enzyme-Linked Immunosorbent Assay (ELISA); 2) quantification of serum IgE, IgG1, IgG4, and inflammatory cytokines IL-4, 5, 13, by ELISA test; 3) count of eosinophils in BAL and in sputum, by FACS (Fluorescent-Activated Cell Sorter) and histochemistry methods, respectively; 4) Basophil Activation Test (BAT) to assess patients' circulating basophil reactivity to allergens.

However, allergic inflammation results not only from an exacerbated Th2-biased adaptive immune response but also from the direct activation of innate immune cells mediated both by allergens themselves and by danger signals (i.e., alarmins) present in the allergen sources (Gabriele *et al.*, 2013; Schiavoni *et al.*, 2017). In this regard, the study of allergen-induced effects on mucosal epithelial cells is a very topical issue. Many researchers have tried to answer the question: "what makes an antigen an allergen?" Several methods have been proposed to discriminate and identify functional and structural features of an allergen, but none of these led to conclusive results, mainly due to biogenic and environmental confounding cofactors (Traidl-Hoffmann *et al.*, 2009). This lack of knowledge hampers the development of *in vitro* methods alternative to animal models. We developed a co-culture system of naive CD4+ T lymphocytes together with allergen-primed Dendritic Cells (DC) plus IL-33, as an alternative method to mouse models of allergic asthma, for the study of innate immunity during allergic sensitization (Afferni *et al.*, 2017; Gabriele *et al.*, 2013; Willart *et al.*, 2012). This novel allergen driven Th2 lymphocytes polarization test allows to evaluate the sensitizing potential of an allergenic molecule or extract and the allergic sensitization mechanisms.

Inflammation of the respiratory tract and relative pathogenic processes involved in respiratory allergies imply cell migration. A technique widely used to analyse cell migration is the culture in the Boyden-type cell culture wells. We set up an *in vitro* model by using the Boyden system to study the infiltration of eosinophils in the lung, which is responsible for remodelling events associated with chronic respiratory allergies (Wilson *et al.*, 2013). In this setting, TC-1 cells (a murine alveolar-derived tumor cell line) were exposed to Der p1 and Papain allergens and the released soluble factors were tested for ability to trigger the migration of eosinophils in the Boyden system (Figure 3).

Furthermore, co-culture of eosinophils with the same TC-1 cells or allergen-conditioned medium allowed to evaluate the cross-talk between lung epithelial cells and eosinophils through the following parameters: *i)* cell-cell interaction time (Andreone *et al.*, 2019), *ii)* epithelial-derived cytokines/chemokines-mediated eosinophils activation, and iii) eosinophilic granule-mediated TC-1 cell death (Andreone *et al.*, 2019; Cañas *et al.*, 2018). Altogether, all the above-mentioned endpoints are implicated in human asthma. Moreover, in the last decades many *in vitro* assays using epithelial respiratory cells grown in the air-liquid interface have been developed to assess the respiratory sensitizing potential of chemicals. To date, this method has been employed only rarely to study respiratory allergies (Willart *et al.*, 2012). Of particular relevance are microfluidic systems that are being developed for both chemical pollution impact and inhaled medicine delivery studies (Benam *et al.*, 2016). In cell cross-talk studies, microfluidic devices unlike other *in vitro* methods, allow the measurement of displacements and interactions between living cells through time-lapse microphotography. Thus, the employment of this technique in respiratory allergy field holds promise in the next future.

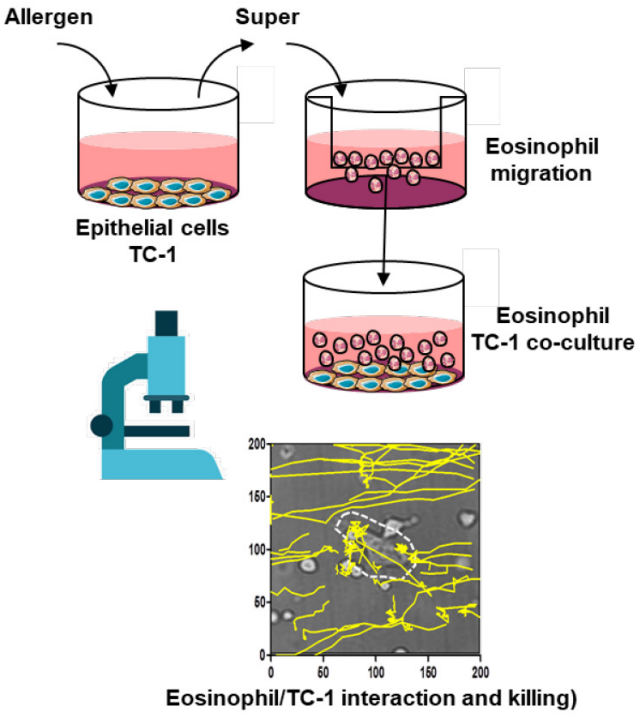


**Figure 3. Boyden type cell culture system for allergen driven eosinophil-respiratory epithelial cells cross talk in respiratory allergies. Airway epithelial TC-1 cells were exposed to allergens and the released soluble factors were tested for ability to trigger the migration of eosinophils in the Boyden system. Allergen-conditioned medium-induced activation of eosinophils was evaluated by co-culture of eosinophils with TC-1 cells and evaluation of interaction times and TC-1 cell death**

# Conclusions

Alternative methods to animal research represent a paramount challenge that will deeply affect the study of immunity in allergy and cancer. Specifically, microfluidic chips and the use of organoids and other advanced *in vitro* systems represent a valid tool to investigate the multifaceted scenarios of immune responses to cancer.

At the same time, validation tools to evaluate *in vitro* the allergic diseases have now reached an adequate affordability. In parallel, animal models have now adopted superior levels of complexity thanks to huge advancements of biotechnologies. Despite the establishment of an increasing number of non-animal methods, we believe these methods will not totally substitute animal experimentation procedures in the near future.

Rather, it is feasible that coupling animal research to *in vitro* advanced methods will represent the future of a multidisciplinary research activity, which takes into consideration the application of the 3Rs principle.

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# ADVANCED THREE-DIMENSIONAL *IN VITRO* TUMOR MODELS AS ALTERNATIVE TOOLS TO ANIMAL EXPERIMENTS IN CANCER RESEARCH

Gabriella Rainaldi
*Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy*

## Introduction

During the past decades, our knowledge on cancer origin has increased immensely, and progress in patient treatments have been appreciably achieved. Nevertheless, cancer remains as one of the leading causes of death worldwide (Hoarau-Véchot *et al.*, 2018), and the high incidence and mortality rates demand for uninterrupted scientific community efforts to deeply dissect the biological mechanisms that underlie its pathophysiology, and to design more effective treatments. Even if hard works have been exerted in cancer research, a relevant open obstacle for the development of novel treatment regimens remains the challenge of translating research breakthroughs from bench to bedside. This hurdle mainly concerns with the fact that, too often, experimental cancer models only poorly mirror the patient's tumors, and as a consequence, many antineoplastic strategies that perform well in tumor models fail in clinical trials.

It is irrefutable that experimental models are essential tools in pre-clinical cancer research. Traditional two-dimensional (2D) *in vitro* cell line cultures and *in vivo* animal models have long been employed to investigate the pathophysiological nature of tumors, and both these experimental models are still considered gold standard in pre-clinical settings in oncology (Hoarau-Véchot *et al.*, 2018; Lv *et al.*, 2017; Huang & Gao, 2018). However, even if these conventional models have yielded notable contribution in understanding the underlying nature of cancer and its response to various treatments, it has become more and more apparent that they are, nonetheless, unsuitable in adequately recapitulate the *in vivo* tumor scenario.

For decades, cancer has been studied primarily by using 2D cell cultures grown in monolayer (Hoarau-Véchot *et al.*, 2018; Lv *et al.*, 2017). This 2D conventional cell model has been extremely useful in basic and applied cancer research and have been instrumental in answering fundamental tumor questions. However, it is now amply recognized that 2D monolayers are inadequate in representing properly the three-dimensional (3D) architecture of *in vivo* solid cancer, its complex microenvironment and cell heterogeneity, and tumor proliferation kinetics. For these reasons, it is becoming more and more clear that 2D tumor monolayers are unsuitable in evaluating, in a realistic manner, the response to anticancer agents. In spite of that, 2D monolayers continue to be routinely used in cancer research due to their well-characterized features, broad availability, simple and efficient culturing workflow, reproducibility, low costs, and because they are amenable of high-throughput screening.

For many years, *in vivo* animal models have been widely employed in cancer research and represent an essential tool to better comprehend the complexity of cancer pathophysiology and to investigate new antineoplastic strategies (Lv *et al.*, 2017). Despite the undeniable importance of animal models in this research area, it must be highlighted that laboratory animal experimentation, like 2D cell cultures, also have a number of drawbacks (Lv *et al.*, 2017; Pinto *et al.*, 2020). It is principally argued that these models often do not faithfully recapitulate both the human tumor microenvironment and the pathogenic processes in human patients. As a consequence, data

obtained from many promising animal studies may not proceed in anticancer development towards clinical applications, reflecting the physiological differences between animal models and humans. In addition, animal models are expensive, and require extensive resources and time to results, all important factors that cannot be ignored in the plan of investigation. Last but not least, ethical issues in relation to the use of animals in scientific research are highly controversial. If investigations with these models continue to be one of the cornerstone experimental approaches in cancer studies, on the other hand, according to the 3Rs principle (Replacement, Reduction, Refinement) the use of alternative biological systems aimed to promote a more responsible employment on animals in research is highly encouraged and demanded (Lv *et al.*, 2017; Pinto *et al.*, 2020).

The relevant issues about 2D *in vitro* cell culture defects and animal model use have prompted cancer researchers to design more realistic *in vitro* tumor cell models able to obtain more coherent data at an early stage of the experimentation, thus, limiting the number of laboratory animals employed in a subsequent experimental phase. To achieve this purpose, more suitable *in vitro* cell models that effectively mirror the spatial architecture of cancer and its pathophysiological behaviour, advanced 3D *in vitro* tumor models have been developed (Lv *et al.*, 2017; Pinto *et al.*, 2020; Santini & Rainaldi, 1999; Weiswald *et al.*, 2015). Nowadays, these are no doubts that *in vitro* 3D tumor systems represent the promising approaches to bridge the gap between traditional 2D cell monolayers and *in vivo* animal models. As a matter of fact, it is hoped that these 3D tumor systems, besides overcoming the limitations and hurdles inextricably associated with 2D monolayers, could reduce, or ideally, replace the utilize of animal models in agreement with the 3R concept. The potential of *in vitro* 3D tumor systems is gaining increasing scientific recognition and the introduction of these models for investigating tumor biology and its response to antineoplastic treatments is rapidly expanding (Hoarau-Véchot *et al.*, 2018; Lv *et al.*, 2017). In fact, it is believed that these 3D culture systems might be the actual promise in experimental translational research aimed to coherently define and speed-up predictions on the clinical efficacy, and to apply adequately to international and national research projects. In this scenario, cancer investigation is rapidly shifting into the direction of 3D *in vitro* tumor models and, surely, this scientific trend is strongly driven by the closely connected open issue to support the 3Rs principles in cancer studies.

In the past decades, 3D *in vitro* tumor models have become a focus of research in cancer cell biology and enormous advances have been made in the development of these systems. To date, numerous types of 3D *in vitro* tumor models have been developed and can be principally distinguished, based on tumor cell sources (cancer cell lines, or patient tumor tissues), culture methods, and time required for establishment. Among them, most extensively *in vitro* 3D tumor systems described thought the literature include 3D MultiCellular Tumor Spheroids (3D-MCTS), tumorospheres, tissue-derived tumor spheres, organotypic multicellular spheroids, tumor organoids, and patient derived 3D models (Weiswald *et al.*, 2015; Boucherit *et al.*, 2020).

This article will focus on the potential of *in vitro* 3D-MCTS, an advanced tumor model, on which bases the author has been conducting her cancer research activity at Istituto Superiore di Sanità for more than twenty years. 3D-MCTS merit special attention, because they are balanced between complexity and reproducibly and, as a consequence, may represent the reliable tumor model to be incorporated into preclinical cancer research.

# 3D MultiCellular Tumor Spheroids

For too long, tumors have been investigated by using 2D cell culture models. It should be recalled that solid tumors grow in a 3D spatial arrangement resulting in a gradient distribution of

oxygen and nutrients, as well as other physical and chemical stresses, including antineoplastic agents. By contrast, in monolayer cultures the growth of cancer cells in a bidimensional 2D array is responsible for their equally exposure to oxygen, nutrients and anticancer treatments. Moreover, it is now amply recognized that the particular cell-cell and cell-microenvironment interactions in a 3D organization affect the tumor cell structure, adhesion properties, mechanotransduction, and signalling in response to soluble agents (Weiswald *et al.*, 2015). All these factors in turn pilot overall characteristics and behaviours of cancer in a way that differ deeply from conventional 2D flat cultures. These aspects are of extreme relevance because it results clear that 2D tumor cultures lack of numerous features that govern fundamental *in vivo* tumor cell processes, and as a consequence, experimental results obtained with these 2D tumor models often differ with large discrepancies to that obtained by using more *in vitro* realistic experimental cancer systems. In fact, it is becoming clearly evident that 2D tumor monolayers are inappropriate in coherently predict the real action of anticancer treatments, thus, invaliding seriously the research data obtained with 2D tumor models.

The crucial issue to design optimal *in vitro* cancer models that accurately capture the 3D complexity nature of tumor biology has urged scientists to create 3D tumor models (Lv *et al.*, 2017; Pinto *et al.*, 2020; Santini & Rainaldi, 1999; Weiswald *et al.*, 2015). To successfully mimic *in vivo* human cancer, the ideal 3D *in vitro* tumor systems should resemble not only the overall tumor cell-cell and cell-extracellular matrix interactions, but also the selective pressure that occur in the human body. This is an enormous and intricate challenge since it must be achieved the correct balance between the capacity of the 3D tumor models to faithfully reflect the distinctive tumor microenvironment, while enabling manageable analyses, low costs, and efficient high-throughput methodologies. At the same time, with focus on 3Rs principle, the more accurate the 3D tumor model is to spatial arrangement and pathophysiological characteristics of tumors, the more it could be relevant as alternative research tool to animal experimentation. Most 3D culture models, currently do not meet all these ideal criteria, but rather have their own limitations and strengths.

Among all several models for 3D tumor cultures, 3D-MCTS have attracted great attention and remain the most widely applied and well characterized 3D *in vitro* system of cancer (Huang & Gao, 2018). They are considered the "classical" 3D cell system in cancer biology and are seen as the gold standard models for anticancer treatment studies. 3D-MCTS are typically grown from cancer cell lines, but rarely from tumor tissue, too (Weiswald *et al.*, 2015). They can be generated from tumor cell alone (homotypic 3D-MCTS) or co-cultured with other cell types (heterotypic 3D-MCTS). However, not all cell lines are capable of forming compact MCTS, as some appear as loose aggregate even after prolonged culture period (Rainaldi *et al.*, 1999). Historically, development of 3D-MCTS was largely due to the pioneering work of Sutherland and co-workers in the early 1970s who demonstrated the validity of this model for fundamental research, including radiation biology studies (Weiswald *et al.*, 2015; Santini *et al.*, 1999). Since then, the application of 3D-MCTS in cancer research has grown exponentially and, surely, the major scientific attractive of this tumor model is its complex 3D network of cell-cell and cell-matrix interactions, and the fact that may contain an extensive extracellular matrix (ECM). In addition, it has been amply reported that they may resemble avascular tumor microregions and micro-metastases with respect to their growth kinetics, concentric arrangement of heterogeneous cell populations, pathophysiological gradients of nutrient and metabolic waste distribution, oxygen concentration, cell proliferation and drug access (Huang & Gao, 2018; Pinto *et al.*, 2020; Weiswald *et al.*, 2015). Indeed, larger 3D-MCTS (critical size, about 400-600 micron) sustain oxygen and nutrient gradients that often result in the formation of a necrotic core similar to those in poorly vascularized tumors (Lv *et al.*, 2017; Pinto *et al.*, 2015).

3D-MCTS are concrete and crucial 3D research tumor models at the Istituto Superiore di Sanità (ISS, the National Institute of Health in Italy), where the author has been using them in a systematic manner, since the late nineties of the last century, to face the complex challenge to investigate cancer with more realistic *in vitro* models. On this focus, 3D-MCTS models have been grown in our laboratory from numerous cell lines, such as, MCF-7 (human breast cancer), LoVo (human colon adenocarcinoma), A431 (human epidermoid carcinoma), MG-63 (human osteosarcoma), HT-29 (human colon adenocarcinoma), and CaSki (human cervical carcinoma), just to name a few. Through these years, different 3D spheroid culturing strategies have been used and well-established in our laboratory at ISS, specifically, anchorage-independent methods, namely, the gyratory rotation system and the liquid-overlay technique (Santini and Rainaldi, 1999; Rainaldi *et al.*, 1999; Santini *et al.*, 1999; Rainaldi *et al.*, 2003), and the anchorage-dependent technique using Matrigel. The core principle of the anchorage-independent methods is to promote MCTS formation by preventing cell adhesion to a surface. After an initial phase of aggregation, cells can start to secret their own extracellular matrix and, gradually, compacted spheroids are formed which start to growth. In our pioneering spheroid works, the gyratory rotation technique has been widely utilized to achieve this purpose. In this agitation induced system, a suspension of single tumor cells obtained from trypsinized monolayer was placed in glass Erlenmeyer flasks and gassed with an air/$CO_2$ mixture. The flasks were then placed in a temperature-controlled rotation incubator, and the culture was maintained until the spheroids reached the features required for the objectives of our cancer studies. However, over the years, the anchorage-independent liquid-overlay technique has played a central role in generating 3D spheroids in our laboratory. This 3D culturing approach involves placing trypsinized, monolayer-cultured single tumor cells in dishes covered with a non-adhesive surface, onto which cell adhesion do not occur and, within 1-3 days they form 3D spheroids. In our experimental protocol, a thin layer of agar has been usually preferred to accomplish this goal. It should be pointed out that, when adopting both these 3D culturing methods, the total tumor cell number, the amount of growth medium, the size of culture plates, and the rotation rate in the gyratory system must be carefully established since they are all critical elements in obtaining MCTS. Indeed, these factors are specific for each cell lines, and only their proper combination results in MCTS generation. In the last decade, the anchorage-dependent technique employing one of the most common naturally derived hydrogels, that is Matrigel, has been amply utilized in our laboratory to obtain different types of 3D tumor models. In our experimental procedure, a single-cell suspension from tumor cell lines is resuspended in the growth medium mixed with a desired percentage of Matrigel. This cell suspension is plated onto a layer of previously solidified Matrigel, and the growth medium is then added to each sample. This protocol favors the growth of spheroids partially embedded into Matrigel, making them particularly advantageous 3D models for diverse cancer research studies conducted in our laboratory, including that focused on co-cultures with immune cells. However, due caution should be exercised when adopting this method. In fact, it requires not only laborious preparation and Matrigel handling but, also with this technique, great care must be reserved to determine the total cell number, and the overall percentage of Matrigel and volume of growth medium used for each 3D sample. Again, all these factors exert a crucial role in the proper spheroid formation because they result specific for each cell line and, as a consequence, culturing protocols must be properly configured, otherwise 3D tumor spheroids will not form.

Over the years, 3D tumor spheroids have allowed our research group to make accurate observations about diverse aspects of the intricate tumor biology and its behaviour. In this context, of particular interest are our studies in which we demonstrate that 3D tumor spheroids of various histotype are markedly dissimilar with respect to the same cells grown in 2D monolayer in many morphological-functional features, such as, cell adhesion molecule expression, and response to Ionizing Radiation (IR) used in radiotherapy (Rainaldi *et al.*, 1999; Santini *et al.*, 2006a; Indovina

*et al.*, 2007). Since topics dealt with these studies are salient issues in the fight against cancer, our results have contributed to underline the crucial importance of utilizing pertinent and more realistic *in vitro* tumor models in cancer research investigations, that is the 3D spheroid models.

Certainly, MG-63 osteosarcoma multicellular spheroids (Figure 1) have been one of the better characterized and applied 3D tumor model used by our research group to investigate several cancer aspects, such as, adhesions and spreading properties (Indovina *et al.*, 2008), and the response to IR (Rainaldi *et al.*, 2003; Santini *et al.*, 2006a; Indovina *et al.*, 2007). In particular, as regards the latter point, along with important morphological analysis aimed to reveal the underlying cell death characteristics, the integrative use of the high-resolution proton nuclear magnetic resonance (1H-NMR) spectroscopy approach has played a notable role in our understanding the features and dynamics of spheroid response to IR antitumor treatments (Santini *et al.*, 2006a; Santini *et al.*, 2006b). Furthermore, in order to gain major insight into the complex tumor biology and its behaviour, our experimental activities have also taken advantage of the use of co-cultures between MG-63 spheroids and primary non-tumor cells (Indovina *et al.*, 2008).

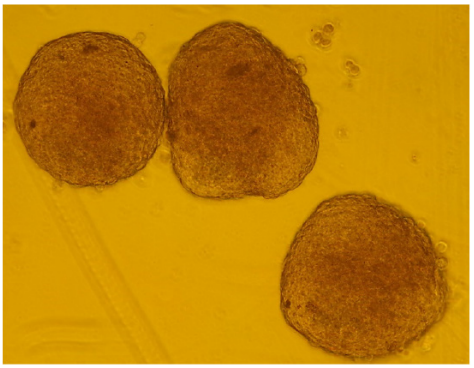

**Figure 1. Light microscopy micrograph of MG-63 osteosarcoma spheroids after 72h of growth**

HT-29 spheroids have been another 3D tumor model extensively adopted and dissected in our cancer research studies at ISS (Santini & Rainaldi, 1999; Rainaldi *et al.*, 1999; Santini *et al.*, 1999; Ferrante *et al.*, 2006). Of special interest, a spatial-temporal sequence of events leading to spheroid formation in HT-29 cells has been observed. In fact, as reported in our works, HT-29 cells first form round-multicellular aggregates by about 48h of culture, and then well-rounded and compacted spheroids by about 72h. At 5 days of culture HT-29 spheroids become highly compacted with well-defined margins. More recently, we have been focusing in a deeper investigation of the internal ultrastructure of HT-29 spheroids by using transmission electron microscopy (TEM). TEM analysis confirms that HT-29 spheroids at 72h of growth appear as 3D well-formed structure composed of numerous intimately-compacted cells (Figures 2A-D). It is interesting to note that at this time of growth, the cellular interrelationships are so highly organized that different cell junctions can already be clearly identified. In particular, the outer edge of spheroids is characterized by a series of tight junctions (TJ) that define the internal microenvironment from the outside one (Figures 2A-C). However, these junctions appear increasingly frequent by moving from the edge toward the more compacted center of spheroids, where cells are more closely packed (Figures 2A-B). Adherent junctions (AJ) (Figure 2C), and desmosome (Figure 2D) are also easily detectable in these spheroids. More in general, in HT-29 spheroids at 72h of growth, there is a clear concentric organization, characterized by proliferating cells in the peripheral area (mitotic cell in Figure 2A), and the gradually disappearing of the intercellular spaces toward the spheroid core (Figures 2A-B). Ultrathin sections of the HT-29

spheroids at 5 days of growth (Figures 2E-G) show an extremely dense 3D spatial structure, especially in the central area where there are very few intercellular spaces and the cellular boundaries are hardly recognizable (Figure 2E). In some cases, it can be observed lumen-like structures rich in microvilli and characterized by TJ that well define the internal/external zones (Figures 2F-G).

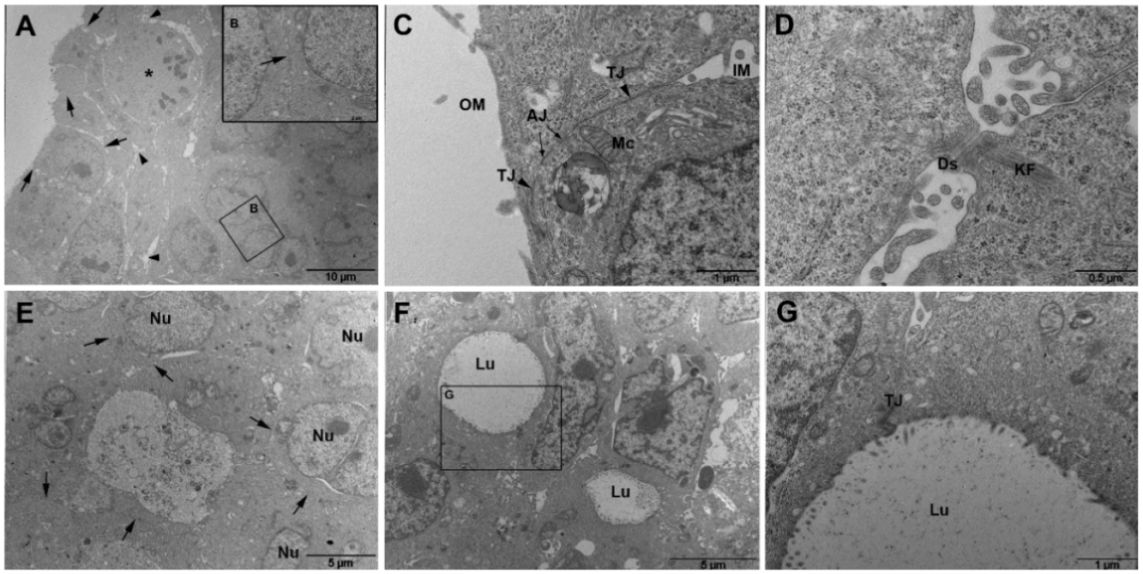


**Figure 2. Representative transmission electron microscopy micrographs of HT-29 spheroids at 72h (A-D) and 5 days of growth (E-G): A. HT-29 spheroids at 72h of growth are characterized by an edge zone in which are evident the abundance of microvilli that extend both on the outer membranes and in the intercellular spaces (black arrowheads), the presence of TJ (black arrows), and a mitotic cell (asterisk). B. Inset of a TJ between two cells at high magnification. C. AJ and TJ between two cells at the edge of spheroid define the outer (OM) and inner (IM) microenvironments. Mc: mitochondrion. D. A plaque desmosome (Ds) linked to numerous keratin intermediate filaments (KF). E. The core of HT-29 spheroids at 5 days of growth is characterized by a considerable reduction of the intercellular spaces due to the progression of cellular junctions and the resultant increase of the 3D structure solidity. Black arrows shown the intercellular boundaries that are hardly recognisable. Nu: nucleus. F. Two lumen-like structures (Lu) in an internal zone between the cells. G. High magnification of the rectangle area in F showing the microvilli protruding into the lumen and one of the TJ that defined the lumen space**

In recent years, the above described Matrigel-based anchorage-dependent technique has been routinely applied in our laboratory to create different *in vitro* 3D tumor models, that is, those formed from human colorectal adenocarcinoma Caco-2 and human cervical carcinoma CaSki cell lines, and from human B lymphoma Karpas 422 cell line, too. As a selected example of 3D tumor models grown in our experimental activities by using this culturing system, scanning electron microscopy micrographs of CaSki spheroids, after 14 days of growth, are depicted in Figure 3. As can be seen, CaSki cells form tightly compacted spheroids, and it is extremely difficult to discern individual cells from each other in their 3D architecture.

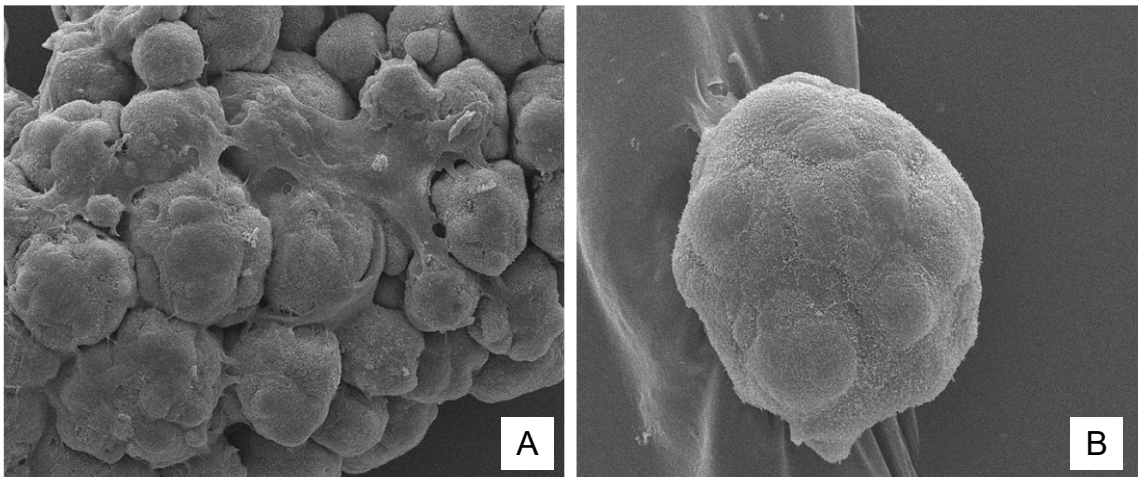


**Figure 3. Scanning electron microscopy micrographs of human cervical carcinoma CaSki 3D spheroids after 14 day of growth using the Matrigel-based anchorage-dependent technique. The tight compaction and close interrelationships between tumor cells are clearly visible in these spheroids. Original magnifications: (A) 1200 x; (B) 2400 x**

The long-lasting research activity and experience gained with *in vitro* 3D tumor spheroid at our ISS laboratory is well in line with two basic concepts that should never be minimized in the experimental world of MCTS: i) different cell types may form spheroids in a diverse manner, and ii) specific temporal dynamic of events leading to spheroids formation can be observed in different cell types. As a consequence, great care should be given to the choice of the cell type to use for a specific cancer study. For example, in those 3D models where compacted spheroids do not form directly, but rather pass through previous 3D aggregate form phases, the appropriate culture time for experimentation should be carefully determined. With reference to HT-29 spheroid growth dynamic described above, in our previous paper we have demonstrated that the compaction cell degree of these spheroids influences their response to IR, thus impacting in spheroids resistance to this stressing agent (Ferrante *et al.*, 2006). On the other hand, another important issue for which the culture time of spheroid formation should be critically evaluated is that larger spheroid, in culture for a longer time, may show a necrotic centre that may interfere with the purposes of our research. However, in MCTS, it is possible to control not only their size, but also the formation of the necrotic central area. This is especially important because apposite spheroids tailored to the specific experimental aims may be correctly designed. Last but not least, the success or failure of tumor spheroid formation may depend on whether, or not, the appropriate 3D method is used.

From all these considerations, it appears clear that 3D spheroid models and methods of growth should be critically evaluated before beginning a study. In this complex 3D experimental scenario, cancer study aims not only dictate which methods is the most appropriate, but, the selection of the correct spatial-temporal frame at which to conduct our experiments may influence in a decisive manner the success of the results obtained.

# Conclusion

In this era of ever-expanding innovative antitumor strategies, in which the biological challenges to address are increasingly intricate, it has become clear that the use of more sophisticated *in vitro* tumor models is indispensable to coherently predict the efficacy of anticancer treatments. In this context, 3D-MCTS are currently considered an extremely attractive 3D tumor model for antineoplastic agent evaluation in oncology fields. The advent of 3D tumor models constitutes an important step forward in cancer research. In fact, these cancer models may fill the gap between traditional 2D cell cultures and *in vivo* animal models, thus, helping to reduce the use of animals with cost and ethical benefits. For now, animal experiments cannot be completely avoided and, therefore, they are still not fully replaceable and continue to be essential for cancer research. However, by producing more realistic data, 3D tumor models could be integrated in the research workflow and may provide robust and economical preclinical insights. Although research on these topics may be experimentally quite complex, every effort should be made to conduct cancer research so that more precise information regarding the effects of antineoplastic treatments can be obtained.

## Acknowlegments

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# *IN VITRO* INTESTINAL BARRIER MODELS: ARE THEY READY FOR STANDARDIZATION?

Olimpia Vincentini (a), Valentina Prota (b), Isabella De Angelis (b)
*(a) Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy*
*(b) Department of Environment and Health, Istituto Superiore di Sanità, Rome, Italy*

## Introduction

*In vitro* model of intestinal barrier formed by differentiated Caco-2 cell line grown on permeable insert is a well consolidate model widely applied in pharmacological and toxicological studies. However, several critical issues are ascribed to this model mainly due to its simplification in respect to the complexity of the human intestinal barrier *in vivo*. To date an improvement of the model has been proposed by co-culturing Caco-2 cells with other human cell lines relevant for intestinal barrier functions. Some studies have been carried out at ISS in order to develop a reliable model of intestinal barrier, suitable for regulatory purposes.

In this report, characteristics and limitations of the Caco-2 based intestinal *in vitro* model are described, in order to provide an overview of the state of the art, with a view to a future standardization of the model.

## Epithelial barriers

Epithelial barriers represent the defensive system of the organism since they separate in a selective way the external body surfaces from the organ luminal compartments. Epithelia actively regulates solutes and nutrients exchange between different body districts and simultaneously prevents the entrance of pathogens and harmful substances. To fulfil this mandate, epithelial cells have a polarized structure and are sealed each other by tight junctions (TJ). Many advanced *in vitro* models of epithelial barriers are now available (as skin, corneal, lung, intestinal, renal, blood-brain, placental) gaining relevance as an alternative to animal experiments in risk assessment, pharmacokinetic, and toxicological studies.

*In vitro* barrier models are generally achieved by culturing epithelial cells on permeable inserts that allow to separate the apical compartment, facing the luminal environment, from the basolateral compartment, facing the mucosal tissue. However, barriers are not only constituted by epithelial cells but an integration of different cell types contributes to carry out their peculiar functions. In particular, in the intestinal barrier enterocytes absorptive cells are the most abundant but, goblet cells, endocrine cells and M cells are also present (Lozoya-Agullo *et al.*, 2017).

Several *in vitro* approaches have been developed to assess the intestinal permeability and absorption of drug/chemical substances. *In vitro* techniques for permeability assessment are less laborious and expensive compared to *in vivo* animal studies and offer benefits in terms of ethical considerations. Absorption results obtained with these *in vitro* systems have shown an overall good correlation (Artursson *et al.*, 2021). The successful application of *in vitro* system to predict drug/chemical absorption depends on how closely the *in vitro* model can mimic the physiology and functionality of the *in vivo* intestinal barrier (Balimane *et al.*, 2000).

Although traditional cell cultures are limited models, being unable to predict multifactorial processes such as bioavailability or immune responses, there are clear advantages of using *in vitro* models for adsorption and translocation studies. On the other side, it is important to improve existing intestinal barrier models using advanced approaches to obtain reliable data. (Antunes *et al.*, 2013; Youhanna *et al.*, 2021).

# *In vitro* model of intestinal barrier

## Caco-2 monoculture model: characteristics and limits

The most widely used *in vitro* model for intestinal barrier is represented by the Caco-2 monolayer. Although these cells derive from a human colon carcinoma, they display features of the enterocytes expressed at 21 weeks of intrauterine gestation. In fact, Caco-2 cells are able to polarize and differentiate in long term cultures on permeable filter support (transwell system), acquiring morphologic and functional characteristics of the enterocytes, such as microvillous structure on the apical surface, mature tight junctions, hydrolysing enzymes and carrier-mediated transport systems for sugars, amino acids, and drugs (Sambuy *et al.*, 2005).

Caco-2 cell model has been accepted as reliable model for prediction drug intestinal permeability in humans by pharmaceutical companies and regulatory authorities because high correlation was reported between Caco-2 permeability coefficient and human absorption data, particularly for molecules transported by passive paracellular mechanisms (Shah *et al.*, 2006). Furthermore, good reproducibility was obtained in inter-laboratory comparison studies confirming the robustness of the model for permeability studies (Prieto *et al.*, 2010).

However, Caco-2 model presents several limitations mainly imputable to:

- lack of complexity of the intestinal epithelium environment composed of multiple cell types such as mucus secreting goblet cells, endocrine cells, M cells and immune cells;
- tighter TJ in respect to small intestine, resulting in less permeability for hydrophilic compounds via the paracellular absorption pathway;
- overexpression of P-glycoprotein which may lead to higher secretion rates of foreign molecules and consequently to their lower absorption;
- lower permeability of compounds transported via carrier-mediated absorptive pathways, compared to the human small intestine, probably due to the colonic origin of this cell line.

In recent years more realistic *in vitro* models of intestinal barrier were developed co-culturing different intestinal-derived cell types, to better mimic the intestinal barrier environment. Consequently, modifications and improvements of Caco-2 monoculture need to be investigated to generate a more predictable and robust model of barrier.

## Relevance of mucus: Caco-2/HT-29-MTX co-culture

Over the past years, it has been proposed to co-culture Caco-2 cells with HT29-MTX mucus secreting cells. HT29 cells, obtained from human colon adenocarcinoma, are differentiated into mature goblet cells using methotrexate (Lozoya-Agullo *et al.*, 2017). As Caco-2 cells, HT29-MTX can be maintained in culture for about three weeks forming on insert supports a polarized monolayer with sparse and short microvilli (Araùjo & Sarmento, 2013).

The main characteristic of the globet cells *in vivo* is the secretion of mucins that, forming a shield of mucus along the gastrointestinal tract, plays a relevant protective role against the ingested molecules. Indeed, mucus protects the intestinal epithelium by separating pathogens and

toxic substances from the epithelial cells, also preventing intestinal inflammation processes (Hansson, 2012). Mucus can also affect chemicals and particles mobility through the intestinal barrier, so mucus absence in *in vitro* models could lead to an overestimation of the translocation process. Moreover, HT29 TJ are looser than those established by Caco-2 cells, i.e., more similar to the TJ established by enterocytes of the small intestine *in vivo*.

Consequently, co-culture of Caco-2/ HT29-MTX cells is closer than Caco-2 monolayer to human small intestinal epithelia, both protecting the cellular layer through the presence of mucus and increasing its permeability *versus* ingested substances.

On this respect, the fraction of globet cells in the co-culture is crucial. A ratio of 9:1 Caco-2/ HT29-MTX seems to be the best co-culture condition (Georgantzopoulou *et al.*, 2016).

### Promoting M cells differentiation: induction by Caco-2/Raji B co-culture

The Caco-2 based intestinal barrier model was also improved co-culturing Caco-2 with Raji B cells, a B lymphocytes cell line derived from a human Burkitt's lymphoma. It has been demonstrated that Raji B cells, added in the basolateral compartment of the transwell system, are able to induce, by soluble factors not yet completely identified, the M (Microfold) cell phenotype in differentiated Caco-2 cells (des Rieux *et al.*, 2007). M cells are characterized by the presence of few and irregular microvilli and a decreased glycocalyx, (García-Rodríguez *et al.*, 2018), and, even if represent only the 10% of follicle-associated epithelium in the human intestine (Ude *et al.*, 2019), they play a crucial role in antigen sampling and in the endocytic absorption of particulate matters as bacteria, viruses, nano and microparticles.

Morphological analysis by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) is the main tool to verify M cells induction. Furthermore, other possible markers for the detection of M cells have been identified, but due to their low specificity for these cells, they are still controversial.

### Increasing the complexity: the Caco-2/HT29-MTX/Raji B tri-culture

The simultaneous presence in a co-culture system of the three cell lines previously described represents the next step for reproducing intestinal epithelium physiology. When cultured together all these cell lines are able to maintain their characteristics and functions mimicking even more the interactions which underpin barrier functions of intestinal epithelium (Antunes *et al.*, 2013; Araújo & Sarmento, 2013). In the set-up of the tri-culture model it is important to preserve the right proportions between the different cell lines, according to the *in vivo* situation, and the right time scan of the co-culturing process, particularly for which concern Raji B cells addition.

Due to the interplay of many and complex factors, the tri-culture model need of a careful characterization before to be applied for permeability studies. In this perspective lymphocyte-enterocyte cross talking, markers of M cells identification, and mucus production characterization are the aspects that more than others require further investigation and standardization.

## Towards the tri-culture model standardization

Any advanced *in vitro* model must balance complexity and robustness, i.e. should be complex enough to mirror the *in vivo* situation but simple enough to be reproducible and transferable. Consequently, a careful definition of the main model parameters as well as of the experimental

conditions is an essential requirement for Non-Animal Methodology application in the regulatory context.

At ISS, in the framework of projects founded by the Italian Ministry of Health and EU, the tri-culture model Caco- 2/HT29-MTX/Raji B cells has been recently implemented. Caco-2 and HT29-MTX were seeded in a 9:1 ratio in the apical compartment of permeable insert, allowing Caco-2 enterocytic differentiation and mucus production. At day 14, 16 and 19 of co-culture Raji B cells were seeded in the basolateral compartment to induce M phenotype in Caco-2 cells (Figure 1).

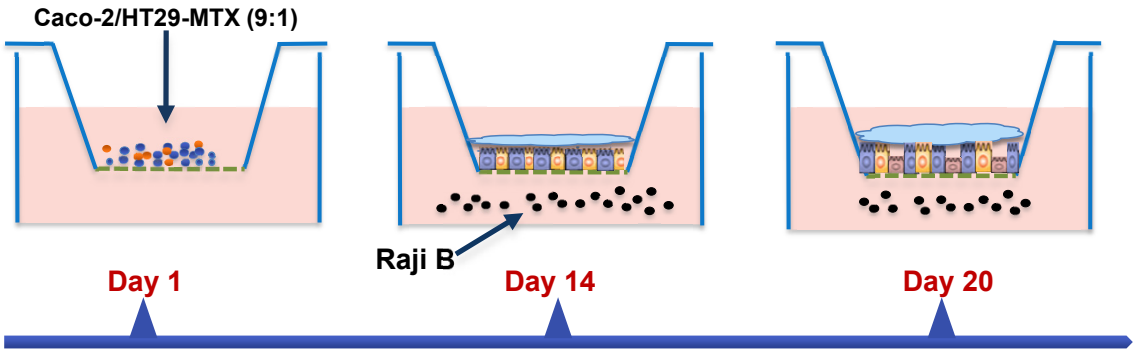


**Figure 1. Experimental design for Caco-2/HT29-MTX/Raji B triculture *in vitro* model**

After about 20 days the tri-culture has been characterized for the following parameters (Table 1):

- barrier integrity, measured by transepithelial electrical resistance (TEER) and by tight junction evaluation, determined by Zonula occludens (ZO-1) expression;
- permeability by paracellular passage of fluorescent markers as Lucifer Yellow or FITC-Dextran;
- M cells phenotype characterization, observed by SEM and TEM analysis and expression of markers;
- mucus production and characterization, established by Alcian Blue or Periodic Acid Schiff staining, ELISA assays of released mucins MUC2 and MUC5 AC and confocal microscopy detection of MUC2 and MUC5 AC.

**Table 1. Endpoints analyzed on day 20 of co-culture and respective methods of detection**

| Endpoint | Methods |
|---|---|
| **Barrier integrity** | TEER |
| | ZO-1 |
| **Permeability** | Lucifer Yellow |
| | FITC-Dextran |
| **M cells characterization** | SEM |
| | TEM |
| | Paracellular markers |
| **Mucus production and characterization** | Alcian Blue |
| | Periodic Acid Schiff |
| | ELISA and immunohistochemistry of MUC 2 and MUC 5 AC |

# Conclusion

Caco-2 monoculture does not entirely represent the complex environment of the intestinal barrier. Thus, the tri-culture model Caco-2/HT29-MTX/Raji-B, improving the physiological relevance of the *in vitro* model, has received growing interest for the qualitative/quantitative assessment of chemicals and drugs absorption and translocation processes.

Although the model has been widely described in literature (des Rieux *et al.*, 2007; Antunes *et al.*, 2013; Araújo *et al.*, 2013), its characterization is still ongoing and some criticalities need to be solved. The tri-culture model has shown good performances in terms of stability and reproducibility, it could therefore be considered a reliable alternative to animal test for the investigation of chemicals gastrointestinal barrier's interaction/adsorbption. Due to its relevance, further effort should be done in order to standardize this *in vitro* model, with the aim to make it suitable for regulatory purposes.

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# REPLACEMENT STRATEGIES IN FISH ECOTOXICOLOGY

Mario Carere, Ines Lacchetti, Laura Mancini
*Department of Environment and Health, Istituto Superiore di Sanità, Rome, Italy*

## Introduction

Ecotoxicology represents a relatively young subject that has the aim to study the adverse effects of environmental chemicals at molecular, cellular, individual and population level. There is a growing recognition that for sustainable development it is necessary to ensure prevention of the adverse effects on environment and human health of traditional and emerging chemicals (Mancini & Zapponi, 2002, Bourdeau *et al.*, 1989).

Ecotoxicology will play a key role in the recent European Strategy on chemicals sustainability that gives a series of urgent actions in order to achieve a toxic-free environment (European Commission, 2020).

The need for rapid test systems to assess the toxic outcome, if any, of chemicals designed and introduced in the market has led to a variety of use of laboratory animals.

The European Commission reports the statistics on the use of animals (in total 10.6 million used for scientific purposes in the Member States of the European Union in 2018, including Norway) (European Commission, 2021) under Directive 2010/63/EU (Europe, 2010): fishes are the second most frequent group of animals used after mice, with a percentage of 26%, and with a growing trend in comparison to the previous report.

As in previous years, in relation to the testing carried out to satisfy regulatory toxicity, safety and efficacy information requirements, in general for all the animals used (included fishes) the main topics in which animals were used concerned medicinal products for human use (64%), veterinary medicinal products (16%) and industrial chemicals (8%).

The main fishes used were zebrafish (17%) followed by salmon, cod and seabass. These data are probably an under-estimation because, for example, for environmental monitoring the statistics are difficult to be collected due to a lack of a reporting systems. Ecotoxicological methods can be applied for different purposes: to evaluate the toxicity of single substances also in a regulatory context or to use them as monitoring methods for aquatic ecosystems (Effect-Based Methods).

In the context of Regulation such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) or, for example, in the environmental risk assessment of veterinary pharmaceuticals ecotoxicological tests (European Union, 2008) the tests on fishes represent a relevant part of the risk assessment. For fish risk evaluation, adult fishes are usually used (e.g., *Danio rerio* or *Oryzias latipes*) for acute or chronic tests.

A key piece of information required for the environmental risk assessment is indeed the concentration at which a chemical causes an adverse effect, in particular the so-called NOEC (No-Observed-Effect-Concentration). This value is used to derive PNECs (Predicted No Effect Concentrations). In environmental risk assessments, PNECs are compared with PECs (Predicted Environmental Concentrations) to establish whether there is any environmental risk. PNECs can be estimated from acute or chronic data.

For environmental monitoring also *in vivo* bioassays with fishes (Effect-Based Methods) are used to evaluate the quality of surface waterbodies together with other aquatic organisms in order to cover all the trophic levels of an aquatic ecosystems.

The species of fishes used for environmental monitoring are several including also marine waters:

- *Oncorhynchus mykiss*;
- *Dicentrarchus labrax*;
- *Oryzias melastigma*;
- *Danio rerio*.

These methods, also called Effect-Based Methods (bioassays *in vivo* and *in vitro*, biomarkers) can have several objectives, for example in the context of the Water Framework Directive of the European Union (EU WFD) (Europe, 2000):

1. as screening tools, as part of the pressures and impacts assessment to aid in the prioritisation of water bodies;
2. to establish early warning systems;
3. to take into account the effects from mixtures of pollutants or not analysed chemicals (e.g., to support investigative monitoring where causes of a decline of specific species are unknown);
4. to provide additional support in water and sediment quality assessment, though not as a substitute for conventional chemical and ecological monitoring under the EU WFD.

Effect-Based Methods are tools used for the monitoring of waterbodies and other environmental compartments with the aim to detect effect caused by pollutants or group of pollutants, these effects can be detected at molecular, cellular, individual or population level (Wernersson *et al.*, 2015).

Effect-Based Methods measure the toxicity of environmental samples, generally under defined laboratory conditions, using a common procedure to measure toxicological endpoints at organism level or in simpler biological organizations. They are preferably applied in a battery, using a large number of test species from several taxa, and across the main ecological or trophic positions (i.e., from bacteria to fish, and from decomposers to final consumers).

Bioassays *in vivo* are tests in which whole living organisms are exposed to environmental samples like surface water, sediment, wastewater, dredge material or extracts from these samples. The endpoints for fishes can be different: mortality, effects on reproduction, effects on growth of individuals, metabolic changes, behavioural changes.

The frequent use of the adult fishes has given rise to ethical concerns since the early 1980s and newer alternative assays are deemed to be more applicable to long-term hazard identification and risk assessment in an environmental context. With the implementation of the European chemical Regulation REACH, there is a clear mandate to strongly promote the development of alternative methods according to the 3Rs principle for "Replacement, Reduction, and Refinement" and to preferentially use data generated by alternative methods whenever validated methods are available.

According to the European Union Directive 2010/63/EU on the protection of animals in research, animals are not protected in their early stages of life. For fish, this can last until 120 hours post-fertilization when they have developed a digestive tract and swim bladder, can ingest food and have the ability to swim and all of the yolk has been consumed, so they have an independent feeding.

In the last years, in particular for fishes, are becoming popular new tests that consider only the early stages, these tests have been proven to have the same reliability of the acute test with adult

fishes and are now used in a wide variety of ways for evaluate the risks of a chemicals and for environmental monitoring.

Given its excellent correlation with the acute adult fish toxicity test and the fact that non-feeding developmental stages of fish are not categorized as protected stages purposes, the FET (Fish Embryo Toxicity) test has been widely used (OECD TG n. 236) (OECD, 2013) for different purposes. Germany replaced adult testing in 2003 by a standardised 48h test with zebrafish (*Danio rerio*) embryos (Braunbeck *et al.*, 2015).

# Zebrafish embryos as adult model alternative

Zebrafish embryo models have been especially successful in drug discovery research for human diseases (Cassar *et al.*, 2020).

Zebrafish assays using larval zebrafish could be used to replace some animal toxicity studies, first establishing that the larval zebrafish is a relevant model for the system (target, gene, pathway, mechanism, tissue, organ, etc.) with validation studies.

It has its wide acceptance and popularity as a replacement model due to many scientific attributes such as small size, ease of maintenance, low cost, rapid growth rate, high fecundity rate, external fertilization, optical transparency of the embryo, ease of genetic manipulations, high genetic similarity to humans and regenerative capacity (Lammer *et al.*, 2009).

At some stages of research, it is possible to use zebrafish larvae to replace higher-order animals (mice, rats, etc.) to study targets and mechanisms in whole organisms. Forty-eight hours after fertilization, the larvae develop a complete central nervous system, as well as endocrine, gastro-intestinal and cardiovascular systems. This allows simultaneous monitoring of behaviour and various physiological parameters.

To use zebrafish embryos can represent an effective way to implement partial replacement: this method makes it possible to evaluate compounds early in drug discovery in lower-order animals, instead of rodents and adult fishes (Strahle *et al.*, 2012).

Additionally, as zebrafish embryos are transparent and develop in transparent eggs, significant amount of information can be collected during their development, before the fish becomes adult. Consequently, zebrafish embryos may also contribute to reduce the number of animals used for research (Sobanska *et al.,* 2018).

In a typical experiment running at the ecotoxicology laboratory of the Ecosystem and Health Unit of the Department of Environment and Health of the Istituto Superiore di Sanità (ISS, the National Health Institute in Italy), breeding fishes are maintained in tanks with a loading capacity of 1 L water per fish at 26 ± 1°C and with a fixed photoperiod of 12:12 (light: dark). Each test can be performed in three replicates. Zebrafish eggs can be exposed in 24-well plates at a developmental stage ranging from 32 to 128 cells of segmentation. Each well contains one egg in 2 mL of the sample. A plate control and internal control are prepared with the test medium. Embryos can be kept in controlled conditions for four days at 26 ± 1°C. The morphological observations of the embryos are made at 96h post fertilisation (Table 1).

Sublethal endpoints are also recorded to improve the evaluation of the sample toxicity with an enhanced level of detail.

The investigated sublethal endpoints can be: spine deformation, hatching delay, general underdevelopment, absence of pigmentation, eye deformation, tail deformation, fin deformation, low heartbeat, head skeleton malformation, oedema. All these endpoints are correlated to several effects.

**Table 1. FET (fish embryo toxicity) test: parameters from OECD Guideline (TG n. 236) as adopted in the ISS**

| Parameters | Description |
|---|---|
| **What is analysed** | Four apical observations are recorded daily as indicators of lethality, being coagulation of fertilised eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. Acute toxicity is determined based on the occurrence of any of the lethal observations, with calculation of $LC_{50}$ value. |
| **Tissue/cells examined** | Whole embryo, somites, tail-bud, heartbeat |
| **Method used** | It is possible to perform the test with exposure in 96-well instead of 24-well plate which reduces the amount of required sample for testing. |
| **Amount sample needed** | About 180 fertilized embryos for the main test (20 embryos per test concentration, and for the control conditions: dilution-water, solvent, positive, and plate controls). When performing a range finding test 10 embryos are recommended per concentration |
| **Possible to use stored samples** | Not for the acute test. Howeve by the end of test the zebrafish embryos might be stored for future analysis with additional methods |
| **Possible to use both in marine and limnic environment** | Zebrafish is a freshwater fish species. However, the FET method can be adapted to estuarine or marine species (e.g,. stickleback). A sediment contact protocol has been developed (Hollert *et al.* 2003) and validated (Feiler *et al.* 2013) |
| **When to take samples** | The test is initiated as soon as possible after fertilisation of the eggs |
| **Sublethal effects detected** | Neurotoxicity, embryotoxicity, genotoxicity, cardiotoxicity, teratogenicity, morphological changes |
| **Response time** | The test is conducted for 96 hours. |
| **Costs** | Low for the FET acute test, however that changes accordingly with complementary methods performed by end of test |

# Modes of action detected by zebrafish embryos

The analysis of multiple endpoints and the assessment of specific Modes of Action (MoA), i.e., toxicity mechanisms, induced by chemicals in zebrafish earliest life stages, allows a detailed detection of different chemical pollution effects. A broad literature review on the use of this method for detecting chemical pollutants in the context of the EU WFD has been performed (Cristiano *et al.*, 2019). In this study a total of 30 different effects at various levels of toxicity (e.g., morphological, molecular, physiological, and behavioural level) were reported. The three main MoA that we identified in this work were DNA toxicity (i.e., genotoxicity, mutagenicity and gene expression alterations), neurotoxicity and developmental toxicity, but also immunotoxicity, reproductive system impairment, oxidative stress and cell cycle impairment have been collected. Each MoA and the relative effects were then associated to the Priority and watch list substances of the EU WFD.

Considering the increasing numbers of environmental contaminants with potential neurotoxic potential, eco-neurotoxicity should be also considered in future risk assessments. In particular the number of potential neurotoxicants in the environment is raising and can pose a risk for humans and the environment (D'Amora & Giordani, 2018). An evaluation of neurotoxicity (including developmental stage) is also performed using non-mammalian species, because the mechanisms underlying the development and function of the nervous system are well conserved across the phylogenetic tree. Many of the basic molecular processes are identical in mammals and in non-mammalian species. Therefore, several alternative species including *Danio rerio* and *Oryzias latipes* are used as vertebrate non-mammalian models and complementary to *in vitro* approaches. The gathering of data from these multiple information sources could be used to develop Integrated Approaches to Testing and Assessment (IATA) designed in a fit-for-purpose manner for different regulatory purposes, including aquatic and human health protection. In particular, for neurotoxicity there are several possibilities to apply the zebrafish embryo model as an early warning system:

- *Locomotor activity* (i.e., swimming behaviour)
  It can be an important endpoint in neurotoxicity detection in zebrafish, with responses in most cases comparable to locomotion analysis in mammals): as in mammals, ethanol-treated zebrafish embryos showed altered locomotor activity (Selderslaghs, 2013).

- *Acetylcholinesterase* (AChE)
  Several studies support the use of zebrafish as a screening tool for neurotoxicity induced by chemicals on the cholinergic system (Koenig *et al.*, 2020). The cholinergic system is associated with several cognitive functions and cognitive processes. In this context, zebrafish is a useful and simple model for neurotoxic studies, as it has a well-conserved amino acidic sequence for the AChE gene.

- *Lateral tail movement*s
  They represent the first spontaneous behaviour observed in zebrafish embryos; at 28.5°C this phenomenon starts at 17 hours post fertilization (hpf), while at 26°C its occurrence has been observed since 23 hpf (Zindler *et al.*, 2019). The tail-coiling test, based on this principle, consists of the evaluation of spontaneous tail coiling frequency in zebrafish embryos, usually aged 24 to 26 hpf, as a possible indicator of neurotoxicity.

# Conclusions

The European legislation is rapidly progressing towards alternative methods in order to reduce the use of animals for laboratory purposes. Also, in the field of ecotoxicology new alternatives have been proposed and applied in research and for regulatory purposes.

In particular, in relation to fishes the possibility to replace the adults with early-life stages for both environmental risk assessment and environmental monitoring is progressing and advancing and high-throughput methods are becoming always more accurate with the possibility to detect MoA of different environmental contaminants also at very low levels and often in mixtures.

The use of zebrafish embryos, for his peculiar characteristics, can represent an excellent model that should be considered in the future in order to protect our ecosystems in compliance with ethical issues related to animal welfare, and application of the 3Rs principle.

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# ORGANOTYPIC BRAIN SLICE CULTURES AS TOOLS TO MODEL NEUROLOGICAL DISEASES AND FOR FUNCTIONAL SCREENING OF DRUGS

Roberta De Simone (a), Caterina Veroni (b), Luisa Minghetti (c), Maria Vinci (d),
Maria Antonietta Ajmone-Cat (a)
*(a) National Centre for Drug Research and Evaluation, Istituto Superiore di Sanità, Rome, Italy*
*(b) Department of Neuroscience, Istituto Superiore di Sanità, Rome, Italy*
*(c) Research Coordination and Support Service, Istituto Superiore di Sanità, Rome, Italy*
*(d) Department of Onco-Hematology, Gene and Cell Therapy, Bambino Gesù Children's Hospital, Rome, Italy*

## Introduction

The 3Rs approach (Replacement, Reduction, and Refinement) is at the basis of national and international legislation and regulations to improve welfare of animals used in research. Although *in vivo* animal models represent the most realistic platform for research and testing of therapeutic approaches, they may also imply stress and/or pain for the animals. This raises ethical issues and arouses intense debate on their necessity, justification, and acceptability. Moreover, *in vivo* studies require considerable financial and time efforts to set up, to be performed and analysed.

On the other hand, *in vitro* cell-based studies fail to replicate the conditions of cells in a tissue, sometimes weakening the predictive value of *in vitro* data. Recent advances in three-dimensional (3D) culture techniques have provided a bridge to fill the gap between *in vivo* procedures and *in vitro* methods, enabling the study of tissue and organ function *ex vivo*. Many successful examples of reconstituting organ and tissue function *ex vivo* are now available for most tissues and organs (Shamir & Ewald 2014), including brain.

Organotypic Brain Slice Cultures (OBSCs) substantially retain the morphological and physiological features of the brain region of origin, including cell-cell interactions, neuronal networks, and synaptic organization, and contain nearly all brain cell types, thus allowing to study different cell populations within their microenvironment.

These cultures can be successfully prepared from almost any region of the Central Nervous System (CNS); however, hippocampus, cerebellum and spinal cord have been so far the most widely studied for their clear implication in several neurological and neuropsychiatric disorders. Co-cultures of two or more regions that take part in a pathway, as well as whole brain coronal or sagittal slices, can also be established to mimic a more *in vivo*-like environment, although their long-term culturing is more challenging and requires specific medium adaptations.

OBSCs are typically prepared from early postnatal rodents (usually P5-8), as brain slices from embryonic and adult stages are more vulnerable to mechanical trauma during preparation and easily lose their cytoarchitectural organization in the long-term. These cultures are suitable for any kind of application and analytical technique (including molecular and biochemical assays, long-term live imaging, or electrophysiology), making them an excellent model system for several areas of neuroscience field.

OBSCs represent a valid method of partial Replacement, which includes "the use of primary cells (and tissues) taken from animals killed solely for this purpose (i.e., not having been used in a scientific procedure that causes suffering)", according to the definition formulated by the UK's National Centre for the Replacement, Refinement and Reduction of animals in research (NC3R).

OBSCs have also been prepared from *Drosophila*, in further fulfilment of partial Replacement (which also includes "the use of some animals that, based on current scientific thinking, are not considered capable of experiencing suffering"), and from human patients, in the fulfilment of the full Replacement principle. However, translational limitations of the circuitry modelled in the fruit fly ganglia in the first case, and technical and methodological limitations in the second, including poor and sporadic availability of human tissues, currently hinder their widespread use.

An additional benefit of OBSCs is related to Reduction, as multiple slices can be prepared from one single animal and used in different experiments, significantly reducing the number of animals to be euthanized compared to an *in vivo* experiment.

Moreover, experimental procedures causing distress and/or pain to the animal, including traumatic lesions, administration of drugs, exposure to neurotoxicants, induction of inflammation, neurodegeneration/neuropathology, demyelination, can be directly applied to the slices, in the fulfilment of the Refinement principle. The recent possibility of genetically manipulating slices after explant, by several transfection techniques, further increases the perspective for expanding the use of these models in neuroscience studies, according to the Refinement principle. For example, the use of recombinant adeno-associated virus (rAAV)-mediated gene delivery to selectively manipulate neurons, astrocytes, microglia, oligodendrocytes, or combinations thereof, now allows to explore cell-autonomous and non-cell-autonomous mechanisms in the different experimental conditions modelled by OBSCs.

Conversely, target innervation and the cross-talk with the systemic milieu and peripheral organs (e.g., pituitary and adrenal glands, intestine) are not represented in OBSCs; similarly, the absence of functional vasculature and intact blood-brain-barrier must be considered when considering drug candidates through to further pre-clinical testing. Future efforts in combining OBSC models with microfluidic engineering technology (such as in body-on-a chip systems) could be the key to increase the translational value of this powerful *ex vivo* platform. Other limitations of the OBSC model include the acute effects of axotomy and slice flattening over time, which can be at least partially overcome by optimization of temperature and incubation media. At the same time, post-dissection events per se can be studied as a model of brain injury and recovery.

Moreover, the initial inflammatory reaction spontaneously subsides after 10-14 days from explant, and the slices are ready to be exposed to different experimental protocols and analysed by biochemical, morphological, and functional assays after one or two additional weeks.

Here we provide some examples of the exploitation of rodent OBSC models, currently used in our laboratories, to investigate the complexity of the healthy and pathological brain.

## Organotypic hippocampal slice cultures as a model to study microglial functional properties and neuroinflammatory processes

Accumulating evidence is showing that neuroinflammation, mainly sustained by the resident immune cells microglia, is involved in a broad context of acute and chronic neurological diseases, including stroke, brain's response to traumatic injury, Alzheimer's disease, Parkinson's disease, epilepsy, Multiple Sclerosis (MS). Moreover, neuroinflammation is a key player in the development of cognitive and emotional dysfunctions in several psychiatric disorders, including anxiety and depression (Gilhus & Deuschl, 2019).

Besides its critical role in host defence and pathology, the brain immune system modulates homeostatic brain processes such as synaptic formation and scaling, long-term potentiation, and neurogenesis. Thus, aberrant microglial activation during critical windows of development may result in neurodevelopmental defects and increased vulnerability to inflammatory stimuli, setting the stage for adult psycho- and neuro- pathologies.

It is now largely accepted that microglia in response to different cues can acquire an array of different functional states, well beyond the classical pro-inflammatory and the alternative anti-inflammatory states, traditionally referred to as M1 and M2 phenotypes, respectively.

The acquisition of the different phenotypes is a dynamic process, whose modulation is likely related to diverse outcomes of microglial activation in terms of brain repair or damage. Another important concept is the existence of "innate immune memory" in microglia, whereby phenomena such as "priming" and "tolerance/sensitization" occur (*see* Neher & Cuningham, 2019 for a recent review).

"Priming" takes place when the exposure to an initial stimulus makes microglia more susceptible to a delayed secondary inflammatory stimulus, which can then trigger a heightened or exaggerated inflammatory response.

"Tolerance/sensitization" is characterized by suppression of pro-inflammatory gene expression on one hand and induction of anti-inflammatory genes on the other, in response to persistent stimulation.

There is still scarce information available on the trajectory of microglial activation in different pathological conditions, on the reversibility of different phenotypic commitments, on the ability to preserve the molecular memory of previous stimuli, and therefore on the functional consequences on the viability and fate of other brain cell populations.

OBSs provide an ideal tool to study these aspects, as they enable the analysis of microglial responses against any treatment in an *in vivo*-like microenvironment and, in parallel, of relative effects of each treatment on the properties of mature and immature neuronal and glial cells (astrocytes and oligodendrocytes), over extended time in culture.

As an example of such an application, we used Organotypic Hippocampal Slice Cultures (OHSCs) (Figure 1) from rat pups (P5-P6), prepared according to the membrane interface technique (350-400 μm thick), to get insight into the process of microglial activation in response to single vs repeated exposure to the bacterial endotoxin lipopolysaccharide (LPS).

LPS, along with several other components of pathogens and endogenous molecules produced in conditions of tissue damage, is specifically recognized by Toll like receptor 4 (TLR4), a key component of the innate immune system belonging to the Pattern Recognition Receptor (PRR) family; LPS is thus a prototypical inflammogen, widely used in studies on central and peripheral inflammation.

Differently from a single LPS challenge, persistent LPS stimulation of OHSCs elicited a potentially protective microglial response, characterized by the elevated expression of anti-inflammatory and neuroprotective molecules (Ajmone-Cat *et al.*, 2013), thus validating and extending, in a more complex physiological context, the data previously obtained in purified microglial cultures (Ajmone-Cat *et al.*, 2003; Cacci *et al.*, 2008). Thanks to their long-lasting viability (up to 5-6 weeks) and ease accessibility, OHSCs offered the possibility to study the consequences of these "acute" or "chronic" preconditioning paradigms on the response to a new hit. This approach evidenced the existence of mechanisms of molecular inflammatory memory that can be relevant in pathological brain conditions characterized by chronic or repeated exposure to pathological noxiae. Important microglial functional aspects, such as phagocytic activity (Figure 1c-d) and motility, could also be analysed into OHSCs (Ajmone-Cat *et al.*, 2013).

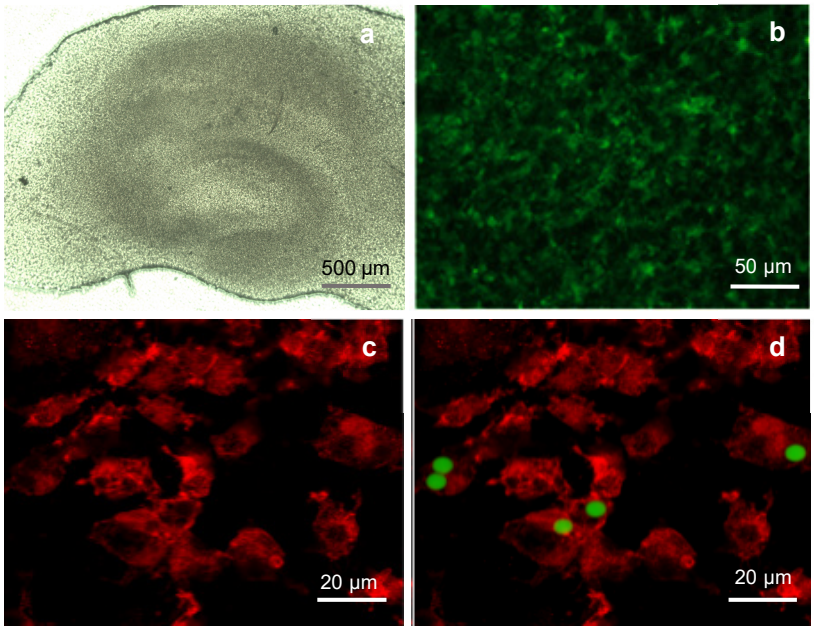


**Figure 1. Microglial reactivity in rat organotypic hippocampal slice cultures (OHSCs): a) Phase contrast microphotograph of organotypic hippocampal slice cultures (OHSCs) from rat pups (P5-P6), imaged at 2 weeks post-dissection. Scale bar = 500 µm. b) Fluorescent staining of microglia in unstimulated OHSC, labelled for the microglial marker Iba-1 (green). Scale bar = 50 µm. c-d) Confocal images showing the ingestion of fluorescent latex beads (6 µm-diameter, green) by Iba-1 (red) positive microglia in OHSCs (modified from Ajmone-Cat *et al.* 2013)**

By taking advantage of the same experimental model, we identified glycogen synthase kinase-3 beta (GSK-3β), a crucial regulator of TLR signalling, as a part of the molecular machinery involved in the adaptive response of microglia to repeated LPS stimulations. Inhibition of GSK-3β by the mood-stabilizer drug lithium chloride (LiCl) enhanced the process of microglial adaptation to repeated stimuli, favouring the acquisition of anti-inflammatory/protective functions and the extinguishing of pro-inflammatory ones. Oligodendrocyte maturation was higher in slices exposed to repeated LPS stimulation than in slice exposed to a single LPS or naive, and further enhanced in the presence of LiCl, as indicated by the expression of Gpr17 and Myelin Basic Protein (MBP), markers of oligodendrocyte maturation (Ajmone-Cat *et al.*, 2016). This and other evidence reinforced the – at that time – emerging idea that the outcome of microglial activation can be protective and favourable to regenerative/reparative mechanisms under specific conditions, and not univocally harmful, as it was previously conceived.

Collectively, this and other studies prove that OHSCs constitute a powerful *ex vivo* tool for dissecting diverse aspects of neuroinflammation at various cellular and molecular levels, and for testing compounds able to modulate microglial reactivity towards protective functions. OHSs, and OBSs in general, are attracting more and more attention due to the possibility of applying and combining new experimental approaches. For example, the application of genetic and/or pharmacological techniques to deplete microglia by OHSCs, followed by microglia replenishment with adoptive transfers, is providing additional insight into microglial biology (Coleman *et al.*, 2020).

More recently, OBSs (hippocampal and hippocampal-entorhinal cortex slices) from adult mice, including genetically engineered mice, have been employed to study aspects of aging-

related neuropsychiatric/neurodegenerative disorders (Humpel *et al.*, 2019), as well as for high throughput screening of potential agents working against such disorders. In addition, behavioural data from specific mouse strains can be correlated with data obtained from the slices of the same animals, such as molecular, functional, or morphological data, increasing the translational relevance of this experimental platform.

## Whole brain organotypic brain slice culture as a model to study tumor biology

Pediatric Glioblastoma (pGBM) and Diffuse Intrinsic Pontine Glioma (DIPG) are amongst the most aggressive tumors of the CNS, characterized by a significant degree of genetic and phenotypic intra-tumor heterogeneity, affecting children and young adults, for which there is no effective treatment (Vinci *et al.*, 2018).

Understanding the mechanisms that regulate the direct or indirect cell-cell communication within genetically and phenotypically distinct subclonal cell populations and with the brain microenvironment could allow the identification of effective therapeutic strategies against these fatal diseases.

We have recently developed a new model of whole brain organotypic slice cultures (wOBS; sagittal or coronal), based on an adaptation of the previously published hippocampal brain slice approach (Ajmone-Cat *et al.*, 2013), to establish a co-culture system consisting of wOBSs with pGBM or DIPG primary patient-derived cell lines (Figure 2).

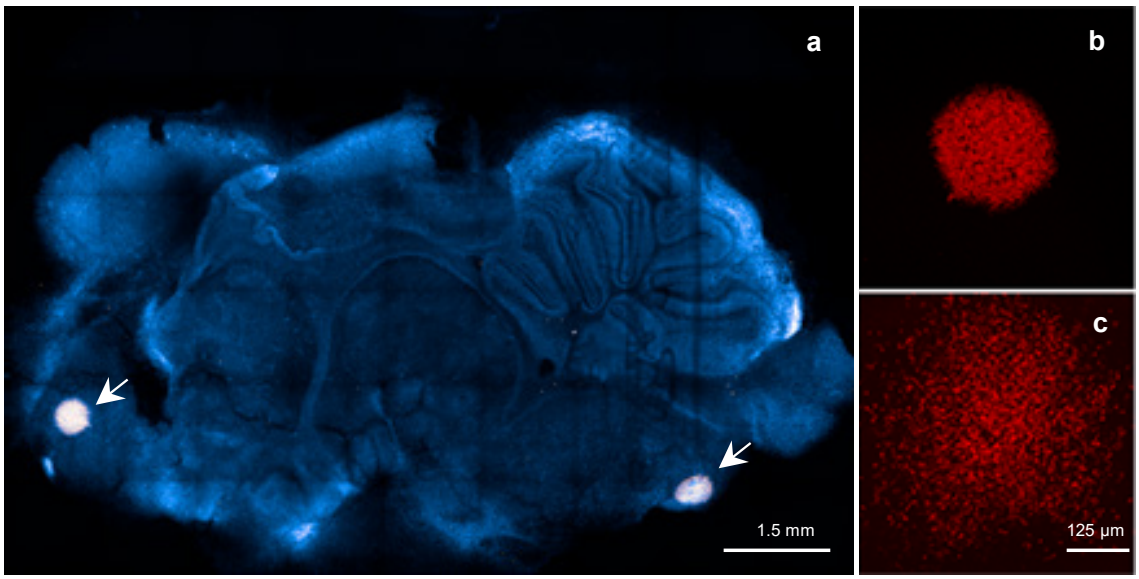


**Figure 2. Co-culture of whole brain organotypic slices with primary patient-derived glioma cells: a) In whole brain sagittal organotypic slice (wOBS; 350 µm thick) from CD1 mouse pup (PND 6–7), neurospheres obtained from a DIPG primary-patient derived cell line were implanted into the cortex and pons (arrows). The image was obtained by Operetta CLS (mosaic 10X). Up to six slices, complete of pons and medulla, can be obtained from each brain and cultivated for at least 3 weeks. b-c) Confocal picture of implanted cells at day 1 (b) and day 7 (c) post-implantation, stained with anti-human nuclei antibody**

Subsequently, multifluorescent marking technology was applied by Vinci's group to derive single cell-derived clones and study their heterogeneous invasion into wOBS once implanted as neurospheres in the pontine area. Different fluorescence analysis platforms (e.g., Confocal microscope, Operetta CLS and Digital slide scanner) were integrated to image and analyse the obtained DIPG/wOBS cultures (Pericoli *et al.*, 2020).

This model will enable future investigations into the cellular and molecular mechanisms of intra-tumor heterogeneity in pGBM and DIPG and will allow exploring how different clones can interact with the microenvironment, including tumor associated-microglia. Tests of promising anti-tumoral compounds, or their combination with emerging approaches such as CAR-T cells therapy, are underway in our laboratories using this model (de Billy *et al.*, 2021), opening new avenues to accelerate translational research in this field.

# Organotypic cerebellar slices as a model for the study of myelination/remyelination processes

Myelin sheath consists of lipid-rich membrane layers wrapped around axons, allowing rapid propagation of action potentials, providing metabolic support to neurons and maintaining axonal integrity. Axon myelination occurs after the differentiation of Oligodendrocyte Progenitors (OPs) in mature oligodendrocytes, the myelin-producing cells of the CNS, and implicates a multistep process finely tuned by intrinsic or environmental factors. Demyelinating disorders, characterized by a chronic or episodic destruction of the myelin sheath resulting in impaired neuronal function, are a leading cause of neurological disability with a major impact on quality of life and public health costs. In MS, a chronic inflammatory disease of the CNS, myelin damage is followed by spontaneous remyelination, however as the disease progresses, this recovery process ultimately fails and persistent demyelination with consequent axonal loss results in progressive and irreversible functional deficits (Franklin *et al.*, 2020). Currently approved therapeutic strategies for MS include a variety of anti-inflammatory and immunomodulatory treatments that effectively control the rate of relapse and delay disease progression; however, therapies that can promote remyelination are not yet available and the molecular and cellular basis of the myelin repair deficiency during the progression of MS are still unclear (Cunniffe & Coles, 2021). Understanding how oligodendrocytes initially produce myelin during development and remyelinate axons in the diseased CNS is of significant clinical interest, as it paves the way for novel possible strategies to stimulate endogenous OP differentiation as means of myelin repair and neuroprotection (Franklin *et al.*, 2020). In recent years, the molecular mechanisms regulating OP activation and differentiation into mature myelinating oligodendrocytes have been extensively studied using both *in vitro* and *in vivo* models, and a reasonable number of interesting drug targets and putative chemical modulators impacting remyelination have been identified.

Also, in this field of research, *ex vivo* cultures represent an excellent compromise between the advantages and disadvantages of both *in vitro* and *in vivo* systems described in the introduction. Cerebellar slice cultures from neonatal laboratory rodents have indeed emerged as a very useful and accessible model to study the molecular and cellular mechanisms regulating OP biology, as well as myelination, demyelination, and remyelination of axons, while maintaining cross-talk between neural cell populations in the context of a preserved cytoarchitecture (Doussau *et al.*, 2017).

It has been shown that slices prepared from post-natal day 7 rodent brains preserve a relative intact three-dimensional cellular environment and all stages of the oligodendrocyte lineage are maintained during subsequent development *in vitro*. In addition, MBP transcript increases over

time (Figure 3A, unpublished data) and partial myelination can be observed after 5 days *in vitro* as revealed by the significant number of MBP-positive processes aligned with axons (Figure 3B, unpublished data).

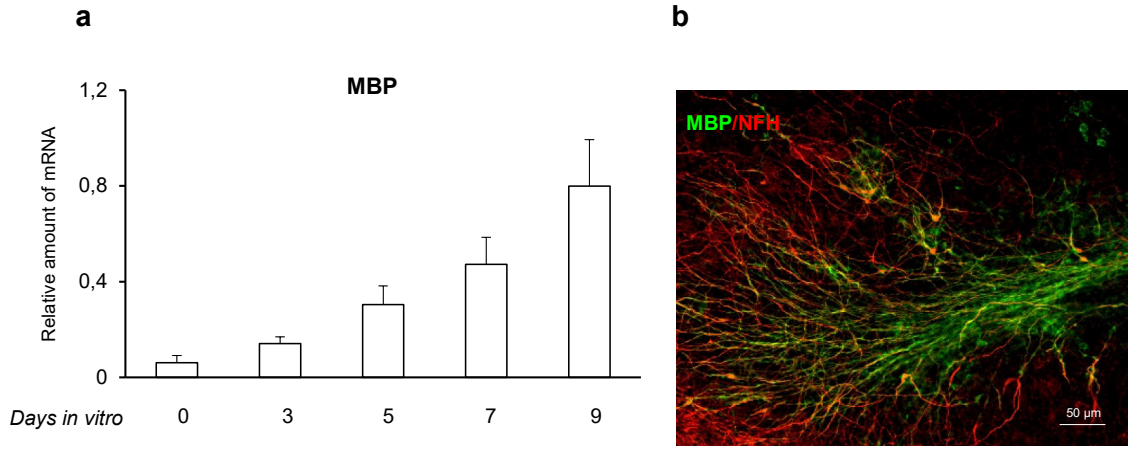


**Figure 3. Characterization of cerebellar slice myelination model: a) Expression of myelin basic protein (MBP) transcript in cerebellar slices during myelination: slices were prepared by 7-day-old mouse cerebellum and grown in culture medium. MBP transcripts increased linearly during the developmental period analysed. Data are expressed as induction of MBP mRNA normalized to GAPDH (2^-ΔCt) + standard error of the mean of 3 experiments. b) Immunohistochemistry characterization of cerebellar slice myelination model: myelin basic protein (MBP, green) and neurofilament heavy chain (NFH, red) staining in organotypic cerebellar slices prepared from P7 mouse cerebellum after 5 days in culture shows preserved neurons and myelin processes aligning with axons**

Based on this evidence, myelinating slices represent a powerful tool to study oligodendrocyte maturation and differentiation over time as well as their response to different stimuli influencing the first stages of myelin development. Demyelination of axons in cerebellar organotypic slices can be induced using the membrane-disrupting chemical lysolecithin, resulting in a rapid loss of myelin, with the subsequent return of myelin sheaths after toxin removal, suggestive of remyelination. This model has been extensively characterized both at protein and gene expression level demonstrating that after toxin removal and during the recovery phase OPs proliferate and differentiate in myelin-producing oligodendrocytes and macrophages are present and activated to clear myelin debris (Zhang *et al.*, 2011; Veroni *et al.*, 2020). Both myelination and demyelination/remyelination models can answer a number of questions. Indeed, they can be used for different experimental approaches, which include the study of factors that are regulated during the processes of myelin development, damage and repair, the analysis of the electrical activity and potential changes of neurons, the possibility to apply genetic manipulation by preparing slices from transgenic mice or using viral vectors and other innovative tools such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and small RNA interference. Recently, a model in which exogenous OPs are cultured in organotypic mouse cerebellar slices depleted of their endogenous oligodendrocytes has been described (Baudouin *et al.*, 2021), offering the challenging perspective of analysing human OP differentiation in an *ex vivo* environment.

Since targeting the endogenous regenerative process is a conceptually attractive approach to enhance remyelination, the lysolecithin model of demyelination has also been widely used to investigate the remyelinating potential of exogenous drugs as well as antagonists/agonists of receptors and proteins involved in OP differentiation. In this regard, in a recent study we have

shown that selective Tumor Necrosis Factor Receptor-2 activation by specific agonistic antibody enhances oligodendrocyte maturation and myelin protein expression and promotes a neuroprotective milieu in mouse cerebellar slices following toxin-induced myelin damage (Veroni *et al.*, 2020).

In addition, by acting as an important link between high-throughput *in vitro* approaches and animal models, lysolecithin treated slices have been also used in several drug repurposing studies, which hold great promise in the field of demyelinating diseases, leading to the identification of several modulators of remyelination (Cole *et al.*, 2017). In 2017, we performed an extensive screening of a library of 2000 drugs and other natural substances to test their remyelinating potential using *in vitro* models and validating the most promising compounds in organotypic cerebellar slice models of both myelination and demyelination/remyelination; we identified two molecules able to promote remyelination, namely the radical scavenger edaravone and the 5-methyl-7-methoxyisoflavone (Eleuteri *et al.*, 2017). These models may thus represent powerful approaches in drug screening studies, avoiding the use of animal models for the validation of a large number of compounds, showing sufficient throughput and enough biological complexity to allow pre-selection of compounds for further *in vivo* studies.

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# FAVOURING AN OPEN DIALOGUE BETWEEN RESEARCHERS AND REGULATORS ON NEW APPROACH METHODOLOGIES AND ANIMAL TESTING: THE ROLE OF THE EUROPEAN NETWORK PARERE

Emma Di Consiglio (a)*, Silvia Dotti (b)*
*(a) Department of Environment and Health, Istituto Superiore di Sanità, Rome, Italy*
*(b) Reference Centre for Alternative Methods, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy*

## Introduction

The need to develop new tools and increase the ability to evaluate the safety of a wide number of substances is an active area within the risk assessment procedure for both chemicals already present in the environment and those requiring pre-marketing approval. The final scope is to ensure a high level of protection of human and environmental health, while encouraging the use of New Approach Methodologies (NAMs) and supporting the development of additional NAMs to be used within several regulatory contexts.

In the toxicological field, in order to assess the (eco) toxicity potential of different chemicals (e.g., industrial chemicals, pesticides, biocides, pharmaceuticals and others), on the basis of scientific informed decision making and reducing or definitely replacing animal testing, a large number of *in silico*, in chemico and *in vitro* methods as well as whole alternative approaches (e.g., Defined Approaches) have been implemented. European (e.g., European Centre for the Validation of Alternative Methods, EURL ECVAM) and international (e.g., Organisation for Economic Co-operation and Development, OECD) bodies lead those processes to evaluate whether currently available valid NAMs can be international accepted and implemented for regulatory purposes, including their characterization, validation, standardization and harmonisation into Test Guidelines (TGs).

Increasing the knowledge and the confidence on the existing alternative strategies, following the 3Rs (Replacement, Reduction and Refinement) principle, is another key aspect to be considered not only among scientists in academia and regulators, but also at the educational level, e.g., in secondary schools, academic and vocational training.

## European regulatory framework

The impact of the Directive 2010/63/EU (2010) on the protection of animals used for scientific purposes, on other pieces of the legislation of the European Union (EU), such as Regulation (EC) 1907/2006 for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and regulations for the authorisation of active substances and products, has provided specific rules concerning the use of alternative strategies, whenever possible – e.g., Regulation (EC) 1107/2009 on Plant Protection Products; Regulation (EU) 528/2012 on Biocides; Regulation

* National Coordinators of the Italian Network PARERE

on Medicinal products; etc.– or as unique possibility (i.e., Regulation (EC) 1223/2009 on cosmetic products) or the avoidance of duplication of studies on vertebrate animals (for example with specific and updated Technical Guidelines as for pesticides).

The long-term vision for the EU's chemical policy has committed to modifying EU legislation to produce the required information, needed for the hazard identification and risk assessment of substances. The final aim should be i) to increase protection of human health and the environment and ii) to remove pollution from all sources and move towards a toxic-free environment. At the same time, it is necessary to maximise the use of NAMs based on the latest scientific advances in the field, avoiding unnecessary animal testing (European Commission, 2020).

All together, these legal constrains have encouraged a change on the vision of evaluation of toxicological critical endpoints: the final objective of the whole process is a full replacement of procedures on animals for scientific, regulatory and educational aims as soon as scientifically achievable.

At present, following different regulatory requirements, the potential hazard assessment of chemicals, for some specific toxicological endpoints, as skin corrosion and irritation, serious eye damage and irritation, skin sensitisation, and mutagenicity and genotoxicity) is more and more often carried out by using non-animal approaches (Pistollato *et al.*, 2021).

In this context, in the last decades the main mandate of EURL ECVAM, as defined in Directive 2010/63/EU, has been to develop, validate and promote the application of new alternative methods/strategies, in the field of EU legislations, research as well as education and training programmes. Before a test can be accepted, it has to be evaluated for its scientific validity and reliability, assessing how the test has been planned and conducted. One of the key elements of this complex and rigorous process is also to demonstrate the intra and inter-laboratory reproducibility of the submitted test with the effort of different teams within the EURL ECVAM network of specialised laboratories placed in the EU member states (EU-NETVAL). An independent robust scientific peer review, development of specific recommendations on the validity of the method, as well as of its integration in existing or novel regulatory testing strategies, are a prerequisite to the final inclusion in the OECD Work plan for the TG Programme and subsequent international acceptance. The whole process requires a close collaboration and connection among various stakeholders and organizations, including different specialists such as scientists, regulatory assessors, representative of EU and international Agencies.

# European PARERE network

A great and collaborative effort is still needed to speed up the entire process, in order to fully characterize the toxicological profile of particular substances, including the assessment of the toxicokinetic behaviour as well as systemic toxicity, relying as much as possible on NAMs based strategies. During the last years, the establishment of dedicated networks, with different tasks and responsibilities, has favoured a continuous and effective communication among experts, succeeding in most of the above-mentioned areas within the EU requirements.

The Preliminary Assessment of Regulatory Relevance (PARERE) Network was set up by EURL ECVAM, as soon as Directive 2010/63/EU entered into force, as required in Article 47; Comma 2. Each Member State (MS) nominated National Contact Points to provide advice on the regulatory relevance and suitability of alternative approaches proposed for validation.

Since 2010, at national level, two experts in the field of regulatory assessment and alternative methods, at the Istituto Superiore di Sanità (Rome) and Reference Centre for Alternative Methods of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia)

have functioned as National Coordinators, after designation by the Italian Competent Authority, Ministry of Health.

From the very beginning, the primary needs to share both the requests and the information, from and to EURL ECVAM, in a policy of constructive and open cooperation, have been tackled and solved, by the establishment of a National Committee. Currently, there are 18 experts, nominated on specific eligibility criteria and endorsed by the National Coordinators and Competent Authority. The Italian PARERE Network is a real example of close collaboration, among regulatory, academic and industry sectors, including experts from the Istituto Superiore di Sanità, the Ministry of Health, the Agenzia Italiana del Farmaco, some of the most eminent Italian Universities, research institutions and industries. Indeed, it can benefit from the presence of experts in cross-sectional regulatory areas (i.e., pesticides, biocides, cosmetics, pharmaceuticals, industrial chemicals, and medical devices, human and veterinary vaccines) as well as widespread expertise: toxicology, *in vitro* toxicology, toxicokinetics (TK), *in silico* models, ecotoxicology, risk assessment).

During the last ten years, all the activities carried out and promoted by PARERE have been essential to facilitate the process of regulatory acceptance of new and alternative methods that can eventually replace tests on animals. Indeed, it is fundamental that regulators have been involved as early as possible in providing a preliminary view on the potential relevance of the submitted test methods and testing strategies or giving advice during the peer review and subsequent evaluation. National Coordinators have shared and discussed among experts a wide number of requests received from EURL ECVAM. The applicability and relevance of each proposal have been assessed, based on the needs of different regulatory frameworks. Several promising methods have been identified and selected for further validation steps; for others, potential shortcomings, limitations for their use for safety assessment purposes or requirements for further development and additional testing, have been pointed out and submitted to EURL ECVAM as comments/recommendations. Up to now, PARERE has been consulted on more than 15 Test Submissions, around half of them (e.g., single tests to address primary key events related to the human health hazard endpoint skin sensitisation) were validated, reviewed and subsequently, approved by OECD as TGs (e.g., OECD TG 442).

Recent advances in toxicological knowledge, predictive models and systems biology and, importantly, the scientific consensus that one single *in vitro* test would have not been sufficient as a stand-alone method to cover complex endpoints (such as skin sensitization, acute toxicity) have provided means to integrate different information/data within specific frameworks and more complex approaches (i.e., Adverse Outcome Pathways, AOPs; Integrated testing strategies). Also in this context, PARERE has helped to monitor and ensure priorities and requirements, set by leading safety assessment European authorities, such as EFSA and ECHA. For instance, some requirements for TK data are present in EU chemicals legislation, as well as the recommendation, in different regulatory guidances, of the use of these data (from *in vitro* measurements or computational predictions) to support the assessment of systemic toxicity. These concepts were well defined in the EURL ECVAM strategy for achieving 3Rs impact in the assessment of toxicokinetics and systemic toxicity (2015).

The status of each method can be monitored using the EURL ECVAM Tracking System for Alternative methods towards Regulatory acceptance (TSAR, https://tsar.jrc.ec.europa.eu/) as well as EURL ECVAM Database on Alternative Methods to Animal experimentation (DB-ALM, https://data.jrc.ec.europa.eu/dataset/b7597ada-148d-4560-9079-ab0a5539cad3), where a collection of alternative method summaries and protocols is available.

Over the years, PARERE members have given also support to evaluate specific EURL ECVAM recommendations (e.g., the recent "Non-animal-derived antibodies and to stop using animals for antibody development and production if not scientifically justified", 2020),

standardisation and validation frameworks for novel technologies and case studies from specific research projects funded by the EU.

One of the main roles of PARERE consists in encouraging an information flow between EURL ECVAM and regulators/researchers, regarding the development and validation of new methods, with involvement of experts, within the National Committee for the Protection of Animals used for Scientific Purposes and in recognising regulatory areas that need specific attention.

Along with these key activities, the dissemination of the 3Rs principles has been encouraged. Specific Workshops ("The 3R principle for a common vision", 2019) were organized thanks to the strict collaboration with various entities fully involved in the promotion of scientific progress in the field of alternative methods, at national level, such as Italian 3Rs Centre, Reference Centre for Alternative Methods, Italian Platform of Alternative Methods.

Some of the decisions, at national (National contact Point of Directive 2010/63/EU), European and international (i.e., National Coordinators OECD TGs programme) levels, have been based on the scientific opinions delivered by PARERE experts; these include harmonization of the criteria for endocrine disruptors, updates of REACH Regulation and the recent Surveyon EU Chemicals Strategy for Sustainability and REACH Information Requirements, promoted by ECHA (2021).

From the time of its establishment, PARERE has always improved the dialogue between regulators and researchers, also encouraging a more informed approach focused to education and training. In collaboration with other colleagues, its members participate to public events that bring researchers closer to the public, such as the European Researchers' Night and teach specific courses in secondary schools and universities, describing the animal use in science and the 3Rs principles (Figure 1).

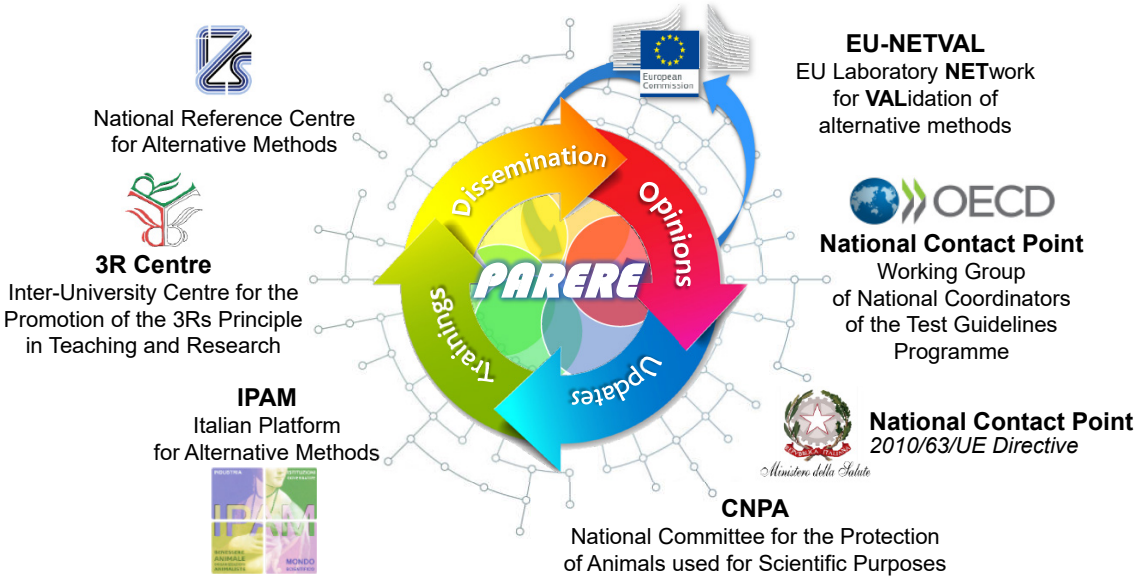


**Figure 1. PARERE links with national and international Entities promoting the 3Rs principle**

One of the great strengths of the Network, its collaborative work, is presented every year, during PARERE and other stakeholders' meetings (https://ec.europa.eu/jrc/en/eurl/ecvam/alternative-methods-toxicity-testing/advisory bodies/parere). On that occasion, National Coordinators have the opportunity to report the activities carried on in the field of alternative

methods in each MS. In 2020, Italy promoted a Survey on the state of the art of NAMs and their regulatory acceptance status at National level. The results of the Survey showed that Italy is very active, with some ongoing activities in the field of vaccines, development of specific AOPs, *in vitro* intestinal barrier models, modelling human variability in TK and toxicodynamic (TD) processes. In the meantime, an update on a possible validation framework for alternative methods in the area of respiratory sensitisation and possible applications of Organ-on-a-Chip (OoC) technologies and their potential for translation into the regulatory contexts was given.

The development of new *in vitro* methods should be purpose-driven and possibly prioritised with respect to specific data requirements. A screening phase could be based on alternative approaches, including *in vitro* methods anchored to key events defined in the existing AOPs. Indeed, AOPs could help to prioritise testing by identifying critical key events to be further investigated. It could also be more efficient to consider validation according to a specific purpose, i.e. research, prioritisation, hazard or risk assessment.

# What is needed to move on to the "next generation risk assessment"

The integration of NAMs, such as *in vitro* testing in cell lines, 3D organoids or OoC, combined with computational modelling, is in line with the general shift in toxicology from testing in whole animal to the so called "next generation risk assessment" (Figure 2), aimed at understanding the mechanisms underlying adverse effects, thus allowing a more informed and targeted evaluation of chemicals (Ingenbleek *et al.*, 2020; Testai *et al.*, 2021).

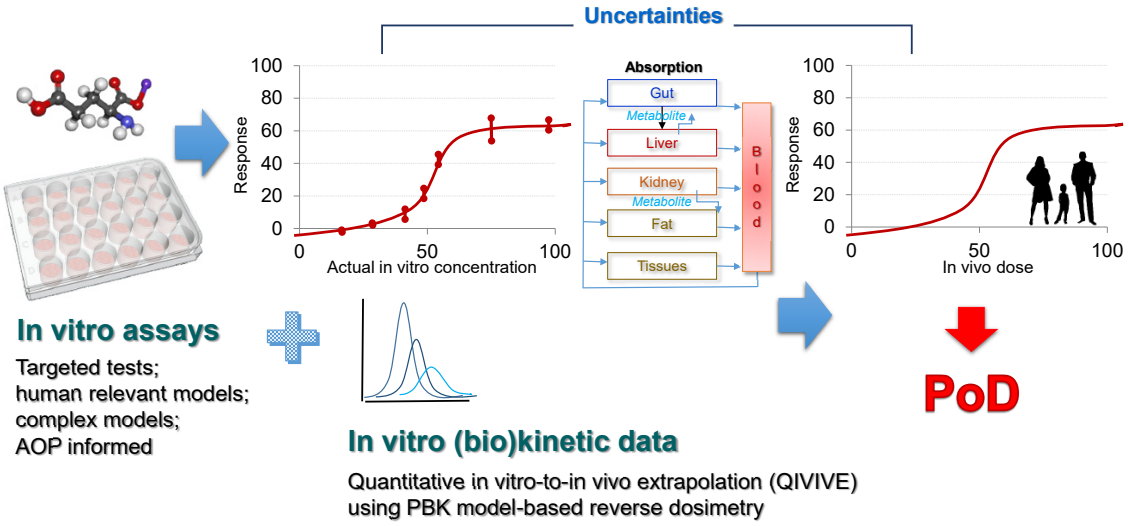


**Figure 2. Schematic representation of the Next Generation risk assessment, by using targeted *in vitro* tests with human relevant models, coupled with Quantitative *in vitro* to *in vivo* extrapolation based on Physiological Based Kinetic models**

A battery of new prediction models, more relevant for humans, considerations of the normal life low-dose exposure as well as of the route, duration and frequency of the exposure should be included to adequately predict the potential risk associated with any substance for the human

population (Zhang *et al.*, 2018). Human induced pluripotent stem cells (e.g., hiPSC-derived neuronal and glial cultures) are considered a proper *in vitro* model to allow mechanistic understanding of adverse effects induced by chemicals, avoiding species extrapolation (Di Consiglio *et al.*, 2020).

Additional steps are needed to further improve these approaches, such as the inclusion of TK information and integration of quantitative data into AOP frameworks.

However, the extrapolation of actual concentrations *in vitro* to *in vivo* exposure remains a challenge in the interpretation of *in vitro* effect data. Reverse dosimetry using physiologically-based kinetic models is able to simulate the behaviour of the chemical in the body, to estimate external exposures from target tissue concentrations (Mielke *et al.*, 2017). These models are increasingly used to perform quantitative *in vitro-in vivo* extrapolations (QIVIVE) for chemical hazard characterisation and identification of the Point of Departure (PoD), as basis to derive Reference Values. However, QIVIVE studies are usually based on *in vitro* nominal concentrations. The differences found from *in vitro* exposure can be partly explained with the lack of consideration of the biokinetic behaviour of the chemicals in the experimental models, i.e. the actual fraction of chemical available to interact at the target site (Pomponio *et al.*, 2015; Kramer *et al.*, 2015). The discussion is ongoing to re-design also default assessment factors, to derive Reference Values, with chemical-specific and/or pathway-related adjustment factors, when specific data on the chemical or of the metabolic pathway is known in humans (Testai *et al.*, 2021; Di Consiglio *et al.*, 2021).

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# MONOCYTE ACTIVATION TEST AS REPLACEMENT OF ANIMAL-BASED METHODS FOR THE *IN VITRO* ASSESSMENT OF HUMAN VACCINE PYROGENICITY

Marilena Paola Etna (a), Fabiana Rizzo (a), Daniela Ricci (a), Ramona Ilari (b), Martina Severa (a), Valentina Salvati (c), Andrea Gaggioli (c), Ingo Spreitzer (d), Christina von Hunolstein (c), Eliana Marina Coccia (a)
*(a) Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy*
*(b) Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy*
*(c) National Centre for Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome, Italy*
*(d) Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Langen, Germany*

## Introduction

The quality of vaccines for human use is assured by several periodic controls performed by the manufacturer under Good Manufacturing Practices on the raw materials and final vaccine formulation. Moreover, in accordance with the European Directive 2001/83/EC on medicinal products for human use, marketing authorization also requires the testing of critical parameters on the final vaccine formulation by the European Official Medicine Control Laboratory (EU OMCL). In this context, safety and potency are key parameters that needs to be monitored by careful and reliable testing that often involve a very significant number of animals.

Being pyrogens one of the principal causes of fever and febrile reactions in humans, it is of key importance to control pyrogenicity in parenterals, including vaccines. Moreover, pyrogens can induce also other physiological reactions ranging from septic shock to multi-organ failure and, sometimes, even death (Prajitha *et al.*, 2018).

The most pyrogenic, abundant and stable exogenous pyrogen is the endotoxin (lipopolysaccharide, LPS) from Gram-negative bacteria (endotoxin, i.e., the "toxin" is part of the bacterium, and not actively secreted). Nevertheless, virtually all gram-positive/gram-negative bacteria, either alive or their break-down products like LPS, muramyl peptide derivatives and peptidoglycans – all of them Pathogen Associated Molecular Patterns (PAMPs), sometimes called non-endotoxin pyrogens (NEP) –, behave as (weaker) endotoxins. In addition, other microbial-derivates, as fungal products (mannan and glucan components of *Candida albicans*), viral RNA, enterotoxins (*Staphylococcus aureus*) and erythrogenic toxins (Group A streptococcus) may act as pyrogens.

Currently, different tests are foreseen in European Pharmacopoeia (Ph. Eu.) for pyrogen testing namely the Rabbit Pyrogen Test (RPT, Chapter 2.6.8), the Bacterial Endotoxin Test (BET, Chapter 2.6.14), the Recombinant Factor C test (rFC, Chapter 2.6.32) and the Monocyte Activation Test (MAT, Chapter 2.6.30).

The RPT is considered the gold standard for pyrogen testing. It is an *in vivo* method where variations of body temperature are measured in rabbit after the injection of the solution to be examined. The rabbit model has been chosen because of the similarity with the human response to endotoxin and this test allows the qualitative measurement of both endotoxin and NEP (Greisman & Hornick, 1969). Another widely used assay is the BET, also known as Limulus Amoebocyte Lysate (LAL) assay. This limit/qualitative test is able to detect only endotoxin contaminants, by using the amebocyte lysate from the horseshoe crab Limulus polyphemus. Different methods are exploitable for this test: the gel-clot method; the turbidimetric method and

the chromogenic method. Although the existence of alternative methods, RPT and BET – both animal-based tests – are still widely diffused in routine testing of medicinal products and, thus, they poorly adhere to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Indeed, the intention of this directive was to improve and enforce animal welfare by giving stricter advice on how to transfer these measures into national law.

The theoretical basis of the above-mentioned EU Directive has been firstly postulated in 1958 in the book "The Principle of Human Experimental Technique" authored by Rex Burch and William Russell where the three principles of replacement, reduction and refinement –known as the 3Rs principle – were introduced (Russell & Burch, 1959). Accordingly, in the field of vaccines, technical progress in analytical methods and their application are currently implemented in quality strategy to adhere to 3Rs principle (Akkermans *et al.*, 2020).

Besides ethical reasons, *in vitro* assays represent a more suitable alternative than animal-based methods by improving the evaluation of critical attributes for the product quality in terms of low variability, high sensitivity, and reduction of time and costs.

In line with the 3Rs principle and thanks also to the advance in science and technology, some alternatives for pyrogenicity testing have been developed. In particular, the rFC test (evolution of LAL test using a recombinant version of the protein purified from the horseshoe crab blood) recently introduced in the Ph. Eu. allows the quantitative measurement of endotoxin contents, thus representing a non-animal alternative for BET. Another attractive possibility for the replacement of the RPT is the MAT, a semi-quantitative/quantitative test, based on the capacity of human monocytes or monocytic cells to release endogenous mediators of inflammation, after the stimulation with either endotoxins or NEP (Hartung, 2021).

This test was originally described and inserted in Ph.Eu. as an alternative for testing parenterals however, in the last years it was applied for vaccine testing too (Figure 1).

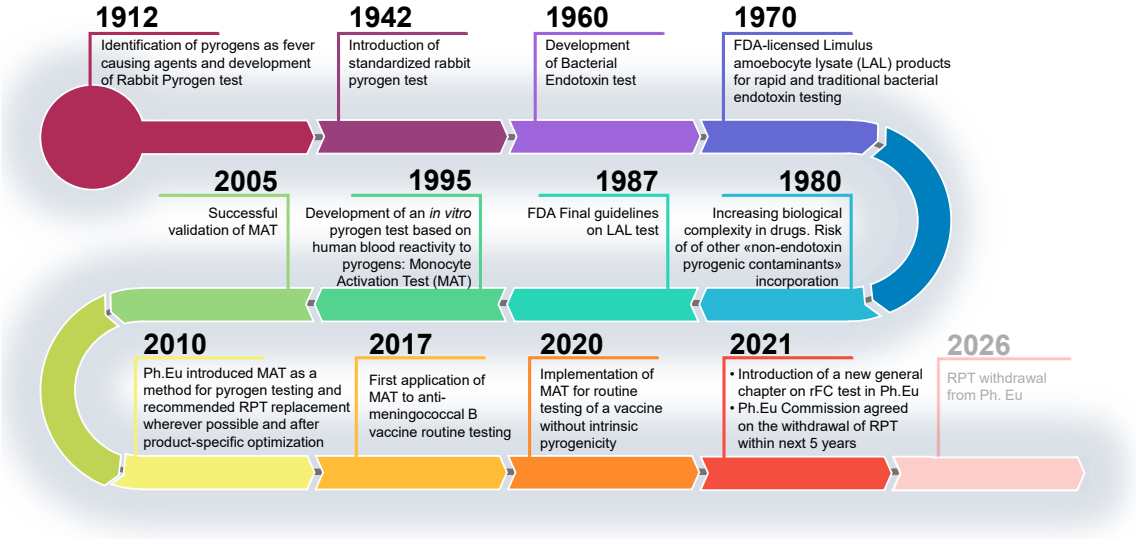


**Figure 1. Main achievements in pyrogen testing**

# First application of MAT to an inherently pyrogenic product: the case of an anti-meningococcal B vaccine

Although rabbits and man similarly react to endotoxins, the response to high levels of pyrogens became more pronounced in human with respect to rabbits (Greisman & Hornick, 1969), likely dependent of differences in the two immune systems.

In light of these differences, the presence of several PAMPs in vaccine formulations has recently raised concerns about the reliable applicability of RPT for pyrogen detection. Accordingly, this issue came up when RPT was applied in the routine testing of an anti-meningococcal B vaccine (anti-MenB), an aluminum hydroxide-adsorbed multicomponent subunit vaccine containing three *Neisseria meningitidis* recombinant proteins, namely the Neisserial adhesin A (NadA), the Heparin-Binding Antigen (NHBA) and the factor H binding protein (fHbp) and the *N. meningitidis* Outer Membrane Vesicles (OMV) (Valentini *et al.*, 2019). The presence of OMV from serogroup B *N. meningitidis*, which contains meningococcal LPS (endotoxin) and several lipoproteins, porins, peptidoglycan and muramyl peptides (NEP) confers to the product inherently pyrogenic properties that made it difficult to adapt the RPT, which resulted in high variability and several false positive results (Vipond *et al.*, 2016). Similarly, also LAL test was not suitable for the anti-MenB vaccine given the presence of NEP (Valentini *et al.*, 2019). Challenges earned with RPT and LAL assays together with growing interest and regulatory requirements in substitution of animal-based methods with *in vitro* approaches opened to the possibility to optimize the MAT as an alternative test for the pyrogen testing of MenB vaccine. Accordingly, thanks to the collaboration of the manufacturer with the OMCLs, MAT was successfully adapted, optimized and validated for assessing pyrogenicity of MenB vaccine batches in routine testing (Valentini *et al.*, 2019). Interestingly, building on anti-MenB vaccine experience, recently the MAT was developed and proposed as replacement of RPT for the consistency/safety testing of Shighella spp. vaccines based on Generalized Modules For Membrane Antigens (GMMA) comprising OMV from genetically modified Gram-negative bacteria and thus being inherently pyrogenic (Carson *et al.*, 2021) (Figure 2).

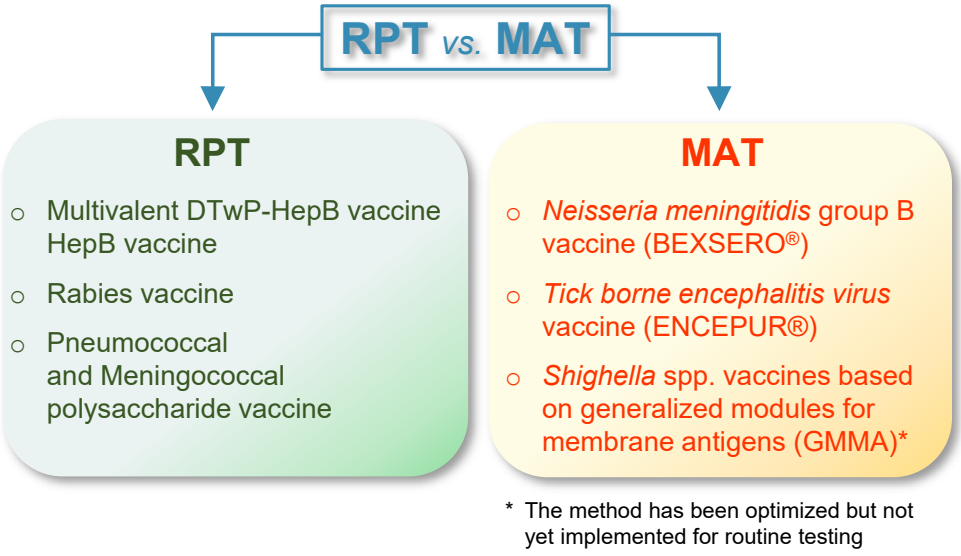


**Figure 2. Application of RPT and MAT in the batch release of the human vaccines**

For intrinsic pyrogenic products the MAT reference lot comparison method was identified as the most suitable. In particular, the estimation of the relative pyrogenicity (RR) of MenB vaccine batches is carried out on four different peripheral blood mononuclear cells (PBMC) donors through the comparison against a vaccine batch used as reference standard. A regression analysis on a multi-dilution dose-response is performed, starting with the estimation of individual slopes and intercepts (for the various preparation) in order to build an ANOVA (Analysis of Variance) table and to estimate the RR. The final result is the Geometric Mean of the four RRs obtained for the different donors are included in the analytical session.

In 2017, MAT assay optimized for the MenB vaccine was approved by the European Medicines Agency (*see* Figure 1) and recently implemented for batch release and post-marketing surveillance controls at ISS by the MAT Unit of Department of Infectious Diseases in collaboration with the National Centre for Control and Evaluation of Medicines.

## Optimization, validation, and implementation of MAT for a not intrinsically pyrogenic vaccine: proposed modifications to MAT monograph

In recent years, a wide-ranging collaborative project funded by Innovative Medicine Initiative 2 (IMI 2), namely "Vaccine batch to vaccine batch comparison for consistency testing" (VAC2VAC), aims to provide the proof of concept of consistency approach for batch release of vaccine by means of *in vitro* analytical methods (http://www.vac2vac.eu/). The ambition of this public-private consortium is to develop and optimize non-animal-based model for demonstrating vaccine batch consistency, safety and efficacy. Accordingly, one of the objectives of ISS Unit within VAC2VAC project is to optimize the MAT for the Tick-Borne Encephalitis Virus (TBEV) vaccine as alternative for the replacement of the currently used RPT test.

Indeed, the anti-TBEV vaccine is among products for which both WHO technical report series and Ph. Eu. product specific monograph foresees the assessment of the pyrogen content as safety test prior to batch release. The anti-TBEV vaccine is composed by the TBEV inactivated by formaldehyde, as active substance, adsorbed onto aluminium hydroxide (Kubinski *et al.*, 2020). Although the active substance by itself does not contain pyrogenic molecules – thus resulting a non-intrinsically pyrogenic product – the production process entails some critical steps, namely the embryo harvest from chicken eggs or the virus propagation that could expose to the risk of bacterial, viral or cellular contaminants entering the final product.

Historically, the pyrogen content of the anti-TBEV vaccine was monitored and assessed by RPT. The possibility to replace the RPT with the MAT when testing for pyrogenicity the TBEV vaccine, was investigated at ISS. Both quantitative Method A (pyrogen content expressed as exact amount of equivalent of endotoxin unit present in the product) and semi-quantitative Method B (pyrogenic level of the product expressed as above or below an established threshold) were used. In this particular case, dealing with a vaccine without intrinsic pyrogenicity and for which the requisite is "not pyrogenic", it was clear that an adaptation of the methods A and B validity criteria was necessary to fulfil at the best Ph.Eur. chapter 2.6.30 requirements (Etna *et al.*, 2020). Along the product-specific optimization it was demonstrated that the pyrogen level of the anti-TBEV vaccine can be established by the MAT with a satisfactory precision as evaluated by repeatability and intermediate precision of the method. However, the experience of MAT adaptation to a not intrinsically pyrogenic vaccine opens the door to: i) overcome the restriction of curve linearity with regards to the product dilution range and, ii) foresee the use of Assay Sensitivity instead of Limit of Detection for the definition of the contaminant limit concentration and product maximum

valid dilution to be tested through MAT (Etna *et al.*, 2020). Of note, the MAT for the anti-TBEV vaccine, optimized and validated at ISS, has been successfully implemented for routine testing by the manufacturer. Moreover, during the MAT optimization, few issues related to Method A and B application came up and were discussed with pharmacopoeia experts leading to an inquiry of revision of MAT chapter 2.6.30 forwarded to the Ph. Eu. Commission (*see* Figure 1).

In line with this, a request of the Italian delegation was submitted at 134th meeting of Group 15 of Ph. Eu to replace in the Ph.Eur. monograph 1375 "tick-born encephalitis vaccine (inactivated)" the current RPT with the MAT (Figures 1-2).

# Past and future of pyrogenicity test

In the last century pyrogen research and testing was mainly focused on endotoxin, as LPS. Therefore, RPT was without a reliable competitor up to 25 years ago when Hartung and Wendel developed a human whole blood cytokine release assay to detect human-relevant pyrogens or PAMPs, as they are called nowadays (Hartung & Wendel, 1995). A long time was, however, necessary from the development to the implementation of MAT into the Ph. Eu. (MAT Monograph 2.6.30, EDQM, 2010) (*see* Figure 1). Since then, MAT was employed as a substitute for detecting Gram-negative endotoxins and NEP alike in injectables on a case-by-case basis. Nevertheless, in spite of positive feature and strength of MAT assay and its applicability to a wide portfolio of parenteral for human clinical use, the 90% of pyrogen testing is still covered by LAL and RPT. However, in the case of vaccine, the applicability of LAL is limited by three main factors: i) the interference of aluminium hydroxide, a widely used adjuvant boosting the immune response; ii) the content of many pyrogenic components different from LPS; and iii) the broad presence of glucans, which are common in fungi but also in cellulose composing filters, that may activate LAL cascade reactions leading to false-positive signals. To our opinion the number of these limitations it is likely to increase given to the ongoing and never-ending development of vaccine and vaccine formulation. At present, human vaccines, whose batch release foresees the pyrogen testing by RPT and MAT, are listed in Figure 2. However, different other types of vaccines are developed and include the following categories: recombinant microbes, purified antigen or so-called subunit vaccines, synthetic antigen vaccines, RNA and DNA vaccines. This new generation of vaccines often requires a delivery carrier (nanoparticles, viral vectors, etc.) and adjuvants displaying strong immunomodulatory capacity, enhanced and long-lasting protective feature (Pilkington *et al.*, 2021). As a consequence, the pyrogenicity test will require continuous update and implementation to face not only with the potential "canonical" pyrogenic contaminants that can be introduced accidentally during the manufacturing process but also with the intrinsic nature of the vaccine itself. For instance, the recent introduction of the so-called nucleic acid nanoparticles (NANPs)-based vaccine as well as the use of adjuvants or amplifiers – such as metabolic and epigenetic modulators (Dominguez-Andres *et al.*, 2020) – enlarges the portfolio of vaccine components that can trigger the innate immune response. Nevertheless, in this context, the versality and broad capacity of human PBMC to detect and sense a wide variety of PAMPs supports the setting for a "next generation" MAT able to evaluate also excessive unwanted pro-inflammatory features of NANPs, novel adjuvant or amplifier formulations (Dobrovolskaia & Afonin, 2020).

# Take home message

MAT is an ever-green assay, whose potential applications are not yet fully exploited for testing both extrinsic and intrinsic pyrogenicity of classical and novel vaccine formulation. In addition of avoiding the use of animal models, the immunological power of this assay relies on the possibility to investigate *in vitro* the main immune cells present in whole blood or PBMC. This represents a win-win feature that makes MAT a malleable *in vitro* experimental setting ready to sense all PAMPs and to face with the forthcoming vaccine formulation to predict their inflammatory nature as well as possible bias of the induced immune response.

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# REPLACEMENT OF ANIMAL TESTING IN ASSAYING POTENCY OF HUMAN VACCINES AT THE ITALIAN OFFICIAL MEDICINE CONTROL LABORATORY

Christina von Hunolstein (a), Maria Jesus Gomez Miguel (a), Cristina Pezzella (a), Luisa Ralli (a), Filomena Nappi (a), Marilena Paola Etna (b), Eliana Marina Coccia (b), Eleonora Puggioni (a), Paola Sestili (a), Valentina Salvati (a)
*(a) National Centre for Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome, Italy*
*(b) Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy*

## Introduction

Several aspects assure human vaccine quality. Briefly, the vaccine production process occurs under Good Manufacturing Practices (GMP), adhesion to which is periodically checked by authorized teams of inspectors. Starting from the raw materials up to the final vaccine product, all production steps are subject to severe quality controls by the manufacturer. Quality testing is an essential element of the process by which medicinal products are released for use. Furthermore, before to enter onto the market, the vaccines for human use have again to be submitted to a control of key parameters. In Europe, the control is performed by a European Official Medicine Control Laboratory (EU-OMCL) according to the Directive 2001/83/EC Article 114 as amended by Directive 2004/27/EC, adopted in the Italian Legislation with the DL.vo 219/2006 (Italia, 2006). The National Centre for Control and Evaluation of Medicines (Centro Nazionale Controllo e Valutazione dei Farmaci, CNCF) of the Istituto Superiore di Sanità (ISS) (Ministero della Salute, 2016) is the Italian OMCL within the European Network as well as a member of the World Health Organization (WHO) National Control Laboratory Network for Biologicals.

For the quality control of medicines, the reference point is the European Pharmacopeia (Ph.Eur.), a compendium of texts on the qualitative and quantitative composition of medicines and tests/methods to be carried out that provide a scientific basis to guarantee the quality during the entire life cycle of a product.

Safety and potency testing are part of the quality control and often involve animal tests. Particularly, for established inactivated vaccines such as diphtheria, tetanus, pertussis, rabies, tick-born encephalitis and hepatitis a large numbers of laboratory animals are used.

Since the elaboration of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes signed in 1986 (Council Directive 86/609/EEC) (Europe, 1986), an intensive activity has been performed by the European Pharmacopeia Commission to review all animal tests present in the various monographs of the Ph.Eur. to apply the 3Rs principle (Replacement, Reduction and Refinement) proposed by Russell and Burch in 1959 (Russell & Burch, 1959). The 3Rs principle provides a strategy for replacement, reduction, and refinement in animal testing and are an internationally accepted methodological approach for manufacturers and national quality control laboratories.

The establishment of the Biological Standardization Programme of the European Directorate for the Quality of Medicines and Healthcare (EDQM) (https://www.edqm.eu/en/biological-standardisation-programme) in 1991 has provided the means to carry out studies to develop and validate methods promoting the application of 3Rs, which were subsequently incorporated into the monographs and chapters of Ph.Eur.

The Bacterial and Viral Vaccines Sections of the Biological and Biotechnological Products Unit of the CNCF-ISS have participated in many BSP studies, in particular in those which objectives were to refine or reduce *in vivo* potency testing or replacing the *in vivo* potency or safety test with an alternative *in vitro* test.

The quality controls to be performed by EU-OMCLs for releasing a vaccine are indicated in the "Product specific guidelines for immunological products consisting in vaccines" published on the web site of the EDQM. Among the tests to be performed, is included the potency test, i.e. assessment of the biological activity of the vaccine.

Vaccines are very complex and heterogeneous products constituted not only by the specific antigen, but also might contain adjuvant/s, excipients and preservatives. The vaccine composition may interfere with non-animal tests determining the difficulty in performing the potency and safety tests by using physico-chemical test and consequently an *in vivo* test is still required. However, very important efforts have been made to replace, reduce and refine the use of animals for potency and safety testing at the European level.

The present review will provide an insight in the progress and achievements of 3Rs application, in particular in the replacement area, in controlling the potency of different kind of human vaccines at the Italian OMCL/NCL.

# Activity performed as OMCL/NCL

At the CNCF, different types of vaccines for human use are controlled before being placed on the market (batch release activity) and/or for post marketing surveillance.

To perform these activities the experts are continuously skilled in ISO 17025 requirements, Ph.Eur., EDQM Guidelines and WHO Technical Report Series.

Below examples of vaccines currently controlled by the CNCF are reported, for which ISS experts have contributed over the years to activities aimed at implementing the 3Rs to *in vivo* assays.

## Meningococcal serotype B vaccine

A meningococcal vaccine against the serogroup B of *Neisseria meningitides* (MenB) is controlled by the ISS. The Outer Membrane Vesicles (OMV) and three recombinant proteins of MenB in the presence of aluminium hydroxide as adjuvant compose the vaccine. The initial potency test, as well as a pyrogenicity test, were designed as *in vivo* tests.

The initial *in vivo* potency assay, after a complex validation study, has been replaced by the manufacturer by an *in vitro* relative potency test (IVRP) based on the Enzyme-Linked Immunosorbent Assay (ELISA), which is also performed by CNCF to release the MenB vaccine.

The rabbit pyrogen test performed to assay the MenB vaccine pyrogenicity has been substituted by the *in vitro* monocyte activation test (for further details, see contribution of M. Etna *et al.* in this report).

## Hepatitis A and B vaccines

There are several types of hepatitis A vaccine.

The *hepatitis A vaccine, inactivated, adsorbed* is a suspension consisting of a suitable strain of hepatitis A virus grown in cell cultures, inactivated by a validated method and adsorbed on a mineral carrier.

The *hepatitis A vaccine – inactivated, virosome* is a suspension of a suitable strain of hepatitis A virus grown in cell cultures and inactivated by a validated method. The virosomes are composed of proteins of an influenza virus strain approved for that particular product and phospholipids are used as adjuvants.

According to the Ph.Eur. (2.7.14 Assay of hepatitis A vaccine) the assay of *hepatitis A vaccine* is carried out either *in vivo*, by comparing, under given conditions, its capacity to induce specific antibodies in mice with the same capacity of a reference preparation, or *in vitro*. The *in vitro* test was approved in June 2014, at the 149th Session of the Ph.Eur. Commission. Until 2014 no standardised *in vitro* test common to all hepatitis A vaccines was available for both manufacturers and NCLs. Indeed, this new method, is based on the determination of the vaccine antigen content by a unique polyvalent enzyme-linked immunosorbent assay (ELISA) developed to appraise all commercially available hepatitis A vaccines. ISS experts participated in the establishment of the hepatitis A reference preparations by using the *in vitro* test and successively replaced the *in vivo* by the *in vitro* test for the control of hepatitis vaccines (Wood *et al.,* 2000; Morgeaux *et al.,* 2015).

Hepatitis B vaccine is a preparation of hepatitis B surface antigen (HBsAg) obtained by recombinant DNA technology.

The assay of hepatitis B vaccine (rDNA) is carried out either *in vivo*, by comparing under given conditions its capacity to induce specific antibodies against the HBsAg in mice or guinea-pigs respect a reference preparation, or *in vitro*, by an immunochemical determination of the antigen content (Ph.Eur., 2.7.15 Assay of hepatitis B vaccine). The Ph.Eur. 2.7.15 does not describe in depth a validated ELISA method applicable to all commercially available HBsAg, therefore manufacturers have developed and validated their own ELISA potency assay and use their own in-house standard preparation.

For Post-Marketing Surveillance, the CNCF tests all hepatitis vaccine by *in vitro* methods.

## Polio vaccines

Oral Poliovirus Vaccine (OPV), which is made from alive attenuated Sabin strains of poliovirus, has been the preferred vaccine throughout the WHO Global Poliovirus Eradication Initiative. However, the intrinsic instability of Sabin poliovirus strains in OPV may lead to the generation and accumulation of spontaneous point mutations in the 5′ non-coding regions of the viral RNA of the vaccine, which have been associated with an increased neurovirulence. For this reason, the maintenance of the attenuated phenotype of OPV strains must be tightly monitored during the production to ensure not only consistency of the vaccine but also the safety of the product (WHO, 2014). This requires that every batch of OPV is tested for neurovirulence in monkeys or transgenic mice susceptible to poliomyelitis.

As a potential substitute of animal based neurovirulence test, a highly sensitive quantitative molecular method called MAPREC (Mutant Analysis by Polymerase chain reaction and Restriction Enzyme Cleavage) was developed to quantify the 5′ UTR revertants in monovalent batches of OPV. The MAPREC test demonstrated that the percentage content of specific mutations directly correlates with the results of the Monkey NeuroVirulence Test (MNVT) (Chumakov *et al.*, 1991). CNCF experts have been involved in the studies for development of the assay and the establishment of the WHO Reference Standards for MAPREC assay (Dunn *et al.*, 2009; WHO, 2012). The MAPREC assay was performed at CNCF as part of the OPV batch release tests, but currently it is performed only upon request by other OMCLs.

## Tick-born encephalitis virus vaccine

Tick-Borne Encephalitis Vaccine (TBEV) (inactivated) is a liquid preparation of a suitable strain of TBEV grown in cultures of chick-embryo cells or other suitable cell cultures and inactivated by an appropriate validated method.

The potency is determined by comparing the dose necessary to protect a given proportion of mice against the effects of a lethal dose of TBEV, administered intraperitoneally, with the quantity of a reference vaccine preparation of TBEV necessary to provide the same protection. Today, two alternative *in vitro* methods are under evaluation in the VAC2VAC project (see below). One is based on an ELISA method and another one aims at identifying novel biomarkers of innate immune response, predictive of vaccine immunogenicity. In particular, a platform based on human peripheral blood mononuclear cells (PBMC) was shown to be able to discriminate among the conforming and not-conforming antigen present in the vaccine final formulation by studying type I interferon (IFN) gene signature analysis by quantitative real time PCR (Signorazzi *et al.*, 2021). This cell-based assay, together with other immunochemical analyses, could be used for batch-to-batch assessment of the TBE vaccine, reducing and eventually replacing *in vivo* assay for potency testing.

## Diphtheria, tetanus and combined vaccines.

The replacement of the *in vivo* potency test for tetanus (T) and/or diphtheria (D) component of monovalent vaccines or combined with other antigens such as pertussis (acellular, aP; whole cell, wP), hepatitis B, inactivated polio viruses and *Haemophilus influenzae* type b glycoconjugate has so far not taken place. These vaccines are quite complex in composition and interference from the different drug substances, adjuvant and excipients increase the difficulty in setting up of *in vitro* tests.

Thus, the potency testing of the diphtheria and tetanus toxoids, active drug substances of anti DT vaccines, is still conducted *in vivo* (Ph.Eur. 2.7. 6 Assay of diphtheria; 2.7. 8 Assay of tetanus). Equal groups of animals are immunized with scalar doses of a reference preparation and with the test vaccine; successively, they are challenge with a lethal dose of the respective toxin. The number of survivals in the different groups is used to calculate the vaccine potency. However, thanks to EDQM collaborative studies, to which also CNCF has participated, the *in vivo* lethal challenge assays have been refined over time with the introduction of serology assay for diphtheria and tetanus, and the use of humane end-points (Hendriksen *et al.*, 2001; Winsnes et.al, 2004). For the serology assay, the immunized animals are not challenged with the toxins, but instead they are bled and the sera tested by an immunoassay for the presence of antibodies against diphtheria or tetanus toxins. Furthermore, in order to reduce the animal use, an *in vivo* single dilution assay, after proper validation, can substitute the *in vivo* multi-dilution assay. WHO also strongly support these alternative tests, but their implementation is not simply since a product specific validation is always necessary.

The CNCF receives at control few batches per year of T or DT vaccine (tetanus max 10; diphtheria <10). A reduction in animal use has been applied by substituting the multiple dilution assay with the single dilution assay, which requires 50% less animals. Furthermore, the end-point for tetanus toxin challenged mice is not anymore, the death, but the paralysis (refinement).

It is a policy of the OMCL network to test for potency only the first final fill lot/batch in case more than one is produced from a final vaccine bulk. Of course, this has brought to a large reduction in animal use respect to more than 20 years ago, when every new fill lot/batch was tested for batch release.

Even if the potency assay for tetanus, diphtheria and acellular pertussis vaccines is still an *in vivo* test, some animal test for these kinds of vaccines has been replaced by non-animal-based test and included in the Ph.Eur. For example, the demonstration of absence of toxin in the toxoid bulks, has been replaced by *in vitro* test: the absence of diphtheria toxin, as well as pertussis toxin, is performed currently using VERO cells or CHO cell, respectively. For tetanus toxin a new *in vitro* assay (Behrensdorf-Nicol *et al.*, 2014) as a potential alternative to the mandatory guinea-pigs test for the absence of toxin in tetanus toxoids (Ph.Eur. 0452 and 0697) is the objective of a forthcoming EDQM collaborative study.

For the non-EU market, DT vaccines are usually combined with wP instead of with aP. The WHO has qualified many DTwP-HepB vaccines that are used in the vaccination campaign of several developing countries. The potency assay for wP foresees an intracranial challenge (WHO, 2013) with a lethal dose of virulent *Bordetella pertussis*. Therefore, in 2008, a proposal for an alternative serology potency test was submitted to the European Centre for the Validation of Alternative Methods (ECVAM), resulting in approval and funding (von Hunolstein *et al.*, 2008). Currently, this serological potency testing for wP is under evaluation by the manufacturers belonging to the Developing Country Vaccine Manufacturer Network (https://dcvmn.org/), which produce vaccines with wP. The evaluation is performed within the remit of a project funded by the National Institute for Innovation in Manufacturing Biopharmaceuticals, USA (https://niimbl.force.com/s/current-projects). A CNCF expert participate to the project as chair of the Steering Committee.

# Discussion and conclusion

A huge effort has been made by the Ph.Eur. to replace, reduce and refine the animal tests for potency and safety testing of human vaccines (Lang *et al.*, 2018). In recent years, good results have been achieved to this aim with the contribution of industries, OMCLs and regulatory agencies.

In the field of batch release, an important contribution to a drastically reduction in animal use has been provided by the requirement of the Directive 2001/83/EC relating to medicinal products for human use. The Official Control Authority Batch Release (OCABR) performed by any given member state shall be mutually recognized by all other member states requiring OCABR for that product. That means that no other OMCLs of the Network controls a specific vaccine batch when already controlled and released by another OMCL.

Substitution of *in vivo* tests is not an easy task, as vaccines are complex and demonstration of equivalence of an alternative method is so far very difficult. To help transition from *in vivo* to *in vitro* methods, the Ph.Eur. Commission developed a new general chapter on the "Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines" (5.2.14, in force since January 2018). In particular, the Ph.Eur. Commission gives advice on how to validate alternative methods in case a direct comparison with the *in vivo* test is not possible, in particular for the vaccines established long time ago (diphtheria, tetanus, rabies, TBEV).

New tests for established vaccines such as diphtheria, tetanus, acellular pertussis, TBEV, rabies, etc., are under evaluation in the "Vaccine batch to vaccine batch comparison by consistency testing" (VAC2VAC) which is a wide-ranging collaborative research project funded by IMI2 which aims to develop and validate quality testing approaches for both human and veterinary vaccines using non-animal methods (www.vac2vac.eu).

The consistency approach has been introduced some years ago and is based upon in depth characterization of the vaccine during development. In addition, the strict application of GMP assures that the quality of batches produced over time are similar for quality, safety and efficacy

to clinically evaluated batches (consistency in production) (De Mattia *et al.*, 2011). The concept is easily applicable to the recently new-generation vaccines that are well characterized as human papilloma virus like-particle or polysaccharide conjugate vaccine for *Haemophilus influenzae* type b, meningococcal serogroup ACWY and pneumococci.

The *in vitro* potency tests, that have been developed based on ELISA assay, are a surrogate of the *in vivo* method, as they do not measure the biological activity of the vaccine, but the content per dose of the antigen/s on the basis of relevant immune epitopes. For example, the IVRP for *N. meningitidis* serotype B, based on ELISA, quantifies the content of the OMV and recombinant proteins (NHBA, NadA, fHbp) present in the vaccine using specific monoclonal antibodies developed to recognize the epitopes relevant in the protection. Monoclonal antibodies are critical reagents, not available commercially as generally developed and produced by the vaccine manufacturer. As all biologicals to be used as reagents in *in vitro* assays, also the monoclonal antibodies need to be well characterized, have assigned a shelf life and be available over the years. Thus, sustainability (and cost) of the reagents for *in vitro* assay are aspects that deserve special attention also from the National Regulatory Authority when approving an *in vitro* assay in replacement of an *in vivo* one.

A great effort to replace *in vivo* potency test is currently ongoing during the development of new vaccines. An example is given by the vaccines against Covid-19, where non-animal methods were identified and proved suitable, during the clinical trials, to indicate immunogenicity and safety of the product.

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# 3R@ISS THE WAY FORWARD

Valentina La Sorsa (a), Eliana Marina Coccia (b)*, Isabella De Angelis (c)*,
Laura Ricceri (d)*, Augusto Vitale (d)*
*(a) Research Coordination and Support Service, Istituto Superiore di Sanità, Rome, Italy*
*(b) Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy*
*(c) Department of Environment and Health, Istituto Superiore di Sanità, Rome, Italy*
*(d) Reference Centre for Behavioural Sciences and Mental Health, Istituto Superiore di Sanità, Rome, Italy*

The 3Rs principle (Replacement, Reduction and Refinement) has gradually become essential when considering animal use in research. It has influenced new legislation aimed at controlling the use of experimental animals in science and in the European Union (EU) it became formally incorporated into the Directive 2010/63/EU on the protection of animals used for scientific purpose. It has also been recognized that the adoption of 3Rs in the experimental design can improve the quality of science. The use of scientifically advanced non-animal methods and testing strategies, as well as appropriately designed experiments that minimize variation, provide standardized optimum conditions of animals' care and minimize unnecessary stress or pain, lead to more reliable data (Caloni *et al*., 2022).

In this context, the Istituto Superiore di Sanità (ISS, the National Institute of Health in Italy) has undertaken, since the early 1980s, several activities aimed at applying internally and disseminating at national and international level the 3Rs approach becoming an important reference institution for the scientific community on this issue.

Few examples of the ISS commitment to the 3Rs principle are: the participation of ISS researchers in the formalization of the CellTox, the Italian association for *in vitro* toxicology (Associazione Italiana Tossicologia *in Vitro*) and the Italian Platform on Alternative Methods (IPAM), currently involved in the promotion of replacement approaches and in the harmonisation of different opinions of the stakeholders interested in animal experimentations, respectively (De Angelis *et al*., 2018); the involvement of ISS researchers in many courses, lectures, seminars and dissemination events (De Angelis *et al.*, 2019) including the European Researchers' Night, initiative promoted by the European Commission, since 2005 in which thousands of researchers in all European countries every year contributed (at ISS in four editions 2016-2019) and training courses during the school-work educational programs at the ISS addressed to high school students (2017-2019); the role of the ISS, on behalf of the Ministry of Health, in the evaluation of research projects under the provision of the DL.vo 116/1992 (the Italian adoption of the EU Directive 86/609 on the protection of animals used in laboratory research). Furthermore, the 3Rs principle has been at the centre of several EU funded projects, in which the ISS was participating as partner, starting from ANIMALSEE looking at new definitions of the 3Rs (Buchanan-Smith *et al.*, 2005), to the most recent ANIMPACT (An ethical, legal and practical perspective on the impact of a new regulatory framework for the scientific use of animals on research and innovation) (2013), looking at motivational factors underlying animal research (Borgi *et al.*, 2021) and including VAC2VAC (Vaccine batch to vaccine batch comparison for consistency testing) looking at developing and validating quality testing approaches for both human and veterinary vaccines using non-animal methods (2016-2022).

* 3R@ISS coordinator group

In 2021, a panel of qualified ISS researchers with different backgrounds and expertise joined together with the intent of identify research activities carried out in the ISS, related to the application of the 3Rs principle, promoting and spreading the knowledge of their implications both for research and regulatory purposes among a larger ISS community, thus giving birth to the 3Rs@ISS working group.

The main objectives of the group are:

- establishment of a forum of in-house researchers, meeting regularly to discuss and share research-related issues concerning the application of the 3Rs;
- organisation of seminars for all internal researchers to allow the exchange of ideas and new perspectives;
- constitution of working groups aimed at dissemination activities such as special issues on relevant topics and guidelines;
- creation of a dedicated section in the institutional website to share documents and initiatives related to the theme and to training courses/webinars dissemination.

The first step taken by the organizing committee was a survey to identify the ISS ongoing projects related to 3Rs application and their level of funding. For this purpose, a census form was distributed to all staff to be filled on a voluntarily basis and the data were collected and analysed. From the analysis it resulted that 2/3 (14 out of 22) of ISS centres and departments were involved in experimental activities and/or regulatory tasks related to the 3Rs. Among all projects identified, basic research was found to be the most represented (41%), followed by preclinical and translational research (27%), regulatory activities (16%) and the third mission (16%), and with most of proposals focused on replacement. There were on average 3 publications for each funded project.

This first year of 3Rs@ISS group activity has resulted in the organization of 6 meetings/seminars on topics that emerged as of interest within the researchers' forum, namely, from more theoretical perspectives to direct application of Replacement, Reduction and Refinement.

The 3Rs@ISS group is by nature an open initiative, always hoping in new participations, with the aims to pave the way to a long and fruitful collaboration among colleagues and external collaborators, to stimulate the research on new methods for 3Rs implementation, to disseminate the current "Best Practice" for animal use and stimulate the continuous updating of such practices (according to EU Directive 63/2010 and DL.vo 26/2014).

On this background, the 3Rs@ISS group proposed to draw up this first report presenting the ISS most innovative activities related to the Replacement issue. In our perspective it represents the first one of a series of editorial initiatives, aimed at disseminating ISS activities for the continuous development of advanced non-animal testing as well as for improved ethical- and scientifically-sounded research on animal models.

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