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Reflection paper on the current regulatory testing requirements for medicinal products for human use and opportunities for implementation of the 3Rs

Draft

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1. Executive summary

The "Reflection paper on the current regulatory testing requirements for human medicinal products and opportunities for implementation of the 3Rs" has been revised to incorporate newly developed approaches in the field of 3Rs that through implementation of revised guidelines are now accepted for regulatory decisions in risk assessment of human medicinal products. It also includes new approaches that are being investigated to appraise their impact on 3Rs in a regulatory context.

2. Introduction

In December 2016, the CHMP and CVMP published the "Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches" (EMA/CHMP/CVMP/JEG-3Rs/450091/2012). This reflection paper has been developed as a follow-up to that guideline and provides an overview of the main animal tests required for the regulatory testing of medicinal products for human use (a parallel document has been developed in relation to veterinary medicinal products [EMA/CHMP/CVMP/JEG-3Rs/740772/2015]). It includes information on opportunities for limiting animal testing that can already be implemented, where appropriate, as well as information on opportunities that may become available in the future. It should be emphasised that the latter comprises areas that are currently under investigation and will necessitate data review and further discussion before a definitive impact on 3Rs can be appraised. This document should encourage sponsors to develop new 3Rs methodologies and submit them for regulatory review and acceptance.

The information is presented in tabular format and divided into sections based on the areas of responsibility of the EMA working parties. In certain areas, essential initiatives or contributions were provided by the European Directorate for the Quality of Medicines & Healthcare (EDQM), in particular the Biological Standardisation Programme (BSP) Group 15 (V) (Vaccines, sera for human and veterinary use).

EMA working parties involved are:

- the joint CHMP/CVMP Quality Working Party (QWP), which develops guidance on quality testing for medicinal products for human and veterinary use;
- the CHMP Non-Clinical Working Party (NcWP, formerly Safety Working Party), which develops guidance on non-clinical testing;
- the CHMP Biologics Working Party (BWP), which develops guidance on quality and safety testing for biological and biotechnological medicinal products;
- the CHMP Vaccines Working Party (VWP), which develops guidance relating to the development of vaccines, including guidance on non-clinical requirements for vaccines;
- the Committee for Advanced Therapies (CAT), which is responsible for assessing the quality, safety and efficacy of advanced-therapy medicinal products (ATMPs) and following scientific developments in the field;
- the CHMP Biosimilar Medicinal Products Working Party (BMWP), which develops guidance on non-clinical and clinical matters relating to biosimilar medicinal products;
- the CHMP Methodology Working Party (MWP), which develops guidance in non-clinical and clinical areas such as biostatistics, modelling and simulation, pharmacokinetics, pharmacogenomics and real-world evidence.

It is important to note that for the tests enumerated in the tables below applicants may deviate from the relevant guidelines in the tables as long as they are able to provide data (new data or published literature) or argumentation to scientifically demonstrate that the 3Rs approach provides an equivalent level of information on quality, safety or efficacy. If an applicant considers that a particular test is not necessary or would like to use a novel 3Rs-compliant methodology (including, but not limited to, those mentioned as newly identified opportunities), advice on the acceptability of the proposed approach can be requested through a scientific advice procedure through EMA's Scientific Advice Working Party

(SAWP). In addition, developers of 3Rs testing approaches or new approach methods (NAMs)[1] can apply to SAWP for a qualification advice or a qualification opinion. Furthermore, developers of NAMs can approach EMA's Innovation Task Force (ITF) for an early dialogue with scientific experts from the regulatory network to discuss regulatory, technical and scientific issues related to method development, the collection of data required for regulatory acceptance of the method, and finally on future qualification of the 3Rs-compliant testing method for a particular context of use.

The current reflection paper provides a snapshot of animal testing requirements and possibilities to include 3Rs-compliant approaches at the time of publication. It is to be expected that, over time, new 3Rs-compliant testing approaches will become accepted, and the tables should be interpreted accordingly.

In reviewing these tables, the reader should remember that while the CHMP is committed to encouraging use of 3Rs approaches wherever possible, these cannot be accepted at the expense of safety and efficacy for patients.

[1] The expression new approach methods/methodologies (NAMs) refers to 3Rs-compliant methods which may be incorporated in the assessment of the safety and efficacy of new medicines to replace or reduce animal use. Examples include *in vitro* (cell-based) systems and computer modelling.

3. Overview of regulatory animal testing requirements

3.1. CHMP/CVMP Quality Working Party and European Pharmacopoeia (Ph. Eur.)

Overview of animal testing requirements for active substances of synthetic, semi-synthetic, fermentation origin as well as medicinal products and radiopharmaceutical preparations (Quality Working Party - CHMP/CVMP)

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Pyrogens (Rabbits)* *see also BWP section for biological products	European Pharmacopeia (Ph. Eur.) chapter 2.6.8 Pyrogens *Note: chapter 2.6.8 will be suppressed from the Ph.Eur as of 1 January 2026* Ph. Eur. chapter 5.1.13 Pyrogenicity *Note: chapter 5.1.13 is to be implemented on 1 July 2025*	Pyrogenicity testing was required for parenteral Amikacin-sulfate, Calcium levulinate dihydrate, Colistimethate sodium, Chloramphenicol sodium succinate, Dicloxacillin sodium, Flucloxacillin sodium, Glucose, Glucose monohydrate, Kanamycin acid sulphate, Kanamycin monosulfate, Polymyxin B sulphate, Sodium citrate. Besides the active substances in the table, the test was used in case of medicinal products derived from these active substances and some older products. *Note: the requirement to carry out the rabbit pyrogen test in*	In June 2021, the European Pharmacopoeia (Ph. Eur.) Commission took the decision to completely replace the RPT 2.6.8 in the Ph. Eur. with MAT (2.6.30) or BET (2.6.14/2.6.32) within approximately 5 years. Subsequently, in June 2024, the Ph. Eur. Commission adopted revised text for 57 monographs where the RPT has been deleted with an implementation date of 1 July 2025. Accordingly, the requirement to carry out the RPT in the monographs for Amikacin-sulfate, Calcium levulinate dihydrate, Colistimethate sodium, Chloramphenicol sodium succinate, Dicloxacillin sodium, Flucloxacillin sodium monohydrate, Glucose, Glucose monohydrate, Kanamycin acid sulfate, Kanamycin monosulfate,	

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		the monographs for the above-mentioned substances has been suppressed. Revised monographs are to be implemented on 1 July 2025.	Polymyxin B sulfate, and Sodium citrate has been deleted. As a result, the new requirements for pyrogenicity in the revised general monograph 'Substances for pharmaceutical use' (2034) will apply. The new requirement in the general monograph 2034 refers to the general chapter 5.1.13 (to be implemented on 1 July 2025), which provides guidance for the selection and implementation of a suitable test for pyrogenicity: MAT (as described in 2.6.30) or BET (as described in 2.6.14/2.6.32).	
Bacterial Endotoxins (amoebocyte lysate from *Limulus polyphemus* or *Tachypleus tridentatus)** *see also BWP section for biological products	Ph.Eur. chapter 2.6.14 Bacterial endotoxins	Test required for active substances of endotoxin-free grade and most medicinal products intended for parenteral administration.	The BET is used to detect or quantify endotoxins from Gram-negative bacteria using Limulus Amoebocyte Lysate obtained from blood cells (amoebocytes) of horseshoe crabs (*Limulus polyphemus, Tachypleus tridentatus*). General chapter 2.6.32 *Test for bacterial endotoxins using recombinant factor C* published in Ph. Eur. Supplement 10.3, describes a test for bacterial endotoxins using recombinant factor C (rFC) that may be used as an alternative to Limulus Amoebocyte	

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			Lysate (LAL)-based methods, thus alleviating the need for the animal resource (i.e. the need for lysate from two species of horseshoe crab known to be endangered).	
Abnormal toxicity test (ATT) (Mice)* *see also BWP section for biological products	Ph. Eur. chapter 2.6.9 Abnormal toxicity was suppressed from the Ph. Eur. in Supplement 9.6 of July 2018 (implementation date 1 January 2019).	ATT was originally developed to detect external contaminants that cause adverse events in biological products.	At its session in November 2017, the Ph.Eur. Commission endorsed the complete suppression of the abnormal toxicity test from the Ph.Eur. The abnormal toxicity test was removed from all Ph. Eur. monographs referring to it. The corresponding general chapter 2.6.9 Abnormal toxicity was suppressed in Ph. Eur. Supplement 9.6 of July 2018 (implementation date: 1 January 2019).	
Physiological distribution	Ph. Eur. General Monograph on Radiopharmaceutical Preparations (human) (0125)	A physiological distribution study (usually in rats or mice) may be required for the following medicinal products (where radiochemical species in a radiopharmaceutical preparation are not sufficiently identified and controlled by other tests, or to confirm batch consistency): Technetium (99m TC) colloidal rhenium sulphide injection,	The test should be avoided whenever possible. According to the scientific development and improved methods of synthesis in the past years, these animal studies are dispensable for new radiopharmaceuticals prepared according to the Ph.Eur**.** General Monograph on Radiopharmaceutical Preparations (human).	

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		Technetium (99m TC) colloidal tin injection, Technetium (99m TC) gluconate injection, Technetium (99m TC) human albumin injection, Technetium (99m TC) macrosalb injection, Technetium (99m TC) microspheres injection, Technetium (99m TC) succimer injection.	After a review by Ph. Eur. of monographs impacted by the latest revision of general monograph on radiopharmaceutical preparation, (human) 0125 (04/2023), the requirement for a physiological distribution test has been deleted for some preparations (updated list is shown to the left).	

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96 *3.2. CHMP Non-clinical Working Party*

97 Overview of animal testing requirements for non-clinical studies for human pharmaceuticals (Non-clinical Working Party - CHMP)

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Repeated dose toxicity (RTD)	ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals (EMA/CPMP/ICH/286/1995) ICH guideline M3 (R2) – questions and answers (EMA/CHMP/ICH/507008/2011) Guideline on repeated dose toxicity (CPMP/SWP/1042/99 Rev 1 Corr) ICH guideline S6 (R1) - preclinical safety evaluation of biotechnology–derived pharmaceuticals (EMA/CHMP/ICH/731268/1998) ICH guideline S9 on nonclinical evaluation for anticancer pharmaceuticals (EMA/CHMP/ICH/646107/2008) Duration of chronic toxicity testing in animals (rodent and	Generally required in two mammalian species (one non-rodent). The recommended duration of repeated dose toxicity studies to support clinical trials and/or marketing depends on the duration of the indicated treatment and ranges from 2 weeks up to 9 months (see ICH M3(R3)). Generally, for rodents, this is 6 months while for non-rodents it can be up to 9 months (see ICH M3 (R2). In the EU, studies of 6 months duration in non-rodents are generally acceptable).	One species could be acceptable on a case-by-case approach, and if clearly justified. Inclusion of additional *in vivo* endpoints in repeated dose toxicity studies to reduce animal use is accepted if scientifically justified, for example by integration of safety pharmacology or genotoxicity endpoints.	Expansion of the concept of integration of additional endpoints in repeated dose toxicity studies if equivalent safety information is supported by retrospective data analysis and/or when sufficient experience has been acquired. Regarding the exposure-based setting of the maximum tolerated dose (MTD), further discussion on the scientific rationale for the required exposure margin is needed. The repeated dose toxicity guideline (CPMP/SWP/1042/99 Rev 1 Corr) could be revised to align with updated regulatory recommendations on selection of animal species (cfr ICH S5 (R3), S1B (R1)). An EMA reflection paper on opportunities to reduce use of Non-Human Primates (NHP) in regulatory testing is under development (1). As per the EMA Non-clinical Domain Work plan (1), the development of a

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	non-rodent) toxicity testing (CPMP/ICH/300/95; ICHS4)			more streamlined non-clinical development plan that includes specific guidance regarding duration of toxicity studies will be considered for other severely debilitating and life-threatening diseases besides cancer. EMA Concept paper on the revision of the Guideline for regulatory acceptance of 3Rs testing approaches includes plan to provide specific Context-of-Use qualification criteria for microphysiological systems for prediction of drug induced liver injury (DILI) that potentially could be used as a complement to *in vivo* RDT testing (2). Additional 3Rs initiatives for consideration include: - A proposal to use a weight-of-evidence (WoE[2]) approach to evaluate the need for 6-month repeated dose toxicity studies of new monoclonal Antibodies (mAbs) where NHP is the only relevant test species (3). Project is funded by EPAA and involves a collaboration of EPAA, MEB, EFPIA

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				& NC3Rs. See also safety testing of biologics. - A methodology for using Virtual control groups to reduce the number of animals in *in vivo* toxicity testing (4, 5). Project is led by the eTransafe consortium. *(While this approach is considered promising both from a non-clinical/3Rs and from a methodology perspective, it would require further assessment and scrutiny of data before integration in a regulatory context).* - The possibility to use only one species for longer-term repeated dose toxicity testing (6). Project led by NC3Rs involving 30 pharmaceutical companies, CROs and regulatory bodies.
Repeated dose toxicity: reversibility	Guideline on repeated dose toxicity (CPMP/SWP/1042/99 Rev 1 Corr) ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for	ICH M3(R2) states the following in Section 1.4, General principles: "The goals of the non-clinical safety evaluation generally include a characterisation of toxic effects with respect to	Guidelines, in particular ICH M3 and ICH S6 give recommendations on the purpose of reversibility and interpretation of toxicity findings after a recovery period. The ICH guideline M3 (R2) - questions and answers document explains in detail reasons	Initiatives from NC3Rs project on best practices to reduce use of recovery animals can be considered (7, 8). For example, that recovery animals should be included for scientific reasons and not by default, that recovery animals at one appropriate dose level should

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	pharmaceuticals. (EMA/CPMP/ICH/286/1995) ICH guideline M3 (R2) – questions and answers (EMA/CHMP/ICH/507008/2011) ICH guideline S6 (R1) - preclinical safety evaluation of biotechnology–derived pharmaceuticals (EMA/CHMP/ICH/731268/1998)	target organs, dose dependence, relationship to exposure, and, when appropriate, potential reversibility.”	where reversibility is not warranted. This Q&A also states that "If a reversibility study is warranted, it is efficient to conduct it as part of a chronic study so that all toxicities of concern can be assessed in a single study, provided that it is not critical to conduct it earlier to support a specific clinical trial."	be sufficient, and consideration of the whole development program when deciding which study recovery animals should be included in.
Genotoxicity	ICH guideline S2(R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use (EMA/CHMP/ICH/126642/2008)	Standard test battery: *in vivo* genotoxicity measurement (e.g., micronucleus (MN) test) can be integrated into a repeated dose toxicity study, when feasible; otherwise, a stand-alone *in vivo* genotoxicity study is requested. Follow up of *in vitro* positives: A single combined *in vivo* genotoxicity study (e.g. MN blood & comet liver) is acceptable.	Standard battery without extra animal study is recommended (*in vitro* tests plus genotoxicity integrated in repeated dose toxicity study).	The possibility to include state-of-the-art genome sequencing techniques as a read-out for genotoxicity in standard *in vivo* tests can be considered.
Carcinogenicity	ICH guideline S1B(R1) on testing for carcinogenicity of	One long-term rodent carcinogenicity study plus one other supplementary study	New addendum of ICH S1B(R1) to be used in close conjunction with ICH S1A *Guideline on the Need for*	Further evaluation of the need for (transgenic) mouse study.

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	pharmaceuticals (EMA/774371/2022)	providing additional information that is not readily available from the long-term assay. Choice of species based upon considerations including pharmacology, repeated dose toxicology, metabolism, toxicokinetics, route of administration. In the absence of clear evidence favouring one species, use of the rat as default.	*Carcinogenicity Studies of Pharmaceuticals*, S1B *Testing for Carcinogenicity of Pharmaceuticals*, and S1C(R2) *Dose Selection for Carcinogenicity Studies*. The addendum is applicable to all pharmaceuticals that require carcinogenicity testing (ICH S1A). Biotechnology-derived pharmaceuticals fall under ICH S6(R1). The addendum expands the evaluation process for assessing human carcinogenic risk of pharmaceuticals by introducing the possibility to use an integrative approach providing specific WoE[2] criteria that inform: 1. Whether or not a 2-year rat study is likely to add value to a human carcinogenicity risk assessment 2. Circumstances where a mouse carcinogenicity study (either a 2-year study in a standard strain of mice or a short-term study in a transgenic model) may not be appropriate.	Appraisal of results of emerging NAMs or 3Rs testing approaches (e.g., *in vitro*, *in silico*, -omics, etc) in informing carcinogenic risk of pharmaceuticals early in drug development programs and in the WoE assessment for carcinogenicity testing.

[2] Weight of evidence (WoE) in the context of non-clinical safety assessment refers to a comprehensive assessment, based on the totality of the *in vitro*, *in silico*, *in chemico* and/or *in vivo* evidence available from relevant non-clinical studies and public sources, including pharmacology, pharmacokinetic (ADME), *in vitro* toxicity, and – where applicable - clinical (safety) data (adult and/or paediatric).

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			Addendum also adds a plasma exposure ratio-based approach for setting the high dose in the rasH2-Tg mouse model, while all other aspects of the recommendations for high dose selection in S1C(R2) guideline still apply.	
Developmental and reproductive toxicity	ICH S5 (R3) Guideline on detection of reproductive and developmental toxicity for human pharmaceuticals. (EMA/CHMP/ICH/544278/1998)	Study of fertility and early embryonic development (FEED) up to implantation: rat (or mouse) default. For biologicals where NHP is the only pharmacologically relevant species: repeated dose toxicity evaluations. Study for effects on embryo-foetal development (EFD): rat and rabbit. Study for effects on pre- and postnatal development (PPND), including maternal function: rat (or mouse).	Defer definitive *in vivo* testing as part of an integrated testing strategy to support limited inclusion of women of childbearing potential (WOCBP) in clinical trials. Inclusion of exposure-based endpoint for dose-selection (testing up to 25-fold exposure margin at maximum recommended human dose (MRHD)). ICH S5(R3) Annex 2 Alternative assays: - Identification of potential use of alternative assays, including specific scenarios. - Qualification criteria for NAMs. - Reference compound list. For biologicals where NHP is the only pharmacologically relevant species:	Maintenance procedure ICH S5 (R4): Potential updates to Annex 1 to provide additional guidance on *in vivo* study designs including: - Reduction and refinement opportunities. - Use of minipig study as possible replacement for NHP, if sufficient data is available. Potential updates to Annex 2 on alternative assays including: - Update of qualification criteria based on acquired regulatory experience. - Update of scenarios under which NAMs can be applied. - Update of reference compound list.

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			histopathology of reproductive organs in sexually mature animals is sufficient. No dedicated FEED required. An enhanced pre-and postnatal development (ePPND) study can be conducted instead of separate EFD and PPND studies.	EMA initiatives: - As per the Non-clinical Domain Work plan (1), an EMA reflection paper regarding alternatives to NHPs in safety testing will be developed, which may affect reproductive toxicological testing.
Toxicokinetics	ICH guideline S3A: Toxicokinetics: a guidance for assessing systemic exposure in toxicology studies (CPMP/ICH/384/95) ICH guideline S3A: Note for guidance on toxicokinetics: the assessment of systemic exposure in toxicity studies - questions and answers (EMA/CHMP/ICH/320985/2016)	Toxicity studies which may be usefully supported by toxicokinetic (TK) information include single- and repeated dose toxicity studies, reproductive, genotoxicity and carcinogenicity studies. Normally, samples for the generation of toxicokinetic data may be collected from main study animals where large animals are involved, but satellite groups may be required for the smaller (rodent) species.	Q&A document describes points to consider before incorporating the microsampling method in TK studies, acknowledges its benefits (and some limitations) for assessment of TKs in main study animals and its overall important contribution to the 3Rs, by reducing or eliminating the need for TK satellite animals. Use of microsampling methods can reduce or eliminate the need for TK satellite animals in any type of non-clinical safety study in rodents and minimise pain and distress in animals. Microsampling is generally applicable to the majority of pharmaceuticals and biopharmaceuticals.	Microsampling could be more widely implemented for GLP non-clinical studies.
Pharmacokinetics	ICH guideline M3(R2) on non-clinical safety studies for the	Information on pharmacokinetics (PK) (e.g.,	Use of standard *in vitro* models for comparison of *in vitro* metabolism and	Complex *in vitro* systems (e.g. Microphysiological systems (MPS),

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	conduct of human clinical trials and marketing authorisation for pharmaceuticals. (EMA/CPMP/ICH/286/1995) Note for guidance on pharmacokinetics: Repeated dose tissue distribution studies (CPMP/ICH/385/95; ICHS3B)	absorption, distribution, metabolism and excretion), in test species and *in vitro* biochemical information relevant to potential drug interactions. Repeated dose tissue distribution studies in rodent or non-rodents (case-by-case).	protein binding across species (including human). Non-clinical evaluation of drug-drug interactions is limited to *in vitro* investigations as per ICH guideline M12 on drug interaction studies (EMA/CHMP/ICH/652460/2022).	Organ-on-Chip (OoC)) have been developed for ADME and could potentially be used to generate data for a regulatory submission or a qualification procedure.
Non-Clinical Evaluation of Anticancer Pharmaceuticals	ICH guideline S9 on nonclinical evaluation for anticancer pharmaceuticals (EMA/CHMP/ICH/646107/2008) ICH S9 guideline on nonclinical evaluation for anticancer pharmaceuticals - questions and answers (EMA/CHMP/ICH453684/2016)2018)	Basic framework for non-clinical evaluation of anticancer pharmaceuticals.	3-month data sufficient for marketing authorisation application (previously 6-month chronic toxicity study needed). ICH S9 *Q&A:* A 3-month study in one species (rodent, if relevant) can be sufficient to support continued clinical development of a genotoxic drug targeting rapidly dividing cells. No need for fertility studies (effect on reproductive organs from repeated dose toxicity studies). No need for pre- and post-natal development studies if embryo-foetal development study is positive, no confirmatory study in 2nd species is needed.	The following emerging concept could be considered: A methodology for using virtual control groups to reduce the number of animals in *in vivo* toxicity testing has been developed (4, 5). Project is led by the eTransafe consortium. *(While this approach is considered promising both from a non-clinical/3Rs and from a methodology perspective, it would require further assessment and scrutiny of data before integration in a regulatory context).*

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			ICH S9 *Q&A:* Reproductive toxicity assessment can in some cases be based on dose range finding studies in a single species and alternative assessments may be used (see also ICH S5(R3)). In cases where NHP is the only relevant species and WoE clearly indicates risk, an EFD study is not warranted. Inclusion of safety pharmacology endpoints in repeated dose toxicity studies (ECG in non-rodents). No need for non-rodent studies for initiation of clinical trials with cytotoxic pharmaceuticals. Need for recovery in general toxicity studies based on scientific rationale. *ICH S9 Q&A:* No need for additional general toxicity studies where a product significantly extends survival or clinical development is extended to oncology indications borderline to ICH S9. Other	

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			toxicology studies are to be conducted on a case-by-case basis. *In vitro* assays of anti-tumor activity could be sufficient for characterization of pharmacology, if they generate relevant data. Supportive care (e.g. antibiotics) can be appropriate in some cases. Abuse liability studies not required for pharmaceuticals covered by ICH S9. Antibody-drug-conjugate (ADC) products should be studied in at least one species. Studies on the mAb alone are not warranted. If additional characterisation of payload is needed, it is sufficient to evaluate it in one species, preferably as a separate arm in the toxicology study of the ADC. Additional toxicological studies with disproportional metabolites are not needed. They should only be considered if the metabolite is not produced in the test species and human exposure is high. Mutagenic impurities are managed as outlined in ICH Q3A/B (ICH M7 not	

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			applicable to anti-cancer products covered by ICH S9). If impurities exceed thresholds, a risk assessment should be conducted. A qualification study is usually not needed.	
Safety testing of biologicals	ICH guideline S6 (R1) – preclinical safety evaluation of biotechnology-derived pharmaceuticals (EMA/CHMP/ICH/731268/1998)	Basic framework for non-clinical safety evaluations of biologicals.	Enhanced pre- & post-natal development study design: Reduction of the need for 2 separate studies (embryo-foetal development and peri-postnatal development studies). Reduction of animal numbers with one treated group and a control group can be accepted based on scientific justification. No need for stand-alone fertility studies in non-human primates if additional relevant endpoints (e.g. histology of reproductive organs in sexually mature animals) are included in repeated dose toxicity studies of at least 3 months duration). Use of only one relevant species for chronic toxicity studies (by default the lowest phylogenetic species) generally acceptable (e.g. similar toxicity findings from biologicals in the same	As per the EMA Non-clinical Domain Work plan (1), reflection paper on opportunities to minimise use of non-human primates (NHP) in regulatory testing is under preparation. Additional 3Rs initiatives for consideration include: - A proposal to use a WoE approach to evaluate the need for 6-month repeated dose toxicity studies of new mAbs, where NHP is the only relevant test species (3). Project is funded by EPAA and involves a collaboration of EPAA, MEB, EFPIA & NC3Rs. See also safety testing of biologics. - A methodology for using Virtual control groups to reduce the number of animals in *in vivo* toxicity testing 4, 5). Project led by the eTransafe consortium.

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			class and findings understood from mode of action). Recovery group sufficient at one (justified) dose level. No need for two-year carcinogenicity studies unless there is concern. Use of a surrogate product in order to avoid use of non-human primates e.g. for reproductive toxicity testing, only if necessary and scientifically justified.	- A project led by NC3Rs has identified opportunities to reduce use of recovery animals in toxicity studies of mAbs (7, 8). For these or other novel approaches, voluntary data submission according to the Guideline on the principles of regulatory acceptance of 3R testing approaches (EMA/CHMP/CVMP/JEG-3Rs/450091/2012) could be considered.
Safety pharmacology	Note for Guidance on the Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarisation (QT Interval Prolongation) by Human Pharmaceuticals (CPMP/ICH/423/02; ICH S7B) ICH guideline E14/S7B: clinical and Nonclinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential - questions and answers (EMA/CHMP/ICH/415588/2020)	*In vivo* and *in vitro* tests as complementary approaches to assess the potential for QT interval prolongation. ICH E14/S7B Q&A describes best practices for *in vitro* and/or *in vivo* tests, in case the assays are to be used as part of an integrated clinical and non-clinical risk assessment for delayed ventricular repolarisation and torsade de pointes, which can inform the design of clinical investigations and interpretation of their results.	Integrated test strategy including *in vitro* tests (e.g. hERG assay) for assessment of QT interval prolongation (ICH S7B). Increased predictive capacity of *in vitro* assays through implementation of best practices for *in vitro* IKR/hERG assays, *in vitro* ventricular repolarisation assays (e.g. human induced pluripotent stem cell derived cardiomyocyte assays). Description of generic principles for evaluating the predictivity of proarrhythmic risk prediction models. This can encompass *in silico*, *in vitro, ex vivo* and, where needed, *in vivo*	EMA is developing regulatory acceptance criteria for qualification of 3Rs proarrhythmic models, including organ-on-chip models, for use as part of an integrated risk assessment for regulatory purposes. This will be included in the Guideline on the principles of regulatory acceptance of 3Rs testing approaches (2). The ICH E14/S7B Implementation Working Group (IWG) has been reactivated in March 2024 to develop second-round Q&As for the ICH E14 and ICH S7B Guidelines. These will address any outstanding gaps not captured in the first round of Q&As,

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			models, to guide possible qualification for the intended context of use as part of an integrated risk assessment for regulatory purposes.	which were finalised in February 2022. Furthermore, they will provide additional clarity aimed at making the testing framework sustainable and flexible enough to limit potential regional differences in the recommended approach to this important safety endpoint for existing and emerging modalities. Issues to be resolved with a 3Rs impact pertain to: - Clarification of modalities in scope of ICH S7A, with consideration for assessment needs in line with the pharmaco-kinetic and -dynamic profile of the test material and aiming for consistency with related ICH guidelines (ICH S7A, and ICH S6). - Clarification of requirements for general toxicology studies with integrated safety pharmacology endpoints when used in to inform an ICH E14/S7B integrated risk assessment. - Clarification of requirements to assess QTc and proarrhythmic liability of novel modalities.

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	Note for Guidance on Safety Pharmacology Studies for Human Pharmaceuticals (CPMP/ICH/539/00; ICHS7A)	"Core battery tests" of CNS and cardiovascular/respiratory function.	Integration of safety pharmacology parameters in repeated dose toxicity studies (see ICH S9).	Inclusion of safety pharmacology endpoints: need for retrospective data analysis to expand concept beyond ICH S9.
Immunotoxicity	Note for Guidance on Immunotoxicity Studies for Human Pharmaceuticals (CHMP/167235/2004; ICH S8)	Non-clinical (generally rodents) assessment of unintended immune-suppression or enhancement.	Specific studies only when standard toxicity studies indicate a cause for concern (WoE approach).	OECD test guidance (TG) 360 *Detailed review paper on in vitro test addressing immunotoxicity with a focus on immunosuppression*': this paper defines an *in vitro* tiered approach to testing and assessment.
Phototoxicity	ICH guideline S10: Guidance on photosafety evaluation of pharmaceuticals (EMA/CHMP/ICH/752211/2012)	Integrated process that can involve an evaluation of photochemical characteristics, data from non-clinical studies and human safety information.	Use of photo-chemical evaluation and *in vitro* tests, in combination with *in vivo* non-clinical or clinical data if deemed necessary based on a WoE approach. No photocarcinogenicity test (see ICH M3(R2)) and no *in vivo* photo-allergy test.	A new OECD guidance document on *Integrated Approach to testing and assessment (IATA) for Phototoxicity is in preparation*. (Public consultation completed in February 2023). According to the draft document, introduces an Adverse Outcome Pathway (AOP) for Phototoxicity, overview of available *in vitro/in silico* tests and a decision tree.
Local Tolerance	Guideline on non-clinical local tolerance testing (EMA/CHMP/SWP/2145/2000-Rev.1. Corr 1).	Local tolerance testing should be included as part of the general toxicity studies; "stand-alone" studies on local tolerance are generally not required.	Extra animal studies are generally not required. *In vitro* local tolerance testing and/or integration of appropriate endpoints into general toxicity studies highly recommended.	Guideline on non-clinical local tolerance testing (EMA/CHMP/SWP/2145/2000-Rev.1) is to be revised, where 3Rs aspects will be considered. Newly identified opportunities for consideration include:

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
				- Increased emphasis on *in vitro* testing, especially with regards to skin and eye irritation. - Integration of NAMs for assessing skin sensitisation potential of topically applied pharmaceuticals in accordance with OECD TG 442C, 442D, 442E, and 497.
Dependence Potential	Guideline on the non-clinical investigation of the dependence potential of medicinal products (EMEA/CHMP/SWP/94227/2004)	Two-tiered approach to investigate the dependence potential of new CNS active substances. In the first tier, studies reveal the pharmacological profile of the active substance. Based on data from the first tier and other early indicators, it should be decided whether subsequent *in vivo* behavioural studies investigating the reinforcing properties and potential to cause withdrawal phenomena are necessary.	Specific studies only when, based on a WoE approach, standard non-clinical studies indicate a cause for concern.	
Testing in Juvenile Animals	ICH guideline S11 on nonclinical safety testing in support of development of paediatric	The conduct of additional non-clinical investigations (e.g. safety studies in juvenile	Specific studies warranted only when taking into account the clinical context	An inventory of regulatory lessons learned after the latest guideline

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	pharmaceuticals (EMA/CHMP/ICH/616110/2018)	animals) should be undertaken only when previous non-clinical and human data are judged to be insufficient to support paediatric studies.	and the need of additional information based on a WoE approach. Detailed information on study design provided, to ensure optimal clinical relevance of juvenile animal study design. A list of core endpoints has been defined. Each additional endpoint should be justified. Overview of age-dependent development of organ systems by species (human, rat, beagle dog, minipig, cynomolgus monkey (NHP)).	revision may provide further improvement of the guideline.
Environmental studies	Environmental risk assessment of medicinal products for human use (CPMP/SWP/4447/00 Rev.1)	Basic framework for environmental risk assessment of human pharmaceuticals Phase II, Tier A: Fish toxicity (Fish Early Life Stage Toxicity test OECD TG 210) Phase II, Tier B Fish bioaccumulation (OECD TG 305)	General recommendations are made that the principles of 3Rs should be followed. Testing strategy (i.e., a tiered approach) and methodologies optimised in line with 3Rs. A tailored testing strategy is applied to active substances with specific mode-of-action. For example, for antibacterials with a predicted environmental concentration (PEC) exceeding the action limit, a testing strategy evaluating the effects on lower trophic levels including bacteria,	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
			algae and aquatic invertebrates is considered sufficiently sensitive and *in vivo* fish tests not required. Applicants are encouraged to share ERA data to avoid unnecessary repetition of (animal) studies.	
Qualification of impurities	Non-mutagenic impurities: ICH Guideline Q3A(R2): Note for guidance on impurities testing: impurities in new drug substances (CPMP/ICH/2737/99) ICH Guideline Q3B(R2): Note for guidance on impurities in new drug products (CPMP/ICH/2738/99) ICH guideline Q3C (R6) on impurities: guideline for residual solvents (EMA/CHMP/ICH/82260/2006)	A general toxicity study (one species, usually 14 to 90 days), if data are unavailable for qualification (new impurity) and guideline criteria are met.		An EMA reflection paper on the qualification of non-mutagenic impurities has been drafted (EMA/CHMP/SWP/545588/2017)(13) that will include general information on how *in silico* and *in vitro* approaches (e.g. TTC, QSAR, read-across (RAX) and *in vitro* assays) can contribute to an integrated risk assessment of impurities (if suitable for the purpose) and in some cases obviate the need for dedicated *in vivo* studies. ICH guideline Q3E on extractables and leachables is being developed (10) that will include 3Rs aspects in safety assessment.
	Mutagenic impurities: ICH guideline M7(R2) assessment and control of DNA reactive (Mutagenic) impurities	In case an impurity tests positive in a bacterial mutagenicity assay - and levels of the impurity cannot be controlled at an appropriate	As an initial step, *in vitro* and *in silico* approaches (e.g. TTC, QSAR and *in vitro* assays) are recommended.	Error-corrected next generation sequencing could be used to replace dedicated *in vivo* studies, as data can be generated from tissues harvested in general toxicology studies.

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	in pharmaceuticals to limit potential carcinogenic risk.	acceptable limit (defined in M7) - an *in vivo* mutation study is recommended.	In the case of nitrosamine impurities, an RAX approach to structurally similar substances can be used if supported by a WoE.	
Safety testing of oligonucleotides				A new ICH guideline (ICH S13) on Nonclinical safety studies for Oligonucleotide-based therapeutics is under development. The guideline will harmonise non-clinical requirements for oligonucleotide-based therapies, taking3Rs aspects into account.

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99 ## *3.3. CHMP Biosimilar Medicinal Products Working Party*

100 Overview of animal testing requirements for biosimilar medicinal products (biosimilars working party (BMWP))

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
General Guidelines for Biosimilar Medicines				
Similar biological medicinal products	Guideline on similar biological medicinal products (CHMP/437/04-Rev.1) Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues (EMEA/CHMP/BMWP/42832/2005-Rev.1)	For all biosimilar medicines stepwise approach is recommended for evaluation of the similarity of the biosimilar and the reference product. Analytical studies and *in vitro* pharmaco-toxicological studies should be conducted first, and a decision then made as to the extent of what, if any, *in vivo* animal studies will be required.	*In vivo* evaluation generally not needed. If an *in vivo* evaluation is deemed necessary, the focus of the study/studies (PK and/or PD and/or safety) depends on the need for additional information. Animal studies should be designed to maximise the information obtained. Depending on the endpoints used, it may not be necessary to sacrifice the animals at the end of the study. The duration of the study (including observation period) should be justified, taking into consideration the PK behaviour of the reference medicinal product and its clinical use.	The value of *in vivo* data in the assessment of biosimilarity is generally considered limited and the need for *in vivo* studies should be thoroughly scrutinised. EMA is drafting an overarching reflection paper on a tailored clinical approach in biosimilar development (11) that will replace most product-specific guidelines. The reflection paper is expected to reflect the regulatory experience that non-clinical *in vivo* studies have limited value in the comparability exercise for biosimilars. An EMA reflection paper regarding alternatives to NHPs in safety testing is under development to minimise use of this species (1).
Specific Biosimilar Medicine Guidance				
Biosimilar FSH	Guideline on non-clinical and clinical development of similar	The Steelman-Pohley assay needs to be performed to		

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	biological medicinal products containing recombinant follicle-stimulating hormone (r-hFSH) (CHMP/BMWP/671292/2010)	establish the *in vivo* potency of both the biosimilar and the reference product. It is included in the current guideline that the number of different assays performed may be reduced by a study design in which the biosimilar and the reference medicinal product are compared and simultaneously calibrated against the reference standard. This reduces inter-assay variation and is more economical with regard to reagents and animals used.		
Biosimilar IFN-beta	Guideline on similar biological medicinal products containing interferon beta (CHMP/BMWP/652000/2010)	Generally, *in vivo* studies in animals are not recommended.		
Biosimilar mAbs	Guideline on similar biological medicinal products containing monoclonal antibodies: non-clinical and clinical issues (EMA/CHMP/BMWP/403543/2010)	Generally, *in vivo* studies in animals are not recommended.		
Biosimilar EPO	Similar biological medicinal products containing recombinant erythropoietins	Generally, *in vivo* studies in animals are not recommended.		

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	(EMEA/CHMP/BMWP/301636/08 Rev.)			
Biosimilar LMWH	Guideline on non-clinical and clinical development of similar biological medicinal products containing low-molecular-weight heparins (EMEA/CHMP/BMWP/118264/2007 Rev. 1)	*In vivo* comparative studies only required in some circumstances. (Separate repeat-dose toxicity studies generally not required).	If physicochemical and biological characterisation of the biosimilar and the reference low-molecular-weight heparins performed using sensitive state-of-the-art methods convincingly demonstrates close similarity, *in vivo* studies are not required as part of the comparability exercise.	
Biosimilar INF-alpha	Reflection paper on non-clinical and clinical development of similar medicinal products containing recombinant interferon alpha (EMEA/CHMP/BMWP/102046/2006)	The pharmacodynamic activity of the similar and the reference medicinal product could be quantitatively compared in an appropriate animal model. Data from at least one 4-week repeated dose toxicity study (including local tolerance data) should be provided.		
Biosimilar GCSF	Annex to guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues - Guidance on biosimilar medicinal products containing recombinant granulocyte-colony stimulating	*In vivo* rodent models, neutropenic and non-neutropenic, should be used to compare the pharmacodynamic effects of the test and the reference medicinal product. Data from at least one 4-week repeated dose toxicity study		

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	factor (EMEA/CHMP/BMWP/31329/2005)	(including local tolerance data) should be provided.		
Biosimilar somatropin	Annex to guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues - Guideline on similar medicinal products containing somatropin (EMEA/CHMP/BMWP/94528/2005)	Generally *in vivo* studies are not required.		
Biosimilar recombinant human insulin and insulin analogues	Guideline on non-clinical and clinical development of similar biological medicinal products containing recombinant human insulin and insulin analogues (EMEA/CHMP/BMWP/32775/2005 Rev.1)	Comparative *in vivo* studies of pharmacodynamic effects would not be anticipated to be sensitive enough to detect differences not identified by *in vitro* assays and are not required as part of the comparability exercise. Generally, separate repeated dose toxicity studies are not required.		

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103 *3.4. CHMP Biologics Working Party and European Pharmacopoeia (Ph. Eur.)*

104 Overview of animal testing requirements for biological medicinal products (Biologics Working Party (BWP) - CHMP)

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Manufacture, characterisation and control of the drug substance	Annex I of Directive 2001/83/EC CHMP guideline on development, production, characterisation and specifications for monoclonal antibodies and related substances (CHMP/BWP/157653/07)	The biological activity should be assessed by *in vitro* and/or *in vivo* assays as appropriate.	The option of using *in vitro* assays already exists. Guideline updated to remove reference to the production of monoclonal antibodies from ascites fluid. An *in vitro* assay should be used to monitor the biological activity of the monoclonal antibody unless thoroughly justified.	EURL ECVAM recommends that animals should no longer be used for the development and production of antibodies including for therapeutic use. Identified 3Rs opportunities include e.g. phage display methodologies. (European commission, 2020) (12).
Manufacture, characterisation and control of the drug substance	Annex I of Directive 2001/83/EC CHMP guideline on potency testing of cell-based immunotherapy medicinal products for the treatment of cancer (EMEA/CHMP/BWP/271475/2006)	Potency testing may be performed by means *in vivo* or *in vitro* tests.	The option of using *in vitro* assays already exists. Guideline has been updated to stress that for routine testing an adequate *in vitro* assay is the preferred option.	
Manufacture, characterisation and control of the drug substance	Annex I of Directive 2001/83/EC CHMP guideline on the quality of biological active substances produced by stable transgene	Strategies for control of virus and viroid adventitious agents may include *in vitro* and *in vivo* tests for the absence of such material.	The option of using *in vitro* assays already exists. In addition, the guideline identifies several other approaches that may also be used.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	expression in higher plants (CPMP/BWP/48316/06)			
Manufacture, characterisation and control of the drug substance	Annex I of Directive 2001/83/EC CHMP guideline on development and manufacture of lentiviral vectors (CPMP/BWP/2458/03)	In relation to delivery of lentiviral vectors, *in vitro* and/or *in vivo* experiments are needed to assess construct of characteristics including risk of replication competent lentivirus generation.	The option of using *in vitro* approaches already exists.	
Manufacture, characterisation and control of the drug substance	Annex I of Directive 2001/83/EC CHMP Note for guidance on production and quality control of animal immunoglobulins and immunosera for human use (CPMP/BWP/3354/99 rev1) [2016] Ph. Eur. chapter 5.1.13 Pyrogenicity *Note: 5.1.13 is to be implemented on 1 July 2025.*	Production of animal-derived immunoglobulins and immunosera involves immunisation of animals. Potency testing may be performed in animals. Pyrogenicity testing is required.	The existing text encourages the use of *in vitro* methods. A cell based *in vitro* potency assay has been included in the guideline as an example of an *in vitro* assay. In June 2021, the European Pharmacopoeia (Ph. Eur.) Commission took the decision to completely replace the RPT 2.6.8 in the Ph. Eur. with MAT (2.6.30) or BET (2.6.14/2.6.32) within approximately 5 years. In June 2024, the Ph. Eur. Commission adopted revised text for 57 monographs where the RPT has been deleted, as well as new general chapter 5.1.13 on *pyrogenicity,* which	Production: EURL ECVAM recommends that animals should no longer be used for the development and production of antibodies, including those for therapeutic use. Identified 3Rs opportunities include e.g. phage display methodologies. (European commission, 2020). (12)

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
			provides guidance for the selection and implementation of a suitable test for pyrogenicity (MAT (as described in 2.6.30) or BET (as described in 2.6.14/2.6.32)), with an implementation date of 1 July 2025. Ph.Eur. 2.6.8 will be suppressed from 1 January 2026.	
Manufacture, characterisation and control of the drug substance	Annex I of Directive 2001/83/EC CHMP guideline on allergen products: production and quality issues (CHMP/BWP/304831/07)	In relation to stability testing, if it is not possible to perform potency tests, *in vivo* immunogenicity tests or validated alternative *in vitro* tests should be performed at the beginning and end of the proposed shelf-life period.	The option of using *in vitro* assays already exists.	
Manufacture, characterisation and control of the drug substance	Directive 2001/83/EC Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells (EMA/CAT/GTWP/671639/2008 Rev.1)	Potency testing: Biological potency tests in animal tissues, maintained *ex vivo* or in whole animals, should only be considered in situations where a suitable *in vitro* method cannot be accepted.	*In vitro* potency testing is standard.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Manufacture, characterisation and control of the drug substance	Directive 2001/83/EC Guideline on quality of biological active substances produced by transgene expression in animals (EMA/CHMP/BWP/151897/2013)	Provides guidance on the use of transgenic animals in manufacturing.	Alternative approaches can equally be used - there is no requirement to use transgenic animals in the manufacture of biological medicinal products.	
Specifications	Directive 2001/83/EC ICH Topic Q6B: Note for guidance on specifications - test procedures and acceptance criteria for biotechnological/biological products (CPMP/ICH/365/96)	Biological activity should be assayed, either by animal-based assays, cell culture-based assays, biochemical assays, or other procedures.	The use of non-animal approaches is referred to in the guideline.	
Specifications	Directive 2001/83/EC Guideline on test samples of biological origin (3AB11a)	In relation to the criteria for validation of test procedures, the guideline indicates that "Each test procedure should be validated for each type of biological sample and each species (animal, human). If the same test procedure has been used during the development of the medicinal product (*in vitro*) and during routine tests (*in*	There is no requirement for an *in vivo* test.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
		vivo), a revalidation is necessary.		
Plasma derived medicinal products	Directive 2001/83/EC CHMP Guideline on plasma-derived medicinal products (EMA/CHMP/BWP/706271/2010)	In relation to hepatitis B virus validation, the guideline indicates that "An animal virus model, the duck hepatitis B virus (DHBV), may be used as a model of human HBV. However, it requires the use of its natural animal host (duck or primary duck cells) for titration. In consequence, there is no general requirement to include DHBV in the virus panel. However, in some specific situations where the efficacy of new inactivation procedures is highly virus-dependent among enveloped viruses and for which inactivation/removal efficacy cannot be extrapolated from limited number of model viruses, the use of DHBV could be requested."	Primary duck cells may be used rather than live animals.	
Plasma derived medicinal products	Directive 2001/83/EC CHMP guideline on the replacement of rabbit pyrogen	The CHMP guideline specifically relates to the implementation	The CHMP guideline specifically relates to the implementation of the bacterial	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	testing by an alternative test for plasma-derived medicinal products. (EMEA/CHMP/BWP/452081/2007) Ph. Eur. chapter 5.1.13 Pyrogenicity *Note: 5.1.13 is to be implemented on 1 July 2025. After this date the CHMP guideline may become redundant.*	of an alternative to rabbit pyrogen testing.	endotoxin test as an alternative to rabbit pyrogen testing. The monocyte activation test (MAT; 2.6.30, Ph.Eur.) provides an alternative to the rabbit pyrogen test. In June 2021, the European Pharmacopoeia (Ph. Eur.) Commission took the decision to completely replace the RPT 2.6.8 in the Ph. Eur. with MAT (2.6.30) or BET (2.6.14/2.6.32) within approximately 5 years. In June 2024, the Ph. Eur. Commission adopted revised text for 57 monographs where the RPT has been deleted, as well as new general chapter 5.1.13 on pyrogenicity, which provides guidance for the selection and implementation of a suitable test for pyrogenicity (MAT (as described in 2.6.30) or BET (as described in 2.6.14/2.6.32)), with an implementation date of 1 July 2025. Ph.Eur. 2.6.8 will be suppressed from 1 January 2026.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Plasma derived medicinal products	Directive 2001/83/EC CPMP Guideline on the investigation of manufacturing processes for plasma-derived medicinal products with regard to VCJD risk (CPMP/BWP/5136/03)	Infectivity assays in animals are accepted as the gold standard for the detection of Transmissible spongiform encephalopathies (TSE) agents as there are no generally applicable *in vitro* tests available to identify presence of infectivity and to quantify the infectivity level.	The use of a biochemical assay for detection of PrP^{sc} (abnormal conformation of prion protein) could be acceptable subject to demonstration of correlation between infectivity in a bioassay and the detection of PRP^{SC} in the biochemical assay.	
Stability	Directive 2001/83/EC Guideline on quality of biotechnological products: stability testing of biotechnological/biological products (3AB5A, CPMP/ICH/138/95, ICH Topic 5QC)	Potency testing can be performed in animals in some cases.	There is no requirement for potency testing to take place in animals. *In vitro* potency tests are encouraged to replace *in vivo* tests.	
Drug product	Directive 2001/83/EC CPMP annex to Note for Guidance on Development Pharmaceutics (CPMP/QWP/155/96) - Development Pharmaceutics for Biotechnological and Biological Products - Annex to Note for Guidance on Development	Potency (biological activity) can be tested in animals in some cases.	There is no requirement for potency testing to take place in animals. *In vitro* potency tests are encouraged to replace *in vivo* tests.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	Pharmaceutics (CPMP/BWP/328/99)			
Adventitious agents, safety evaluation, viral safety	Directive 2001/83/EC ICH guideline Q5A (R2): Guideline on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. (EMA/CHMP/ICH/804363/2022) Ph. Eur. chapter 2.6.16 Tests for extraneous agents in viral vaccines for human use Ph. Eur. chapter 5.2.3 Cell substrates for the production of vaccines for human use	Animal testing is needed for detection of some viruses.	Detection of extraneous agents in vaccines (and viral vectors used in gene therapy): Relevant Ph.Eur. texts (Ph. Eur. chapters 5.2.3 and 2.6.16) have been revised to introduce the risk assessment upon which the strategy for extraneous agent testing should be based. This allows the use of broad molecular methods such as high-throughput sequencing (HTS), which may be used as an alternative to *in vivo* tests and allows the removal of *in vivo* tests in adult mice and in guinea pigs. In addition, the decision to maintain or introduce an animal test (test in suckling mice, test in fertilised eggs) in a testing strategy must be justified by the risk assessment (if, taking into account the overall testing package, the risk assessment indicates that the animal test provides a risk mitigation).	Guideline ICH Q5A Rev.2 was adopted in November 2023. The revision introduces high-throughput sequencing (HTS) for virus detection and highlights specific opportunities to replace existing methods with HTS. These include the replacement of *in vivo* assays and rodent antibody production tests by HTS.

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Investigational Medicinal Products	Directive 2001/83/EC CHMP Guideline on requirements for quality documentation concerning biological investigational medicinal products in clinical trials (EMA/CHMP/BWP/534898/08)	Potency testing may take place in animals.	There is no requirement for potency testing to take place in animals – other approaches can also be accepted.	
Investigational Medicinal Products	Directive 2001/20/EC CHMP Guideline on virus safety evaluation of biotechnological investigational medicinal products (EMEA/CHMP/BWP/398498/05)	Tests for infectious retroviruses and *in vivo* tests may be needed depending on the cell type used in manufacturing. Testing for viruses may use animals.	Alternatives to the use of animals may be available.	
Potency testing of botulinum toxin A	Ph. Eur. Monograph 2113 Botulinum toxin type A for injection	An *in vivo* LD50 test has previously been required for potency testing.	Botulinum toxin type A for injection monograph strongly encourages manufacturers to validate alternative 3Rs assays in the interest of animal welfare. It also provides updated information on alternative *in vitro* potency assays to reflect the current situation for authorised products i.e., focus on cell-based assays and includes recommendations on the application of humane endpoints to reduce animal suffering where the test in mice is still being performed.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Potency testing of Diphtheria antitoxin (DAT)	Ph. Eur. monograph 0086 Diphtheria antitoxin	The potency of DAT is determined *in vivo* using guinea pigs or rabbits according to Ph.Eur. 0084	The option of using *in vitro* assays already exists, e.g., Vero cell assay or a PEI in-house toxoid inhibition assay (please refer to WHO/BS/2021.2407).	Besides the longer recognised Vero cell assay, the PEI in-house toxoid inhibition assay has provided very similar results compared to *in vivo* and *in vitro* (Vero cell assay) assays in the scope of the collaborative study for the calibration of the 2nd WHO International Standard for DAT (see WHO/BS/2021.2407).
Pyrogenicity testing of biologicals including vaccines and animal sera for human use	Ph. Eur. chapter 2.6.8 Pyrogens *Note: this chapter will be suppressed from the Ph.Eur. by 1 January 2026* Ph. Eur. chapter 5.1.13 *Note: chapter 5.1.13 is to be implemented 1 July 2025* Ph.Eur. chapter 5.2.11 Carrier proteins for the production of conjugated polysaccharide vaccines for human use. General monograph 'Vaccines for human use' (0153) Ph. Eur. monograph on 3-O-Desacyl-4'-monophosphoryl lipid A (MPL) (2537), and	RPT previously required at various stages of development and as final lot release test.	In June 2021, the European Pharmacopoeia (Ph. Eur.) Commission took the decision to completely replace the RPT 2.6.8 in the Ph. Eur. with MAT (2.6.30) or BET (2.6.14/2.6.32) within approximately 5 years. Subsequently, in June 2024, the Ph. Eur. Commission adopted revised text for 57 monographs, where the RPT has been deleted with an implementation date of 1 July 2025. Accordingly, the requirements to carry out the RPT in monographs for vaccines for human use have been deleted. As a result, the new requirement for pyrogenicity in the revised general monograph 'Vaccines for human use' (0153) will apply. The	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	specific monographs on individual vaccines and animal immunosera for human use: Animal immunosera for human use (0084) HepB (1056) Hib (1219) DTaP-Hib (1932) DTaP-IPV-Hib (2065) Hib-MenC (2067) Men PS vaccine (0250) MenC conjugate vaccine (2112) MenACWY conjugate vaccine (3066) Pneumo PS vaccine (2150) Pneumo conjugate vaccine (0966) Rabies vaccines (0216) Tick-borne encephalitis vaccine (1375) *Note: the monographs above have been revised to delete*		new requirement in the general monograph 0153 refers to the general chapter 5.1.13 (to be implemented on 1 July 2025), which provides guidance for the selection and implementation of a suitable test for pyrogenicity: MAT (as described in 2.6.30) or BET (as described in 2.6.14/2.6.32). Likewise, the general monograph 'Animal immunosera for human use' (0084) has been revised to replace the requirement to carry out the RPT by a new requirement for pyrogenicity, referring to the general chapter 5.1.13.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	reference to 2.6.8, the updates come into effect 1 July 2025.			
Vaccine-specific topics				
Bordetella pertussis vaccine testing	Ph. Eur. chapter 2.6.33 Residual pertussis toxin	Animal tests are no longer required for toxicity testing of acellular pertussis vaccines. *In vivo* potency testing is not required but may still take place.	The Histamine sensitisation test (HIST) has been replaced with a standardised CHO cell clustering assay (Ph. Eur. general chapter 2.6.33) in ten individual monographs on acellular pertussis vaccines. The test for irreversibility of pertussis toxoid has also been removed for acellular pertussis vaccines. Changes were introduced in Ph. Eur. Edition 10.0 (applicable as of January 2020).	Antigenicity assays have been developed by the Vac2Vac consortium that may be considered as a replacement of the serological potency assay.
Diphtheria vaccine testing	Ph. Eur. monograph 0443 Diphtheria vaccine (adsorbed)	Animal tests are no longer required for toxicity testing of Diphtheria vaccines. For potency testing, an assay in guinea pigs (challenge test or determination of antibody titres) is prescribed in the monograph. Use of serological testing is encouraged.	Monographs on diphtheria vaccines published in Ph-Eur. Supplement 10.8, effective Jul 2022, have been revised to delete the test for specific toxicity [test in guinea pigs] performed on the final lot as part of the validation of the production process.	An antigenicity assay has been developed by the Vac2Vac consortium that may be considered as a replacement of serological potency assay.

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Poliomyelitis vaccine (inactivated) testing	Ph. Eur. monograph 0214 Poliomyelitis vaccine (inactivated) Ph. Eur. Chapter 2.7.20 *In vivo* assay of poliomyelitis vaccine (inactivated)	*In vivo* potency assay is currently required.	Monograph 0214 foresees that the *in vivo* assay may be omitted once it has been demonstrated for a given product that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch.	Monograph 0214 is under revision with scope to delete the *in vivo* assay.
Rabies vaccine testing	Ph. Eur. monograph 0216 Rabies vaccine for human use prepared in cell cultures	Potency testing: A challenge study in mice ("NIH test") where rabies virus is injected intracerebrally has previously been required.	Abnormal toxicity testing (former Ph. Eur. 2.6.9): is no longer required as test has been suppressed from Ph. Eur. (see QWP section, topic on ATT for details.) Pyrogenicity: In June 2021, the European Pharmacopoeia (Ph. Eur.) Commission took the decision to completely replace the RPT 2.6.8 in the Ph. Eur. with MAT (2.6.30) or BET (2.6.14/2.6.32) within approximately 5 years. Subsequently, in June 2024, the Ph. Eur. Commission adopted revised text for 57 monographs where the RPT has been deleted with an implementation date of 1 July 2025. Accordingly, the requirement to carry out the RPT in the monograph on	A standardised rabies glycoprotein ELISA with well-characterised commercially available reagents is under study in an EDQM-EPAA project run by EDQM Biological Standardisation Programme (BSP148). It has successfully completed a large collaborative study, and the reporting phase is ongoing.

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
			rabies vaccine (0216) has been deleted. As a result, the new requirements for pyrogenicity in the revised general monograph 'Vaccines for human use 0153) will apply.	
Smallpox vaccine testing	Directive 2001/83/EC CPMP Note for guidance on the development of vaccinia virus-based vaccines against smallpox (CPMP/1100/02) Monograph 0164	Possible animal use includes production and testing procedures.	Pyrogenicity: In June 2021, the European Pharmacopoeia (Ph. Eur.) Commission took the decision to completely replace the RPT 2.6.8 in the Ph. Eur. with MAT (2.6.30) or BET (2.6.14/2.6.32) within approximately 5 years. Subsequently, in June 2024, the Ph. Eur. Commission adopted revised text for 57 monographs where the RPT has been deleted with an implementation date of 1 July 2025. Accordingly, the requirement to carry out the RPT in the monograph on smallpox vaccine (0164) has been deleted. As a result, the new requirements for pyrogenicity in the revised general monograph 'Vaccines for human use (0153) will apply.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Tetanus vaccines testing	Ph. Eur. Monograph 0452 Tetanus vaccine (adsorbed)	Potency testing: A vaccine challenge study (in guinea pigs or mice) or serological test (in guinea pigs) is prescribed in the monograph. Toxicity testing: test for absence of tetanus toxin (toxicity) in guinea pigs is still required.	Sixteen monographs on tetanus vaccines have been revised and published in Ph. Eur. Supplement 10.3. Requirements for the following animal tests have been removed: The *test for specific toxicity* performed on the final lot for human vaccines (performed as a validation of the production process). The *test for irreversibility* of tetanus toxoid carried out on the bulk-purified toxoid.	The BINACLE (Binding and Cleavage) assay is a promising *in vitro* alternative method for in process testing for absence of tetanus toxin). The method has been found suitable for the detection of residual tetanus toxin in certain toxoid in the EDQM Biological Standardisation Programme project BSP136. Product-specific validation of the test will be required.
Tick-borne encephalitis vaccines testing	Ph. Eur. Monograph 1375 Tick-borne encephalitis vaccine (inactivated)	*In vivo* study required for potency testing.		An *in vitro* antigenicity assay (ELISA) has been developed by the Vac2Vac consortium that may be considered as a replacement for the *in vivo* potency assay.

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3.5. CHMP Vaccines Working Party

Overview of animal testing requirements for vaccines (Vaccines Working Party (VWP) - CHMP)

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Non-clinical testing of vaccines	EMA guidance 'Note for guidance on preclinical pharmacology and toxicological testing of vaccines' was replaced in 2016 by WHO guideline on non-clinical evaluation of vaccines (WHO Technical Report Series No 927)	Animal studies are generally required to provide support for the efficacy and safety of the product. This includes characterisation of the immunological response, and in some cases challenge studies to demonstrate protection in relevant models, repeat-dose toxicity studies and, depending on target population, relevant reproductive toxicity testing.	No need for single dose toxicity studies or stand-alone safety pharmacology studies. Safety pharmacology and local tolerance can be evaluated as part of toxicity studies. Toxicity studies in one species is generally sufficient if relevant. In addition, development and validation of *in vitro* studies to reduce/replace animal testing is encouraged.	Pending the outcome of the EMA reflection paper on 'lessons learned from Covid-19' as per the EMA Non-clinical Domain Work plan (1), there may be 3Rs aspects that could be implemented in future guideline revisions.
Non-clinical testing of adjuvants	Guideline on adjuvants in vaccines for human use (EMEA/CHMP/VEG/134716/2004) *Note: Non-clinical and clinical sections in the EMA guideline are superseded by WHO Guideline on the non-clinical evaluation of vaccine adjuvants and adjuvanted vaccines. Annex II, WHO Technical Report Series No. 987, 2014, which have been considered here.*	The increased immunological response to the adjuvant/antigen combination should be shown in a relevant animal model. Toxicity program in general similar to the program for a vaccine, with the combination of adjuvant and antigen. Separate genotoxicity studies may be required for novel adjuvants.	Toxicity testing in a single species is generally acceptable. Separate safety studies of adjuvant only are generally not required. If adjuvant is novel, additional investigations may be performed in separate arm(s) with adjuvant alone. Non-human primates should only be used if no other relevant animal species is available.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Non-clinical testing of influenza vaccines	Guideline on influenza vaccines. Non-clinical and clinical module. (Draft CHMP guideline)	In addition to safety testing, in accordance with the guideline on non-clinical testing of vaccines, animal studies on protection are required for some vaccines. The most appropriate animal model for these studies is the ferret.	None.	None.

111 *3.6. Committee for Advanced Therapies (CAT)*

112 Overview of animal testing requirements for non-clinical studies for cell-based and gene therapy medicinal products

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
General guidelines for Advanced therapy medicinal products (ATMPs)				
Risk-based approach	Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products (EMA/CAT/CPWP/686637/2011).		This guideline should be consulted when planning non-clinical regulatory testing of Advanced Therapy Medicinal Products	
Requirements to support clinical trials	Regulation (EU) No 536/2014 on clinical trials and ATMP Regulation (EC) No 1394/2007 and the Directive 2009/120/EC amending directive 2001/83/EC of the European Parliament and of the Council on the community code relating to medicinal products for use as regards advanced therapy medicinal products. Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA/CAT/123573/2024, draft).	Animal testing requirements are highly specific to the type of product, see topics below.	A risk-based approach may be applied to determine the need and extent of pharmacological and toxicological evaluations.	The draft Guideline provides 3Rs recommendations referring to animal testing requirements given in topics below for cell-based and gene therapy medicinal products. This includes recommendations on the use of a risk-based approach to identify which non-clinical data is needed, that the utility of animal models should be carefully considered, relevance of models justified, and studies planned that take 3Rs into consideration. If no relevant model is available, a WoE approach can be used built on *in vitro/ex vivo* models, *in silico* analysis, literature-based evidence and clinical experience with related products.

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Cell-based medicinal products				
Non-clinical pharmacology and toxicology	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006) Reflection paper on stem cell-based medicinal products (EMA/CAT/571134/2009) Reflection paper on *in vitro* cultured chondrocyte containing products for cartilage repair of the knee (EMA/CAT/CPWP/568181/2009) Guideline on xenogeneic cell-based medicinal products (EMEA/CHMP/CPWP/83508/2009)	Framework for testing requirements for cell-based medicinal products acknowledging that conventional pharmacology and toxicology studies may not be appropriate. For proof-of-concept studies, suitable *in vitro* and/or *in vivo* models should be used. Homologous models or immunocompromised models can be used. Small animal models are usually not sufficient for proof of concept for *in vitro* cultured chondrocyte products. An orthotopic large animal model should be used. Single and/or repeated dose toxicity studies depending on the intended clinical use (single administration or multiple administrations). Relevant animal models should be used, determined on a case-by-case basis. The number and sex of	Risk-based approach as defined in the Annex I, Part IV of Directive 2001/83/EC can be applied. Non-clinical testing should be proportional to the risk expected with clinical use. If relevant animal models cannot be developed, *in vitro* studies may replace animal studies. For proof-of-concept studies, 3D cell culture models can be used. Clinical experience might substitute for some parts of the non-clinical development, determined on a case-by-case basis (EMA/CAT/CPWP/568181/2009). For stem cells, *in vitro* models may provide additional and/or alternative ways to address some specific aspects (EMA/CAT/571134/2009). Safety/toxicology studies can be combined with proof-of-concept studies, efficacy studies, local tolerance studies and incorporate safety pharmacology endpoints.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
		animals as well as the frequency and duration of monitoring should be appropriate to detect possible adverse effects. Due to species specificity, more than one animal species or strains may be needed to address all safety aspects related to stem cells.		
Biodistribution - *kinetics, persistence, migration*	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006) Reflection paper on stem cell-based medicinal products (EMA/CAT/571134/2009) Reflection paper on *in vitro* cultured chondrocyte containing products for cartilage repair of the knee (EMA/CAT/CPWP/568181/2009) Guideline on xenogeneic cell-based medicinal products (EMEA/CHMP/CPWP/83508/2009)	Tissue distribution, viability, trafficking, growth, phenotype, or any alteration of phenotype due to factors in the new environment should be evaluated. Biodistribution studies in small animals (rodents) are recommended. For stem cells, studies on biodistribution, differentiation and possible ectopic tissue formation are required.	Biodistribution studies may not be necessary when cells are physically retained.	
Genotoxicity	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006)	Not required unless the nature of any expressed product indicates an interaction directly		

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
		with DNA or other chromosomal material.		
Carcinogenicity	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006)	Conventional carcinogenicity studies not feasible.		
Tumorigenicity	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006) Reflection paper on stem cell-based medicinal products (EMA/CAT/571134/2009) Reflection paper on in-vitro cultured chondrocyte containing products for cartilage repair of the knee (EMA/CAT/CPWP/568181/2009) Guideline on xenogeneic cell-based medicinal products (EMEA/CHMP/CPWP/83508/2009) Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials EMA/CAT/123573/2024, draft)	Risk of tumorigenicity arising from the cell product or due to neoplastic transformation of host cells should be considered on a case-by-case basis. For stem cells, evaluation of tumour formation including *in vitro* and/or *in vivo* studies is essential.	Tumorigenicity assessment can be integrated in chronic disease or toxicity models.	A stepwise testing strategy for mesenchymal stromal cells is proposed in a publication from regulators and scientists in the field (Barkholt et al, 2013: Cytotherapy. *Risk of tumorigenicity in mesenchymal stromal cell-based therapies - bridging scientific observations and regulatory viewpoints*). *In vitro* studies are normally sufficient, *in vivo* studies only necessary if *in vitro* assays suggest an increased risk for tumour formation. Draft guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA/CAT/123573/2024, draft) clarifies that the extent of non-clinical data is dependent on the perceived risk of tumour formation and should be based primarily on *in vitro* and *ex vivo* analysis, which in some cases may

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
				need to be supplemented with *in vivo* data.
Reproductive and developmental toxicity	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006)	Generally not needed, should be considered on a case-by-case basis.		
Local tolerance	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006)	May be required.	Tissue compatibility and tolerance to excreted substances can be evaluated in single or repeated dose toxicity (safety) studies.	
Immunogenicity, immune response	Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006) Reflection paper on stem cell-based medicinal products (EMA/CAT/571134/2009) Guideline on xenogeneic cell-based medicinal products (EMEA/CHMP/CPWP/83508/2009)	Possible immunogenicity should be considered. For xenogeneic products, studies addressing the immunologic response of the host with or without suppression to the xenogeneic cells, including their bioactive products, are needed.		

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Gene therapy medicinal products				
Non-clinical pharmacology and toxicology	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006) Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells (EMA/CAT/GTWP/671639/2008 Rev.1) Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products (CPMP/BWP/3088/99)	The nature and extent of pharmacological and toxicological evaluation considered on a case-by-case basis. Relevant animal models should be used, i.e., should be permissive for the viral vector and/or mimic the disease or condition to be treated. One relevant species usually suffices. The study duration and sex of animals should be in line with the ICH M3. Single or repeated dosing should mimic the clinical dosing regimen. For genetically modified cells, *in vitro* models can be used when appropriate animal models are not available.	Risk-based approach as defined in the Annex I, Part IV of Directive 2001/83/EC can be applied. Non-clinical testing should be proportional to the risk expected to be associated with clinical use. In cases where there is extensive experience (preclinical and/or clinical) with the vector by a particular route of administration, information from the literature could be used to replace some studies. Studies can be combined with proof-of-concept studies, efficacy studies, or safety pharmacology studies and incorporate other endpoints. Where appropriate, animal testing should be replaced by *in vitro* or *ex vivo* studies. To this end, the development and use of cell- and tissue-based models including 2D and 3D tissue-models, organoids, and microfluidics, *in silico* models or other non-animal approaches are encouraged and they should be used when appropriate and applicable.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
			When necessary, *in vivo* animal studies should be carefully planned to ensure generation of robust data while considering the 3Rs principles. Where feasible, several aspects can be addressed in one study.	
Biodistribution - *kinetics, persistence, migration*	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006) Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors (EMEA/273974/2005) ICH guideline S12 on non-clinical biodistribution considerations for gene therapy products (EMA/CHMP/ICH/318372/2021)	Biodistribution of gene therapy vector to all organs listed in the Annex to the Guideline on repeated dose toxicity (CPMP/SWP/1042/99-Rev & Corr*) should be evaluated including persistence, mobilisation and shedding. Distribution, exposure to, clearance and transcription of the nucleic acid should be investigated. Biodistribution studies in at least two species, one of which should be a non-rodent species, with two dose levels at minimum, should be conducted (EMEA/273974/2005).	Can be included in toxicity/safety studies. Existing biodistribution data with same vector but with a different transgene can potentially support waiving a biodistribution study with new product if scientifically justified. A stepwise approach is followed to address germline transmission with biodistribution studies being conducted first in line with ICH S12. Only in case concerns are identified, can germline transmission studies be requested.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Genotoxicity	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal product (EMEA/GTWP/125459/2006)	Conventional genotoxicity studies generally not needed.		
Carcinogenicity	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal product (EMEA/GTWP/125459/2006)	Conventional carcinogenicity studies generally not needed.		
Tumourigenicity/ oncogenicity	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal	Tumourigenic potential of expressed transgene product may need to be evaluated. Oncogenic potential to be addressed *in silico*; if potential identified, it should be evaluated in *in vivo*/*in vitro* models.	Use of alternative non-animal methods is recommended.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	product (EMEA/CHMP/GTWP/125459/2006)			
Insertional mutagenesis	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006) Reflection paper on management of clinical risks deriving from insertional mutagenesis (EMA/CAT/190186/2012) Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors (EMEA/CHMP/GTWP/587488/2007-Rev.1)	Required for integrative gene therapy vectors. *In vitro* and/or *in vivo* evaluations needed.		

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Reproductive and developmental toxicity	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA/CAT/123573/2024, draft)	The need to be decided based on the possible distribution of gene therapy product to the gonads. Effects on fertility and general reproductive function may be needed. Embryo-foetal and perinatal toxicity studies may be required if WOCBP are to be exposed.		Draft guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA/CAT/123573/2024, draft): The need for non-clinical *in vivo* data should be based on a risk-based approach. Studies may not be needed if no effect on reproductive and/or development is anticipated.
Local tolerance	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014)	May be required, in one species.		
Immunogenicity, immune response	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006) Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically	Should be considered on a case-by-case basis.	Immunotoxicity endpoints can be integrated in the toxicity studies.	

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	modified cells (EMA/CAT/GTWP/671639/2008)			
Germ line transmission	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014) Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006) Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors (EMEA/273974/2005) Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors (EMEA/CHMP/GTWP/587488/2007-Rev.1)	Non-clinical germline transmission studies may be required if biodistribution data indicate cause for concern. If so, one animal species may be sufficient.	A stepwise approach is recommended. If biodistribution studies do not indicate persistent distribution into the oocytes or semen, *in vivo* studies or germine transmission is generally not required.	
Shedding	General principles to address virus and vector shedding (EMEA/CHMP/ICH/449035/2009)	Assessment of virus/vector shedding to tissues and excreta should be conducted in animals to guide the clinical shedding monitoring plan.	Non-clinical evaluation of shedding can be integrated into other animal studies.	Draft guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
	Oncolytic viruses (EMEA/CHMP/ICH/607698/2008) Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA/CAT/123573/2024, draft)			(EMA/CAT/123573/2024, draft): Information on shedding may be based on human data, published data and/or a justification.
Reactivation and latency of virus	Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors (EMEA/CHMP/GTWP/587488/2007-Rev.1)	Maintenance and potential for reactivation or induction of persistence should be evaluated in non-clinical studies, the design of which needs to be scientifically justified.		

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