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ICH guideline S11 on nonclinical safety testing in support of development of paediatric pharmaceuticals

Step 5

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Official address Domenico Scarlattilaan 6 ● 1083 HS Amsterdam ● The Netherlands
Address for visits and deliveries Refer to www.ema.europa.eu/how-to-find-us
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ICH guideline S11 on nonclinical safety testing in support of development of paediatric pharmaceuticals

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List of abbreviations

ADME	Absorption, Distribution, Metabolism, and Excretion
CNS	Central Nervous System
CYP	Cytochromes P450
DRF	Dose Range-Finding
ePPND	Enhanced Pre- and Postnatal Development
FIH	First in Human
FOB	Functional Observational Battery
GABA	Gamma Aminobutyric Acid
GFR	Glomerular Filtration Rate
GI	Gastrointestinal
HPG	Human Pituitary Gonadotropin
ICH	International Council on Harmonisation
JAS	Juvenile Animal Study
NHP	Non-Human Primate
NOAEL	No Observed Adverse Effect Level
PND	Postnatal Day
PPND	Pre- and Postnatal Development
PK	Pharmacokinetics
PD	Pharmacodynamics
TDAR	T-Cell-Dependent Antibody Response
TK	Toxicokinetic
WoE	Weight of Evidence

1. Introduction

1.1. Objectives of the guideline

The purpose of this document is to recommend international standards for, and promote harmonisation of, the nonclinical safety assessments to support the development of pharmaceuticals intended for paediatric use. Harmonisation of the guidance for nonclinical safety studies will define the current recommendations and reduce the likelihood that substantial differences will exist among regions. It should facilitate the timely conduct of paediatric clinical trials and reduce the use of animals in accordance with the 3R (replace/reduce/refine) principles.

1.2. Background

Guidelines have previously been issued by various regulatory agencies and are not in complete agreement on whether a juvenile animal study (JAS) is advisable and its timing and design.

This guideline is intended to complement and expand on existing ICH guidelines (e.g., ICH E11, M3, S5 and S9) and reflects current thinking based on collations of examples by regulatory agencies, by industry surveys, and literature.

1.3. Scope

This guideline recommends an approach for the nonclinical safety evaluation of pharmaceuticals intended for development in paediatric populations. This can include products with prior adult use, as well as products being considered for initial human use in paediatrics (see Section 4).

The ICH S9 guideline should be consulted for recommendations on whether to conduct a JAS for those pharmaceuticals included in the scope of the ICH S9 guideline, i.e., anticancer pharmaceuticals. The ICH S11 guideline should be consulted for study design in all cases, including oncology indications.

Small molecule therapeutics and biotechnology-derived pharmaceuticals as defined in ICH S6 are within the scope of this guideline. Tissue engineered products, gene and cellular therapies, and vaccines are excluded from the scope of this guideline because dedicated juvenile animal safety studies are generally not warranted for such products. However, some of the thinking outlined in this document about evaluating safety with existing information can apply.

1.4. General principles

Paediatric patients who can receive pharmaceuticals during periods of rapid growth and/or postnatal development of several organ systems, represent a distinct population when compared to adults. Immaturity of organ systems and maturation of systems during drug treatment can affect drug pharmacokinetics (PK), pharmacodynamics (PD), and/or off-target effects of pharmaceuticals, potentially leading to differences in safety and/or efficacy profiles between paediatric populations (as described in ICH E11) and/or when compared to adults.

An understanding of the overall clinical development plan is needed to design an appropriate, efficient nonclinical plan. A weight of evidence (WoE; see Section 2) based decision should be made to determine whether additional nonclinical investigations are warranted to support the paediatric population. As clinical development progresses, adjustments to the WoE can be made based on all the available data at that time. The outcome of a WoE assessment can be different for different applications of the same pharmaceutical depending on paediatric age, indication and duration of

treatment. Note that in accordance with ICH M3, juvenile animal toxicity studies are generally not considered important to support short-term PK studies in paediatric populations.

An early consideration of nonclinical support for paediatric pharmaceutical development is recommended. In this respect, changing the design and/or timing of the traditional nonclinical program is one way to address potential safety concerns for the paediatric patient. For example, dosing can be initiated at a younger age in a repeated-dose toxicity study to support the corresponding developmental stages in paediatric patients. Another approach could be to conduct the Pre- and Postnatal Development (PPND) study earlier than the normal drug development paradigm (see ICH M3 and S6), with modifications such as toxicokinetics (TK) in offspring and additional endpoints. These changes can replace or refine the design of a JAS.

The conduct of additional nonclinical investigations should be undertaken only when previous nonclinical and human data are judged to be insufficient to support paediatric studies. A JAS is designed to address safety concerns that cannot be adequately addressed in other nonclinical studies or paediatric clinical trials, including potential long-term safety effects. This guideline recommends a customised JAS that comprises core design endpoints and potential additional endpoints driven by specific concerns.

Some regulatory agencies have procedures for defining paediatric development plans (See ICH E11). Early regulatory interaction, prior to initiating a JAS, is encouraged for efficient paediatric drug development.

2. Considerations for additional nonclinical safety investigations

2.1. Clinical context

The paediatric clinical development plan for a pharmaceutical is discussed in the ICH E11 guideline and needs to be understood before an appropriate nonclinical plan can be designed. The paediatric clinical plan includes the indication/condition, the intended paediatric age group(s), and the treatment regimen (particularly, the duration of dosing during the stages of development). The clinical development of a pharmaceutical for paediatric patients usually follows initial adult clinical studies but can occur in parallel or can be conducted without any adult clinical studies. Whether additional nonclinical investigations are advisable, and their design and timing, will depend on the identified safety concerns and the intended paediatric clinical use.

For severely debilitating or life-threatening diseases, or diseases with serious unmet medical need in a paediatric population, the sponsor and regulatory agencies should consider the benefit of producing data in addition to existing studies versus the potential delay in patient access to a pharmaceutical caused by additional nonclinical testing. The decision whether to perform nonclinical testing and its timing should be based upon a thorough risk-benefit evaluation. In these conditions, if a safety concern is identified as new information is generated, appropriate nonclinical studies should be considered, and could potentially be conducted in parallel with ongoing paediatric clinical investigations.

2.2. Weight of evidence approach

The nonclinical development plan for a paediatric pharmaceutical depends on an integrated assessment based on the totality of the evidence, including the clinical context together with the pharmacology, pharmacokinetic (ADME), and nonclinical *in vitro* and *in vivo* animal, and clinical safety data (adult

and/or paediatric), i.e., a WoE approach. A WoE approach considers multiple factors evaluated together and, therefore, a single factor should not be considered in isolation. The importance of each factor should be considered such that the final decision concludes whether available data adequately address safety concerns in the proposed paediatric population or whether additional nonclinical studies would address those concerns. In addition, the translatability and biological relevance of the JAS data to humans should be considered.

The WoE evaluation should be conducted when designing the initial paediatric development plan, but reassessed if there are new safety signals in nonclinical or clinical studies, changes in age ranges, route of administration, treatment duration, drug product formulation and/or indications. The WoE outcome can be different for each trial depending on the paediatric population and the disease to be treated.

Figure 1 shows key factors that should be considered as part of the WoE evaluation. The most important factors (i.e., the most highly weighted) are the youngest intended patient age, and whether there are suspected adverse effects on developing organ systems of the patients. The other factors are not listed in order of importance in the figure. The list is not all inclusive for every situation, as there may be additional specific factors to consider (e.g., risk mitigation). The WoE factors are further described in the following sections.

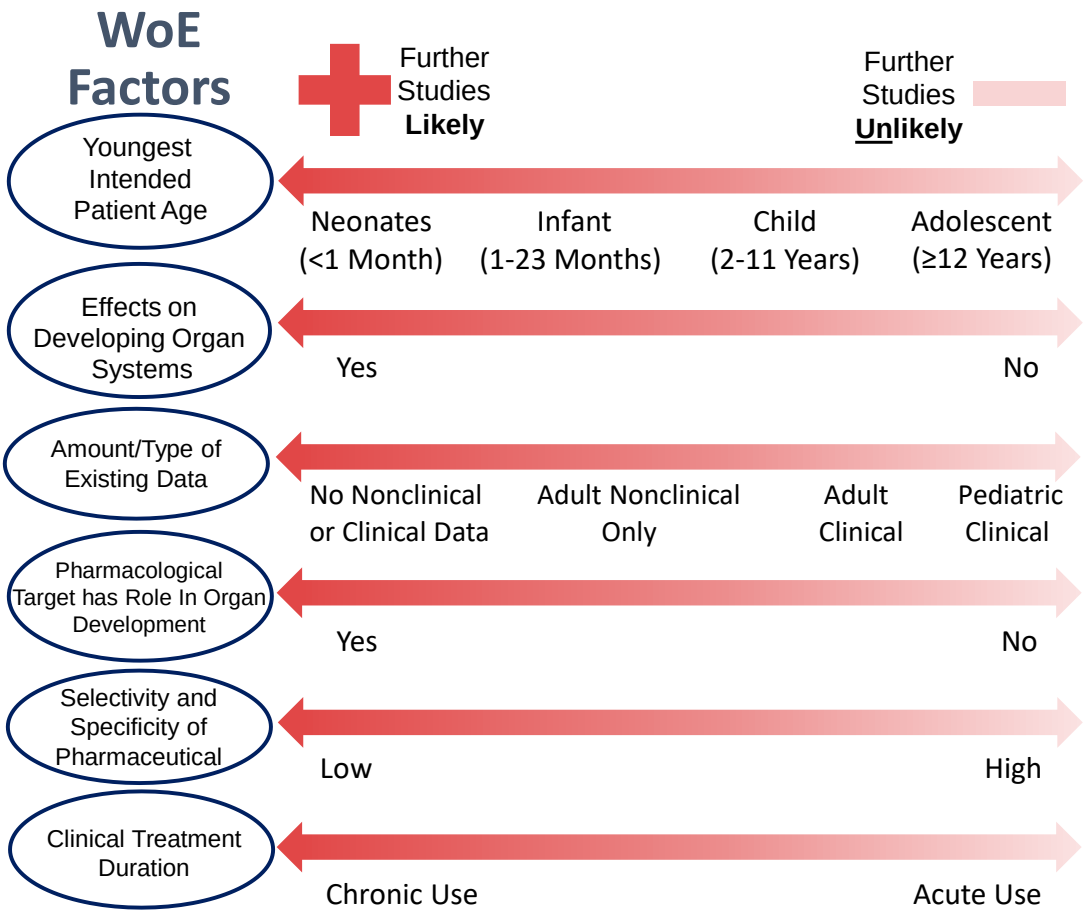


Figure 1: Key WoE factors to be considered in determining if nonclinical studies are warranted. The most important factors that should be highly weighted (listed first) are the youngest intended patient age and whether there are suspected adverse effects on developing organ systems of the patients during the conduct of the paediatric trial. The other factors are not listed in order of importance.

2.3. Considerations to inform the weight of evidence evaluation

2.3.1. Clinical information (WoE factors: youngest intended patient age; amount/type of existing data; clinical treatment duration)

The youngest intended patient age is one of the most important factors to be considered. As stated in ICH E11, any classification of the paediatric population into age categories is to some extent arbitrary, but a classification such as the one in Figure 1 provides a basis for thinking about the nonclinical testing to support safety in such patients. Decisions on how to stratify by age should focus on developmental biology. Additional nonclinical studies are more likely to be warranted to support younger paediatric age ranges.

The existing clinical data relevant for intended paediatric patients come from other paediatric subpopulations (if available) and adults exposed to the pharmaceutical. Therefore, this established clinical safety profile is usually one of the first points to consider when determining if additional nonclinical studies are warranted.

The duration of clinical treatment is another factor in determining whether additional nonclinical studies are warranted. Longer durations of treatment (e.g., 3-month, 6-month, chronic intermittent) are more

likely to expose a paediatric subject during a developmentally sensitive window and are, therefore, more likely to warrant further nonclinical studies than short-term treatments. Short-term exposure to a pharmaceutical is less likely to affect aspects of development such as growth. However, even a short-term exposure can have deleterious effects if it occurs at a vulnerable time of organ development.

Additional nonclinical studies are not warranted when existing clinical safety data and risk mitigation strategies are considered sufficient to support paediatric use. In addition, a JAS is not warranted to confirm toxicity in target organs in which sensitivity to toxicity is not expected to differ between adults and paediatric patients. Differences in target or off-target tissue development are a concern that should be considered.

If adult clinical data are available and exposure is not occurring at a vulnerable time of organ development, a JAS is not considered important to support initiation of short-term PK studies in paediatric patients (see ICH M3).

2.3.2. Pharmacological properties (WoE factors: effects on developing organ systems; pharmacological target has role in organ development; selectivity and specificity of pharmaceutical)

Primary or secondary pharmacological properties of a pharmaceutical can be responsible for unwanted side effects. This can raise concerns for paediatric use if effects occur in systems/organs in development or if developing organs have a different sensitivity from mature organs. A review of the literature on the developmental expression and ontogeny of the pharmacological target(s) (e.g., receptors, enzymes, ion channels, proteins), or the known or potential role of the target during development is recommended. Existing data from genetically modified animals (e.g., the knock-out of a receptor) might also identify developmental effects of potential relevance for the paediatric population, which could be included in the WoE evaluation.

If the known pharmacology of a pharmaceutical has the potential to impact development in the intended paediatric population, or the role of the pharmacology on development is not understood or not reasonably predictable, further nonclinical investigations should be considered. Potential adverse effects of pharmaceuticals with high selectivity and specificity for their target (e.g., monoclonal or bispecific antibodies) are more likely to be related to exaggerated pharmacology and therefore can be more predictable than effects of pharmaceuticals with lower selectivity or specificity for their pharmacological target. Pharmaceuticals with lower selectivity or specificity can have secondary pharmacodynamic effects and thus are more likely to warrant further nonclinical investigations. *In vitro* or *ex vivo* investigations using juvenile (i.e., animal) or paediatric (i.e., human) tissues or matrices (e.g., serum, urine) might be useful to determine potential age-related differences in sensitivity.

Further nonclinical studies might not add value when the existing pharmacology information has already identified a particular hazard unless a more detailed understanding of the dose-response relationship or differences in sensitivity between adult and juvenile animals is warranted.

2.3.3. Pharmacokinetic data (WoE factors: amount/type of existing data)

Maturation of systems important for ADME such as the gastrointestinal, liver and renal systems can result in rapidly changing systemic exposures in humans and in animals, leading to potential age-related differences in efficacy and toxicity. In humans, these differences are usually most prominent in neonates and infants.

Clinical pharmacology and modelling and simulation tools are considered useful to contribute information on the pharmacokinetics, and also pharmacodynamics, efficacy and safety of a drug in paediatric subjects (see ICH E11). In general, a JAS is not informative in predicting or recapitulating age-related differences in human ADME.

2.3.4. Nonclinical safety data (WoE factors: effects on developing organ systems; amount/type of existing data)

Existing nonclinical data should be evaluated for findings that could indicate potential effects in organs undergoing development in paediatric subjects. Findings occurring in animals at similar exposures as those likely to be achieved in paediatric subjects are of increased concern, particularly if the findings occur in organs/tissues that undergo critical postnatal development at the intended paediatric age (see Appendix A). Safety signals in adult animals in more than one species are of increased concern. It can still be appropriate to evaluate the potential impact on paediatric subjects of a toxicity that occurred in adult animals that did not translate to adverse effects in adult humans if the target organ/system is undergoing development in the relevant paediatric population. Depending on the age of the animals at initiation of dosing and the endpoints included, some of these concerns may have been addressed in existing toxicity studies.

Genotoxicity testing and safety pharmacology investigations are normally conducted to support adult clinical trials and, therefore, should be available before paediatric clinical trials commence. If a safety pharmacology study shows an effect in an organ system undergoing structural or functional development and maturation in the intended paediatric patient population, the possible impact of the effect should be considered. Additional genotoxicity and safety pharmacology assessments in juvenile animals are generally not appropriate to support paediatric indications.

Reproductive and developmental toxicity study data may also be available and can be informative. If PPND/ePPND study data are available and have shown clinically relevant systemic exposures in offspring during the postnatal period, these data can contribute to the WoE evaluation (see Section 1.4). For ePPND studies conducted in the non-human primate (NHP), the data from the offspring can characterise toxicity during early postnatal development, provided relevant exposure and/or PD effects are confirmed in the offspring.

When available, PPND/ePPND data should be evaluated in combination with data from the general toxicity studies in assessing the potential added value of conducting additional nonclinical investigations. Maternal and fetal tolerance of the drug should be considered because they could influence interpretation of the findings in offspring. Observations of adverse effects in offspring would not, on their own, indicate that a JAS is recommended. However, if a safety concern was identified in the PPND/ePPND study, it should be considered in the WoE evaluation. In rodents, these data are primarily relevant to preterm and term neonates if exposure is demonstrated. However, the species-specific development of an organ system should be considered when determining human relevance.

If data from a previously conducted JAS are available, they should be considered in the WoE.

2.3.5. Feasibility

The decision to conduct an additional animal study should also consider the technical and practical feasibility of the study design and endpoints. Some endpoints might not be practical in some species (see Section 3 for further discussion of this point).

In addition, if a dose range finding (DRF) study in juvenile animals indicates that a definitive JAS cannot be conducted at appropriate systemic exposures or relevant ages in the range of those

expected in paediatric patients, such a study might not be informative or warranted (see also Section 3.2 and 3.6).

2.4. Application and outcome of the weight of evidence evaluation

The WoE approach should be applied to determine whether additional nonclinical investigations are warranted, with emphasis on the factors considered most important to inform the clinical risk assessment. When a study is deemed warranted, the specifics of the identified safety concerns should define the aim of the nonclinical investigation; this could be a JAS or another study (e.g., *in vitro* or *ex vivo* investigations). For a JAS, the study objectives should be aligned with the WoE outcome and the intended paediatric use. This is essential to appropriately design and customize the JAS with regard to the treatment period and endpoints to be included.

Examples of applying the WoE approach are in Appendix B.

3. Design of nonclinical juvenile animal studies

3.1. General considerations/study objectives

This section contains recommendations on study design, core endpoints, and additional endpoints that can be included to address specific concerns. A JAS design including all potential additional endpoints is not recommended without a rationale for each additional endpoint. A JAS should be conducted according to Good Laboratory Practices.

The stage of maturation of human and animal organ systems can influence susceptibility to toxicity. Understanding the relative level of maturity and function across species during development is needed not only to design the appropriate JAS but also to aid the translation of nonclinical toxicity findings to human age categories. Comparison of development across species can be challenging and is not uniform across different organ systems. For example, the relative maturity at birth, rate of postnatal maturation, and/or regulation of maturation are quite different between humans and animals. While not comprehensive, Appendix A, Tables A1-A5, provide an overview of comparative development of organ systems by species.

3.2. Dose range-finding studies

A DRF study with small group sizes of juvenile animals is recommended to assess tolerability in relation to exposure and age. This is particularly valuable to design a definitive JAS when dosing starts prior to weaning to avoid unexpected mortality or excessive toxicity, often due to irrelevant exposures. Dosing should include the youngest planned starting age of the animals in the definitive JAS to evaluate the most critical period for tolerability and exposure differences. DRF studies typically are of short duration, have limited endpoints and are not expected to include all core endpoints (e.g., pathology). DRF studies can also be used to explore particular endpoints and thus refine the study design of the definitive JAS. DRF studies are not necessarily conducted according to Good Laboratory Practices.

A DRF study can reveal important information for paediatric development. Differences in exposure between age ranges can be identified in a DRF. This might necessitate an adjustment to the dosing regimen in the definitive JAS (See Sections 3.4 and 3.7).

Alternatively, lack of tolerability in a DRF at anticipated paediatric clinical exposure might indicate a significant concern for the corresponding clinical age range (i.e., the juvenile animals are unexpectedly sensitive, potentially related to their immaturity, which can have clinical paediatric relevance). When the reason for greater sensitivity or significant differences in toxicity is not understood, additional

investigations guided by review of available ADME, safety and developmental biology knowledge can be useful for the interpretation of these differences. This situation might warrant a customized investigative JAS to further define the sensitive age window and/or understand the possible mechanism of toxicity. Results could have safety implications for a specific paediatric age which might alter the intended paediatric clinical age range, and thus the WoE should be revisited (See Section 2.2).

3.3. Animal test system selection

When a JAS is warranted, in most cases a single species is considered sufficient. In principle, the same species as used in adult repeated-dose studies should initially be considered as the species for a JAS, preferably a rodent. In all cases, the selected species should be justified, as nonclinical studies in a pharmacologically non-relevant species can give rise to misinterpretation and are not recommended.

The following factors should be considered when selecting a relevant species:

- An understanding of the ontogeny of the pharmacological or toxicological target (e.g., the receptor) in animals in comparison to that in the intended paediatric population
- Preference for a species and strain for which adult repeated-dose toxicity data are available to facilitate a comparison of the toxicity and systemic exposure profiles between juvenile and adult animals
- Toxicological target organs
  - the relative stage of organ/system development in the juvenile animal as compared to the intended paediatric population (see also Section 3.4)
  - the ability of the animal model to detect toxicity endpoints of concern
- Similarity to human ADME characteristics
- The technical/practical feasibility to conduct the study in the selected species

Advantages and disadvantages of using different rodent (rat, mouse) or non-rodent (rabbit, dog, minipig, NHP) species are outlined in Appendix A, Table A6.

While NHPs are pharmacologically relevant in many cases for biopharmaceuticals, the conduct of a JAS in NHPs prior to weaning can be challenging for both scientific and practical reasons (e.g., breeding/transport and management of dam/infant pairs). There is limited added value of performing a JAS in postweaning NHP. In postweaning NHPs, organ system maturity is generally beyond that relevant for many paediatric ages (see Appendix A). Only in rare cases is the value of a JAS conducted in preweaning NHP justifiable (e.g., intended neonatal paediatric use and inadequate exposure from an ePPND study). Therefore, alternative approaches are encouraged (also see Section 4).

Use of an available homologous protein, as discussed in ICH S6, can be considered for the purposes of hazard identification in the juvenile rodent or non-rodent species.

JAS in two species can be warranted in a paediatric-first situation (see Section 4) or where there are multiple specific concerns for postnatal development and one species alone is not able to address them. Biopharmaceuticals should only be assessed in pharmacologically relevant species, consistent with ICH S6. The conduct of a JAS in a second species to confirm findings in the first species is generally not warranted.

If an animal model of a paediatric disease exists (e.g., for enzyme replacement therapy) and is being used to support pharmaceutical development, appropriate safety endpoints (e.g., histopathology,

clinical pathology) can be incorporated in these studies. This information could contribute to the WoE evaluation, potentially providing sufficient information without conducting a dedicated JAS.

3.4. Age of Animals, Dosing Period, and Dosing Regimen

The age of animals at dosing initiation should developmentally correspond to the youngest intended patient age and will depend on a human-to-animal comparison of developmental periods of organ system(s) of toxicological concern. As comparative organ system correlations are not aligned for each organ across species, priority should be given to any target organ/system of potential concern or to particularly vulnerable developing systems in the intended patient population. The animal age at dosing initiation should be justified using relevant information (such as provided in Appendix A).

In contrast to nonclinical studies for adult populations (see ICH M3), the recommended dosing period is not always directly related to the clinical treatment duration for the paediatric population. When determining the duration of administration in a JAS, it is important to consider the paediatric age range and the shorter developmental period of animals compared to humans, the safety concern for the intended paediatric population, and the relevant period of organ development for the target organ of concern. Dosing in a JAS should usually occur during the critical and active periods of growth and development identified in the tables in Appendix A for a system of concern.

Thus, for example, a pharmaceutical with only the kidney as target organ of concern, could be supported with a rat JAS of limited duration focusing on the relevant period of renal development irrespective of the clinical treatment duration. Alternatively, even if the clinical treatment duration is short, a longer dosing period in animals can be appropriate to address concerns of organ systems that develop at different times or over an extended time. For example, a pharmaceutical with a CNS concern with a 10-day clinical treatment duration intended for patients more than 2 years of age could be supported with a rat JAS with animals dosed from weaning to maturity (see Appendix A).

If clinical treatment is intended through adolescence, dosing to maturity is typically conducted in rodents. The interval between birth and maturity for NHPs is several years, making dosing to maturity impractical. Furthermore, NHPs show considerable inter-individual variation in the age of onset of puberty and maturity. Dosing up to maturity can, however, be possible in other typical non-rodent species when these species mature over a period of a few to several months and with relative consistency (e.g., minipig, see Appendix A).

The dosing regimen should be designed to achieve and maintain relevant exposures during developmental periods of concern. Therefore, dosing regimens in a JAS may not be exactly the same as in the clinic. For example, even though a clinical regimen is once a week, more frequent dosing in juvenile animals might be more appropriate. If drug accumulation is a concern in juvenile animals, dosing could be less frequent than in adult toxicity studies (e.g., every other day vs. daily).

When data demonstrate that animals at different ages have different tolerability or exposures to the drug, dose adjustment over time can be considered to provide information with clinically relevant exposures at the appropriate developmental stages.

If the duration of dosing is not expected to be tolerable in a JAS, it may be possible to achieve the clinically relevant exposure by separating the dosing period into different subgroups (e.g., a 6-week JAS dosing period is split into two subgroups of 3 weeks dosing, each starting at different ages). If subgroups with different dosing periods are used, all subgroups may need to be followed through to maturity to detect late effects. The benefits of this approach should be considered along with the

drawbacks, such as substantially increasing the number of animals and difficulties interpreting data at different ages.

3.5. Post-treatment period assessments

Inclusion of an evaluation period after treatment has stopped in a JAS is generally recommended to help address two issues: 1) whether any effects observed during treatment are reversible, persistent, or progressive and 2) whether any effects emerge later in development as a result of early life exposure (i.e., delayed onset).

Whether a post-treatment period is advisable is dependent on the outcome of the WoE assessment and the endpoints to be evaluated in the study. The principles of evaluation of the potential for reversibility in ICH M3 apply. The duration of the post-treatment period should be sufficient to allow the potential recovery of the effect and should take into account the elimination of the pharmaceutical. However, the demonstration of full reversibility is not considered essential. A trend towards reversibility (e.g., decreased incidence and/or severity) and a scientific assessment that this would eventually progress to full reversibility could be sufficient. Likewise, if irreversibility of a specific effect is well characterised in adult animals, it is generally not necessary to confirm this in a JAS. There are endpoints in a JAS that are not amenable to the classic approach of reversibility assessment, such as the timing of onset of puberty. Additionally, the timing of the post-treatment period in relation to the life stage of the animals should be considered. If the post-treatment period begins prior to maturity, the capacity for recovery can be influenced by the continued growth and development of some organ systems and should be carefully interpreted.

Some alterations can only be identified following an appropriate post-treatment period to allow maturation of an organ system and expression of the alteration. Therefore, some assessments can only be meaningfully performed after a certain level of maturity is reached (e.g., learning and memory, immunological function). These assessments can be conducted in post-treatment periods after exposure has covered all developmental windows relevant to the clinical use. This is especially relevant in cases when the JAS dosing duration would cease at an immature age and the animals will continue to mature during the post-treatment period to an age at which an appropriate assessment can be conducted.

Conducting assessments in the post-treatment period can also address delayed onset changes that can be a result of early life exposure, especially in cases when the JAS dosing stops at an immature age.

In non-rodents, depending on the species, the addition of post-treatment groups for a JAS is generally less useful due to the more protracted development period, high inter-individual variability, and fewer and less well-established assessments available to identify delayed or altered development (e.g., learning and memory tests).

3.6. Route of Administration

The intended clinical route of administration should be used when feasible, but obtaining adequate systemic exposure is paramount (see Section 3.7).

Alternative administration routes should be considered in cases of practical difficulties (e.g., use of oral route in preweaning rats for a dermal product); changing routes during the course of the study can also be considered (e.g., subcutaneous injection until intravenous is feasible). The validity of using an alternative dosing route should be justified (e.g., supported by exposure data in representative juvenile animals).

If the pharmaceutical is intended for use by two or more clinical routes of administration, a JAS with a single route of administration is sufficient but should provide adequate exposure in juvenile animals for all intended clinical routes of administration.

3.7. Dose selection

It is desirable to establish a dose-response relationship for adverse effects and to determine a no-observed adverse effect level (NOAEL) in juvenile animals. Dose levels should be selected to achieve some overlap in the range of exposure in adult animals to enable comparison of effects between young and adult animals. However, the high dose should not result in marked toxicity that can confound the growth and development endpoints and complicate the assessment. Body weight loss or lack of weight gain during rapid growth periods has the potential to confound results, and is therefore not desirable in a JAS. At least one dose should result in exposure levels similar to the anticipated exposure in the intended clinical population if tolerable. For small molecules, selection of the high dose in accordance with ICH M3 applies. For biotechnology-derived products, the principles for dose selection described in ICH S6 apply.

Dose adjustment (increase or decrease) during the course of a JAS should be considered in cases of substantial changes in systemic exposure due to maturation of the ADME systems. Adjusting doses is intended to keep the exposures somewhat consistent and clinically relevant. Generally, more than one dose adjustment during a JAS would not be expected.

3.8. Endpoints

Generally, each JAS should include the core endpoints defined in Section 3.8.1 below. Each additional endpoint (see Section 3.8.2) should be considered and justified to address an identified safety concern. In some cases, such as a follow-up investigational JAS, all core endpoints might not be included when justified.

The justification for including an endpoint should consider that invasive or prolonged procedures should be limited as much as possible during preweaning and at the time of weaning as they can contribute to mortality.

3.8.1. Core endpoints

3.8.1.1. Mortality and clinical observations

Mortality should be evaluated throughout the experimental period. Clinical observations, including physical examinations, should be conducted both during treatment and post-treatment as they can identify overt behavioural effects.

Clinical observations of the maternal animals should include assessment of nursing behaviour and maternal care of offspring when treatment of juvenile animals is initiated prior to weaning. Clinical observations can be different between suckling animals and adults and have different implications on the overall health status of the animal (e.g., hydration status, which reflects nutritional status in pups). Therefore, clinical observations of offspring should also capture observations specific to suckling animals. After weaning, clinical observations should be recorded as is appropriate for adult animals.

3.8.1.2. Growth

Growth should be assessed by body weights in conjunction with long bone length. As body weight increases dramatically during the early postnatal period, individual weight measurements should be

assessed at intervals appropriate for informing dose calculations. Generally, one long bone (e.g., femur) measured for length at necropsy is sufficient (See Section 5).

3.8.1.3. Food consumption

Food consumption during the postweaning period should be assessed as appropriate for the species and housing conditions.

3.8.1.4. Sexual development

The physical indicators of onset of puberty (e.g., for rodents, the age of vaginal opening in females and balanopreputial separation in males) are recommended when the study design encompasses the relevant developmental window.

3.8.1.5. Clinical pathology

Standard clinical pathology examinations (clinical chemistry and haematology) should be assessed as a terminal endpoint at necropsy if evaluation is planned at an age at which clinical pathology ranges are known and can support interpretation of histopathology findings.

3.8.1.6. Anatomic pathology

At the end of the treatment and/or post-treatment periods, gross pathology, organ weights, and comprehensive collection and preservation of tissues should be conducted for animals allocated to necropsy. Microscopic evaluation should be performed on major organs (e.g., bone/marrow, brain, gastrointestinal tract, heart, kidney, liver, lung, ovary, testis with qualitative evaluation of spermatogenic progression in mature males), those with macroscopic lesions and previously identified target organs.

If a JAS is supporting first-in-paediatric trials (see Section 4) then a standard set of tissues as used in adult toxicity studies is recommended for histopathology.

3.8.1.7. Toxicokinetics

When designing the TK component of a JAS, the use of microsampling and sparse sampling (see ICH S3A) is strongly encouraged.

Toxicokinetic sampling should be conducted near the beginning and end of the dosing period. If dosing is started preweaning, interim TK assessment(s) should be considered. If dose levels are adjusted during the study, additional sampling for TK is recommended. A DRF JAS with TK assessment (see Section 3.2) will inform on the sampling day and the timepoints of sample collection. The TK assessment should consider both active pharmaceutical ingredient and relevant major human metabolites.

For biopharmaceuticals, samples for anti-drug antibodies should be collected, and evaluated if appropriate (see ICH S6).

3.8.2. Additional endpoints to address identified concerns

The decision to include additional endpoints should be based on the type and strength of the concern identified in the WoE evaluation.

3.8.2.1. Other growth endpoints

As appropriate for the species, crown rump length, body length (e.g., nose/tail), and/or withers height can be used as an indicator of growth. Serial non-invasive measurements of long bone length using suitable imaging techniques (e.g., X-ray) can be useful in non-rodents in addition to a direct measurement at necropsy.

3.8.2.2. Bone Assessments

When there is an identified concern specific to bone metabolism or structure, additional endpoints should be considered. Examples include assessments of bone mass and geometry using densitometric techniques, serum and urinary biomarkers of bone formation and resorption, and bone histomorphometry.

3.8.2.3. Clinical pathology

Additional haematology, serum chemistry, and/or biomarkers can be considered to further characterise identified target organ/tissue concerns. Other parameters such as urinalysis or coagulation assessments can be added when warranted and feasible.

Due to the limitation in obtaining adequate sample volumes from juvenile animals (especially rodents), additional samples can require additional animals and, therefore, are only recommended when critical to address a concern. When sample volume constraints exist, the parameters to be measured should be selected according to a priority based on the identified concern(s).

3.8.2.4. Anatomic pathology

Additional tissues/organs can be evaluated to address specific concerns. Immunohistochemical or other special staining methods for tissue sections, electron microscopy, histomorphometry, or other imaging techniques can be used for further characterisation when warranted.

3.8.2.5. Ophthalmologic examinations

Standard ophthalmologic examinations (e.g., palpebral reflex, ophthalmoscopy) are not routinely included in a JAS, because structural development of the eye is largely completed during the prenatal period in humans. However, when there is concern for ocular toxicity, assessment of ocular endpoints should be considered.

3.8.2.6. CNS assessments

There are different categories of CNS assessments, such as:

- detailed clinical observations
- behavioural tests
- learning and memory tests, and
- expanded neurohistopathology evaluations.

The selection of any additional CNS assessments should be based on concerns identified in the WoE evaluation. The timing of any such additional assessments within the JAS should take into consideration whether the results will be used to investigate adverse effects that are due to exaggerated pharmacology, developmental neurotoxicity (i.e., effects that are still present or emerge after the cessation of treatment), or both.

If a compound has a target in the CNS, the extent of distribution across the blood-brain barrier and which region of the brain is potentially affected (e.g., by target distribution and related functional pathways) should be considered. Such information, if known, can help inform selection of the appropriate additional CNS endpoints (e.g., when determining whether assessments of learning and memory or other endpoints are warranted).

Detailed clinical observations are a key component of CNS assessment and, therefore, should be assessed as appropriate throughout the study during the treatment and post-treatment periods. These observations should document the severity, the time of onset and the duration of the clinical signs relative to the time of dosing to determine whether or not effects are temporally associated with exposure.

There are many different behavioural tests, including assessment of locomotor activity, evaluation of coordination and reflexes, and/or acoustic startle response (e.g., habituation or prepulse inhibition). The functional observational battery (FOB) or modified Irwin test are considered to have relatively low sensitivity in juvenile rodents and are of limited utility. The selected test(s) should be appropriate for the species being tested and the timing of these assessments should consider the level of maturity in the test species at the age of the assessment. Before deciding whether behavioural testing is performed during the treatment period, the potential for confounding pharmacological effects (e.g., sedation, decreased motor coordination) should be considered.

When specific aspects of learning and memory have been identified as areas of concern in the WoE evaluation, then an appropriate complex learning task capable of assessing such aspects should be selected. For evaluation of persistent or delayed effects on learning and memory, these assessments should be conducted in the post-treatment period.

Postnatal CNS assessments are most commonly conducted and characterised in the rodent. For those pharmaceuticals where the rodent is an inappropriate species, some behavioural tests are also available in other species (e.g., dogs, minipigs). In NHPs, behavioural observations in a JAS or ePPND study can provide an assessment of potential CNS effects. Learning assessments similar to those used in paediatric subjects have also been developed for NHPs, but these are infrequently conducted because of study design complexities and substantial interindividual variability.

Lastly, any CNS regions or components (e.g., hippocampus, myelin) identified in the WoE evaluation as potentially affected should be assessed with expanded neurohistopathological evaluations as appropriate (e.g., additional sections examined, immunohistochemistry, special stains). These assessments are typically performed at the scheduled necropsy at the end of treatment and following a post-treatment period. Imaging technologies can also be useful in specific circumstances.

3.8.2.7. Reproductive assessments

If there is an identified concern for effects on female and/or male reproductive organs or function, histopathology examinations and organ weights can be expanded to include reproductive and/or endocrine tissues in addition to the gonads. It is not important to confirm in a JAS reproductive system effects that were identified as irreversible in adult animals.

In rodents, for concerns relevant to females, assessment of estrous cyclicity is recommended for assessment of reproductive and endocrine function. For concerns relevant to male rodents, sperm analysis (e.g., counts, motility, morphology) and/or testicular immunohistochemistry (e.g., apoptosis) can be considered if of value to further characterise effects.

The timing of the treatment and assessments in relation to that of sexual maturation in the species tested is critical. The timing of folliculogenesis and spermatogenesis should be considered in the study

design and timing of reproductive assessments. Assessment of reproductive organs or function (e.g., estrous cyclicity, sperm count, or qualitative histologic assessment of spermatogenesis) can only be conducted in sexually mature animals. If the clinical age range includes prepubertal stages, there can be a concern whether the pharmaceutical could cause any delayed effect on sexual maturation or reproductive function in adulthood. If the clinical treatment is only during prepubertal stages, a JAS should be designed to treat only during immaturity, and then allow the animal to mature without further treatment and conduct assessments after maturation is reached.

Mating assessments are not generally recommended in a JAS. Most effects on reproductive organs related to male fertility are detectable by histopathology. In female rodents, assessment of estrous cyclicity and ovarian histology can identify many developmental reproductive hazards. In dogs and NHPs, mating assessments are difficult due to the protracted duration of development and high degree of individual variability.

Hormonal assessments are generally not recommended in a JAS as there is considerable variability in hormone levels, especially during puberty. Therefore, any hormonal assessment should be justified, and the timing and specific hormones assessed should be well characterised for the age at which the assessment is conducted.

The feasibility of reproductive assessments is such that the large majority are conducted in rodents, although they can be considered for those non-rodent species that achieve maturity during the conduct of a JAS.

3.8.2.8. Immunologic Assessments

If the pharmacological class or data in animals or humans gives cause for concern for the development of the immune system, assessments for immunotoxicity should be considered as outlined in ICH S8. Such concerns, when considered developmentally important, can include a transient, prolonged or permanent decrease or increase in the number or function of a lymphocyte subtype, or a sustained increase or decrease in immunoglobulin class. Functional assays should be performed at appropriate stages of development, e.g., for T-Cell-Dependent Antibody Response (TDAR) after PND 45 for the rat. Confirmation of immunotoxicity is generally not warranted in a JAS if the toxicity is already well characterised.

3.9. Allocation of animals to study groups and endpoint subsets

3.9.1. Preweaning allocation

A definitive JAS can become large and complex, therefore it is important that the study design balances scientific rigor against animal use. Before designing the study, investigators should know all the planned endpoints (core and additional). Efficiency in study design is critical to reduce animal use as per the 3R principles and should be measured by the total number of maternal animals (and litters) needed to supply the study, including pups that are not used in standardised litters and pups that are not assigned to specific endpoints.

There are different allocation methods for litter management of multiparous non-rodent animals. For species with single offspring or small and variable litter sizes (e.g., NHP, dogs), group allocation design can be modelled following the principles applied in general toxicity studies.

In most species, initiation of a JAS during the preweaning phase presents a unique situation for dosing offspring within a litter. Although the maternal animal is a critical component of the study providing nutrition and care, only the offspring are the test system. The study should be designed to reduce

potential confounders. For JAS, maternal care and litter size are generally considered more important confounders than genetics. Reduction of confounding can be achieved by the way the litters are constructed and standardised in combination with how they are assigned to dose groups and how the individual pups are assigned to endpoints.

When constructing study litters, select dams demonstrating good maternal care and pups in apparent good health. It is advisable that study litters be standardised with respect to number of offspring and sex ratio (i.e., 4 to 5 pups/sex/litter in rats) at the same postnatal age. This can be accomplished by either fully fostering (arbitrarily mixing up all birth litters) or minimally fostering (keeping the birth litter as intact as possible and fostering only as necessary to obtain study litter size and sex ratio requirements). When feasible, standardise study litters at a time that allows some acclimation to the new litters prior to the first dose. As a change in litter size can alter pup growth rate, maintaining a consistent litter size across and within dose groups is recommended during the preweaning phase.

For assigning study animals to dose groups, it is preferred that each standardised litter be assigned to a single dose group to minimise the risk for cross-contamination and to avoid treated and control offspring competing for suckling position and time.

When assigning individual animals to endpoints in definitive studies, it is recommended that litter mates of the same sex not be assigned to the same endpoint to avoid maternal care biases (see Appendix C).

The fostering approach, litter standardisation, dose group, and endpoint allocation methods should be clearly described in the study plan/protocol and report. Appendix C provides a case study using one allocation approach for a rat JAS that minimises potential for genetic, maternal care, and litter size confounders. There are other ways to successfully allocate litters, depending on the study objectives and endpoints, but other approaches should also consider and avoid these biases.

3.9.2. Postweaning allocation

In multiparous animal species, it is recommended to design the study with consideration of potential confounders when possible. In particular, when dosing starts in the early postweaning phase, and, when offspring are supplied from a limited number of natural mothers, the study should be designed in consideration of the potential confounders similar to those at preweaning allocation.

3.9.3. Animal numbers and sex

A JAS should use a group size that is considered generally appropriate in a definitive toxicity study based on the selected endpoints (e.g., approximately 10 animals/sex for end-of-treatment necropsy endpoints similar to repeated-dose toxicity study for rats). To reduce the number of animals, combining assessment of endpoints in the same subset of animals can be effective (See Appendix C). It is recommended that a JAS generally be performed in both female and male animals.

4. Considerations for paediatric-first/only development

A common clinical approach for the development of a paediatric-first/only pharmaceutical starts with a First in Human (FIH) study in healthy adult volunteers prior to any paediatric trial. As *per* ICH M3, this approach generally includes repeated-dose toxicity studies in rodent and non-rodent animals, as well as safety pharmacology and genetic toxicology studies. The principles of ICH S6 can also apply. The toxicity studies to support the FIH adult study could be performed as standard repeated-dose toxicity studies in two species. Alternatively, one or both of these studies could be initiated in juvenile animals, and treatment can be continued into maturity in some species (see Section 3.4). Studies in juvenile

animals should include additional relevant endpoints (see Section 3.8). The approach that includes juvenile animals can be more efficient, as it could support initiation of clinical trials in paediatric patients shortly after the adult FIH study.

There are cases, however, where paediatric patients are treated without any prior adult patient or healthy volunteer data (e.g., for a life-threatening or debilitating disease that only exists in children or when the pharmaceutical cannot be given safely to adult volunteers). In these cases, the FIH trial would be in paediatric patients and the nonclinical program would generally include one JAS in a rodent and one JAS in a non-rodent species. Safety pharmacology and genotoxicity testing would be conducted as appropriate for adult use, although these studies need not be conducted in juvenile animals (see Section 2.3.4).

If the pharmaceutical is intended to treat a chronic paediatric disease, chronic toxicity studies should be conducted in one rodent and one non-rodent species. In at least one of these studies, dosing should start at an age developmentally matched to the lowest age of the intended patient population. In principle, chronic studies that start dosing from ages that developmentally correlate to the youngest paediatric patient age can be sufficient to cover all ages and durations of paediatric use. These can replace adult animal chronic studies and a separate JAS. Further nonclinical assessments of reproductive toxicity and carcinogenic potential can be warranted.

When studies in juvenile animals are warranted for biopharmaceuticals, these should be limited to relevant species, as *per* ICH S6. Non-invasive safety pharmacology endpoints can be included in the juvenile or standard NHP repeated-dose studies. Genotoxic and carcinogenic potential should be addressed as outlined in ICH S6.

A JAS in postweaning NHP is typically conducted in animals starting at 10 to 12 months of age, thus limiting the coverage of the lowest paediatric age ranges. A JAS in preweaning NHP should only be conducted in the situation of pharmaceuticals with first and primarily neonatal clinical use, and where alternative approaches to nonclinical safety assessment are not feasible. Studies with direct dosing of NHPs prior to weaning can require large numbers of mature dams to populate even a relatively small JAS. Therefore, the design and endpoints should be clearly justified based on the clinical concern. Design expectations should also be flexible because, for example, variability in sex distribution and starting weights of offspring is expected. In cases where a JAS is not feasible to support the youngest paediatric age, alternative approaches (e.g., *in vitro* assays, genetically-modified animal models, surrogate molecules) should be considered if available and relevant.

Section 3 should be consulted to determine study designs.

5. Data interpretation

5.1. Considerations for endpoint interpretation

Many observations in a JAS (e.g., body weight, clinical pathology) are age, sex and species/strain dependent. Age-matched concurrent control data are, therefore, critical for interpretation. If available, appropriate historical control data or reference materials (e.g., tissue database or atlas) can also be helpful to interpret results, especially in cases of low incidence findings or unscheduled early deaths (i.e., when control data are often lacking or insufficient).

Interpretation of clinical observations can differ between juvenile and adult animals and assessments in preweanlings should also consider maternal care and the overall health status of the litter.

An assessment on growth should typically be considered a key objective of a definitive JAS and should preferably be conducted at necropsy by an evaluation of long bone length in conjunction with body

weight, and food intake if available. The latter parameters are needed to differentiate between potential direct effects on skeletal development or indirect effects (secondary to toxicity causing malnutrition and body weight loss/decrease) on long bone length. An effect solely on decreased body weight gain is not necessarily an effect on growth. Frequent assessments of long bone length for 'transient' effects on growth are challenging to interpret and offer limited value because of the inter-individual variability in growth rate during development.

Assessment of organ weight data and the onset of sexual development should be performed in the context of growth. Organ weight changes are not always proportional to body weight changes because some organs grow at different rates throughout development (i.e., allometric versus isometric growth). Additionally, organs have different sensitivity to growth effects (e.g., the brain can be less affected than other organs). Thus, interpretation of absolute and relative organ weights should consider these aspects.

For statistical analysis, data collected from offspring while part of a study litter should not be considered an independent variable (see ICH S5).

5.2. Overall interpretation

An integrated assessment should be made across all appropriate studies, comparing available findings in juvenile and adult animals, and evaluating clinical relevance. Relevant findings in juvenile animals not observed in adults should be discussed, as well as any marked differences in sensitivity compared to adults. The overall interpretation of relevant findings should consider the type, severity and recovery (if known) of the effects, the age of the animals and the exposures and/or doses at which any effects were observed, and relate these to the intended paediatric use.

6. Other considerations

6.1. Excipients

Pharmaceutical formulations occasionally contain excipients for which only limited experience exists in paediatric populations. To assess the safety of the excipients in a paediatric clinical formulation, available information on the excipients should be evaluated and a WoE approach should be followed (see Section 2). If there are insufficient data to support the use of the excipient in the intended paediatric population, further safety evaluation can be warranted, for example, an additional group evaluating the excipient alone in a JAS.

6.2. Combination pharmaceuticals

The development of combination pharmaceuticals for paediatric use should have a nonclinical evaluation consistent with the principles outlined in ICH M3 for combination products in general, together with the WoE principles outlined in this guideline. Consequently, a JAS of a combination pharmaceutical should be considered only if previous human and animal data are determined to be insufficient to support paediatric development, and the WoE evaluation suggests that a JAS would address identified concerns. If additional nonclinical information is warranted, the study design should consider which endpoints are appropriate to address any concerns of administering the particular combination. If a JAS is considered appropriate, assessment of the combination as it is to be used clinically might be sufficient and testing of the individual active ingredients might not be critical. Alternatively, an extra group with the combination could be added to a JAS that is already being conducted with one of the single entities. This could provide information that would otherwise be obtained in a separate study with the combination product

Glossary

Allometric and isometric growth

Isometric growth occurs when proportional relationships are preserved as size changes during growth. Allometric growth is any deviation from isometric growth. With allometric growth, properties such as bone length, organ weight and body surface area can change according to an exponential function of body mass.

Endpoint Subset:

A set of individual animals within a dose group that are assigned to the same endpoint.

Enhanced Pre- and Postnatal Development Study (ePPND):

This study design is based on biopharmaceutical experience, often in NHP, and is a PPND study which includes elements of the embryofetal development (EFD) study in newborns and infants instead of the fetus.

Fostering:

The act of nurturing or offering parental care to offspring that are not genetically related. The fully fostering technique arbitrarily mixes up litters with the intent not to have dams with their genetic pups. The minimally fostering technique retains the natural litter as intact as possible, fostering only as necessary to achieve desired litter size and sex ratio.

Juvenile Animal:

An animal in any postnatal stage not fully matured in terms of organ or system morphology and function.

Juvenile Animal Study (JAS) – A nonclinical safety study typically conducted with the objective to provide an assessment of the toxicity profile of a pharmaceutical in juvenile animals.

Paediatric-First Development:

Paediatric-first development describes development for treatment of paediatric patients before any development for an adult indication.

Paediatric-Only Development:

Paediatric-only development describes development for treatment exclusively in paediatric ages (e.g., neonatal respiratory distress syndrome).

Weight of Evidence:

An approach that evaluates information from several sources to decide if there is sufficient evidence to support the development of pharmaceuticals for paediatric use or whether additional nonclinical testing is warranted to address potential safety concerns.

The weight given to the available evidence depends on factors such as the quality of the data, consistency of results, nature and severity of effects, and relevance of the information. The weight of evidence approach requires use of scientific judgment and, therefore, should consider the robustness and reliability of the different data sources.

References

1. ICH E11 Guideline: Clinical Investigation of Medicinal Products in the Paediatric Population; August 2017
2. ICH M3 Guideline: Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals; June 2009.
3. ICH S3A Guideline: Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies; October 1994
4. ICH S5 Guideline: Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility; November 2000
5. ICH S6 Guideline: Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals; June 2011
6. ICH S8 Guideline: Immunotoxicity Studies for Human Pharmaceuticals; September 2005
7. ICH S9 Guideline: Nonclinical Evaluation for Anticancer Pharmaceuticals; October 2009

Appendix A: overview of age-dependent development of organ systems by species

These tables reflect a high-level overview of organ system development by species to illustrate similarities and differences between the commonly used toxicology species, as compared to humans, for the timing and relative duration of development.

The tables are intended to aid in the assessment of the relevance of existing nonclinical data, as well as the selection of species, starting age, and dosing duration for a JAS. They are based on a review of current knowledge, but are not comprehensive. Species-specific and/or organ system reviews in the literature can provide additional detail and should be consulted for each specific situation. Factors such as strain, breeding, and animal supplier can impact age-dependent development and should also be considered.

The following legend applies to Tables A1-A5 and Figure A1:

	Critical period of structural and functional growth and development
	Active period of growth and/or functional maturation
	Slow continued growth and/or refinement of function
	Structurally and functionally fully mature

The human age categories are aligned with those described in the ICH E11 guideline, with approximate milestones (e.g. birth, introduction of solid foods, weaning, puberty, and adulthood) corresponding to development in nonclinical species. The tables only provide an approximation of the ages when these milestones occur. For example, the active developmental period of several organ systems can last until 12 or 18 months of age in humans, which coincides with the regionally and culturally diverse milestones of introduction of solid food and weaning in humans.

Developmental toxicity is of particular concern during critical and active periods of functional and structural growth and less so during periods of slow growth or refinement of function. In some organ systems, such as the immune system, the tissues are largely competent to respond at birth, but then undergo substantial expansion in the immediate postnatal period in parallel with environmental stimulation. Likewise, some organ systems such as the CNS are highly complex, with different timelines for establishment of reflexes, pain pathways, sleep patterns, myelination, coordination and cognitive function. This complexity can influence periods of susceptibility during development, so the milestones listed provide only a general guide. Functional maturation occurs into adulthood in humans for some aspects of CNS development, and cannot be fully modelled by animal test systems. Puberty is a period of intense endocrine activity with important structural and functional maturation of the reproductive system, but also with effects on musculoskeletal growth and CNS development in humans. The timing of puberty can be variable across all species, particularly in primates, where onset and progression are typically monitored by external sex characteristics, as in Tanner staging in humans. Females typically achieve full reproductive functionality prior to males across species. Finally, slow continued growth with refinement of function occurs into adulthood for several organ systems, as is indicated in the tables.

Table A1: age-dependent development of human organ systems

Milestones:	Birth		1st Solid Food	Weaning		Puberty	
Age Categories: System:	Premature through full term birth	Neonate (Term Birth-27 days)	Infant/Toddler (28 days-23 months)	Child (2-11 years)		Adolescent (12-18 years)	Adult (> 18 years)
Cardiovascular	Critical neonatal physiologic transitions (pulmonary and vascular resistance, closure of fetal shunts)		Progressive increase in ion channels/conductance	Adaptive myocardial and vascular changes			
Endocrine	Fetal hormone production begins second trimester & structurally well developed at term Endocrine functions critical for growth/development			Adrenarche in late childhood		Pubertal sex hormone surge and maturation	
Eye	O_2-sensitive retinal angiogenesis	Morphologically well developed at term birth	Development of retina (fovea), lens, iris pigmentation complete by 1 year	Training of vision important between 1-4 years (focus and track at 1-4 months; color vision starts at 3 months)		Star sutures of lens form continuously during adolescence and into adulthood	
Gastrointestinal	Variable preterm suck/swallow reflex Critical functionality present in term neonates Acidification, transit, microbiome		Increased digestive functionality, and absorptive capacity with growth	Progressive adaptations in digestive function to accommodate shift in diet/complexity			
Hepatobiliary	Structurally well developed at term Important neonatal transitions in bile production and elimination		Important increases in metabolic and elimination capacity	Continued refinement of metabolic and elimination function and capacity			
Immune	Neonatal structural expansion of primary and secondary immune tissues		Progressive population of immune tissues and development of memory as a function of time and environment				
Integument	Cornification & vernix	Critical neonatal function (barrier, water, thermoregulation, sensation) High surface area relative to weight as compared to adults		Progressive surface acidification, local microbiome and immune function Pubertal hair growth, oil production			
Nervous	Neuronal subsets defined and active excitatory signaling initial myelination	Maximum neuron count and brain:body weight at term birth Myelin and glia present at term birth with continued refinement Postnatal neuronal apoptosis, synapse pruning, migration and circuit integration Metabolic functions, neurotransmitter and conduction systems mature at variable rates		Refinement of sensorimotor control and expanded capability for complex learning and memory function			CNS development continues into adulthood
Pulmonary	Critical liquid → air transition at birth Canalicular→saccular→alveolar structure Surfactant made preterm, secreted at term		Alveolization progresses	Increased alveolar surface area with growth to maximum aerobic function in adolescence			
Renal	Nephrogenesis not complete until term		Increases in renal function GFR precedes concentration	Tubular growth and refinement of function including erythropoietin and angiotensin axis			
Reproductive	Oocyte meiosis & testicular descent	Testes descended Postnatal HPG surge of sex hormones ('mini-puberty')	Quiescent period			Sertoli cell expansion Breast development Spermarche/Menarche	
Skeletal	Growth plate development	Growth plates present at term birth Critical period of rapid growth driven by growth hormone and thyroid hormone		Slower growth		Pubertal growth	Growth plate closure

Table A2: age-dependent development of rat organ systems

System	General Considerations	Neonate (~ PND 1-10)	1st Solid Food (~ PND 15)	Weaning (~ PND 21-25)	Puberty (M ~ PND 42, F ~ PND 35)	Adulthood (~ PND 70)
Cardiovascular	• Critical neonatal physiologic transitions (pulmonary and systemic vascular resistance) • Adaptive myocardial and vascular changes • Progressive increase in cardiomyocytes and ion channels to PND 21					
Endocrine	• Most glands are well developed at birth and critical for growth					
Eye	• Morphological development of retina, lens, iris, cornea and adnexa ongoing until PND14 • Eyelids open on PND14 • Eyes morphologically fully developed by weaning; continued growth and refinement of vision through puberty					
Gastrointestinal	• Immature at birth; lack gastric acid and poor pancreatic enzyme production until PND 14 • Highly permeable proximal small intestine initially allows absorption of intact proteins • Adaptations in 3rd week of age to accommodate shift in diet					
Hepatobiliary	• Structurally immature at birth • Progressive development of organized hepatic cords and plates, with increase in metabolic functionality, over first 4 weeks of age					
Immune	• Progressive population of secondary immune tissues and development of memory as a function of time and environment • TDAR typically assessed after PND 45					
Integument	• Critical neonatal function (barrier, water and thermoregulation, conductance, sensation); thicker epidermis first 2 weeks of age • Adnexa and hair develop postnatally, structurally resembles adult by PND 21 • Sexual dimorphism by PND 35 to 42					
Nervous	• Structural maturation of olfactory bulbs, cerebellum, hippocampus, and cerebral cortex occurs over first 3 weeks of age • Maximum neuron count and brain:body weight at PND7, with extensive postnatal apoptosis, pruning and migration • Postnatal myelination of spinal cord caudal to cranial; brain for reflexes, sensorimotor, then learning and memory • Conduction systems, opiate receptors/metabolism, GABA, serotonin & noradrenalin pathways mature at different rates					
Pulmonary	• Saccular at birth • Alveolization occurs over first 2 to 3 weeks of age					
Renal	• Nephrogenesis incomplete at birth • Progressive increase in GFR and renal function over first 3 to 5 weeks of age					
Reproductive	• Period of decreased androgen production by Leydig cells during 3rd week of age necessary for expansion of Sertoli and germ cells • Remaining reproductive changes and appearance of sexual dimorphism occur at onset of puberty (5 to 7 weeks of age)					
Skeletal	• Rapid postnatal growth through adulthood • Long bone growth plate structure not evident until PND 14 to 21, and remain open into adulthood					

Table A3: age-dependent development of beagle dog organ systems

System	General Considerations	Neonate (< 3 weeks)	1st Solid Food (~ 3 weeks)	Weaning (~ 8 weeks)	Puberty (M ~ 5-8 months, F ~ 6-12 months)	Adulthood (> ~ 12 months)
Cardiovascular	• Critical neonatal physiologic transitions (pulmonary and systemic vascular resistance) • Adaptive myocardial and vascular changes • Significant increase in blood pressure and decrease in heart rate from week 1 to 6 months of age					
Endocrine	• Endocrine tissue development and initial hormone production occurs in utero • Endocrine functions critical for growth/development and progression through puberty					
Eye	• Morphological development almost complete at birth; eyelids first open at 10-14 days of age • Mature retina by 7 weeks; corneal transparency and iris pigmentation complete by ~ 8 weeks • Vision fully mature by 12-14 weeks					
Gastrointestinal	• At birth gastrointestinal tract is fully formed; suck/swallow and rooting reflexes fully functional within 1-2 days postnatal • Colostrum stimulates intestinal maturation and growth • Metabolic functional development primarily between birth and weaning	?				
Hepatobiliary	• Hepatobiliary structural maturation by 1 week of age • Bile secretory function matures more slowly (30 to 70 % of adult at 4 to 6 weeks of age)					
Immune	• Agammaglobulinemic at birth; need IgG transfer from dam via colostrum within 12-24 hours postnatal • Immunologic tissues are largely structurally mature and functional at or shortly after birth • Rapid postnatal growth of thymus to maximum size at weaning, with involution at puberty					
Integument	• Critical neonatal functions (barrier, hydration, conductance, sensation) develop in initial postnatal phase (~2 weeks) • Initial haircoat and adnexa at birth; planum and pads thicken through weaning, and transition to adult coat					
Nervous	• Hypertonic flexion at birth, then extension develops first week; neonatal (primitive) reflexes disappear at ~ PND 28 • Functional locomotor development occurs postnatally with rapid progression through 8 weeks • Spinal cord reaches structural maturity by 6 weeks postnatal, but nerve conduction speed increases over 6-12 months • Rapid cognitive development with critical developmental period for learning at approximately PND 18 to 28					
Pulmonary	• Lung maturation continues postnatally, and is generally comparable to a term human neonate by 1-2 weeks postnatal • Regular daily rhythm of respiratory rate (RR) by 8 weeks; RR decreases with growth through puberty • Continued development through 8 weeks and growth to maximum alveolar capacity by ~1 year of age					
Renal	• Kidney is structurally and functionally immature at birth, with completion of nephrogenesis at approx. 2 weeks of age • Tubular growth and corpuscle maturation continues through 3 weeks postnatal, reaching maturity by weaning • Concentrating ability develops prenatally, but acid-base homeostasis develops postnatally					
Reproductive	• Testes descend postnatally at 5 to 6 weeks of age • Males reach sexual maturity at ~ 5 to 8 months of age; Females at ~ 6 to 12 months of age					
Skeletal	• Appearance of long bone ossification centers between 1 to 10 weeks of age • Limbs cannot support weight until 2 weeks of age • Most rapid long bone growth is complete by 5 months of age, with slower continued growth until ~ 18 months of age					

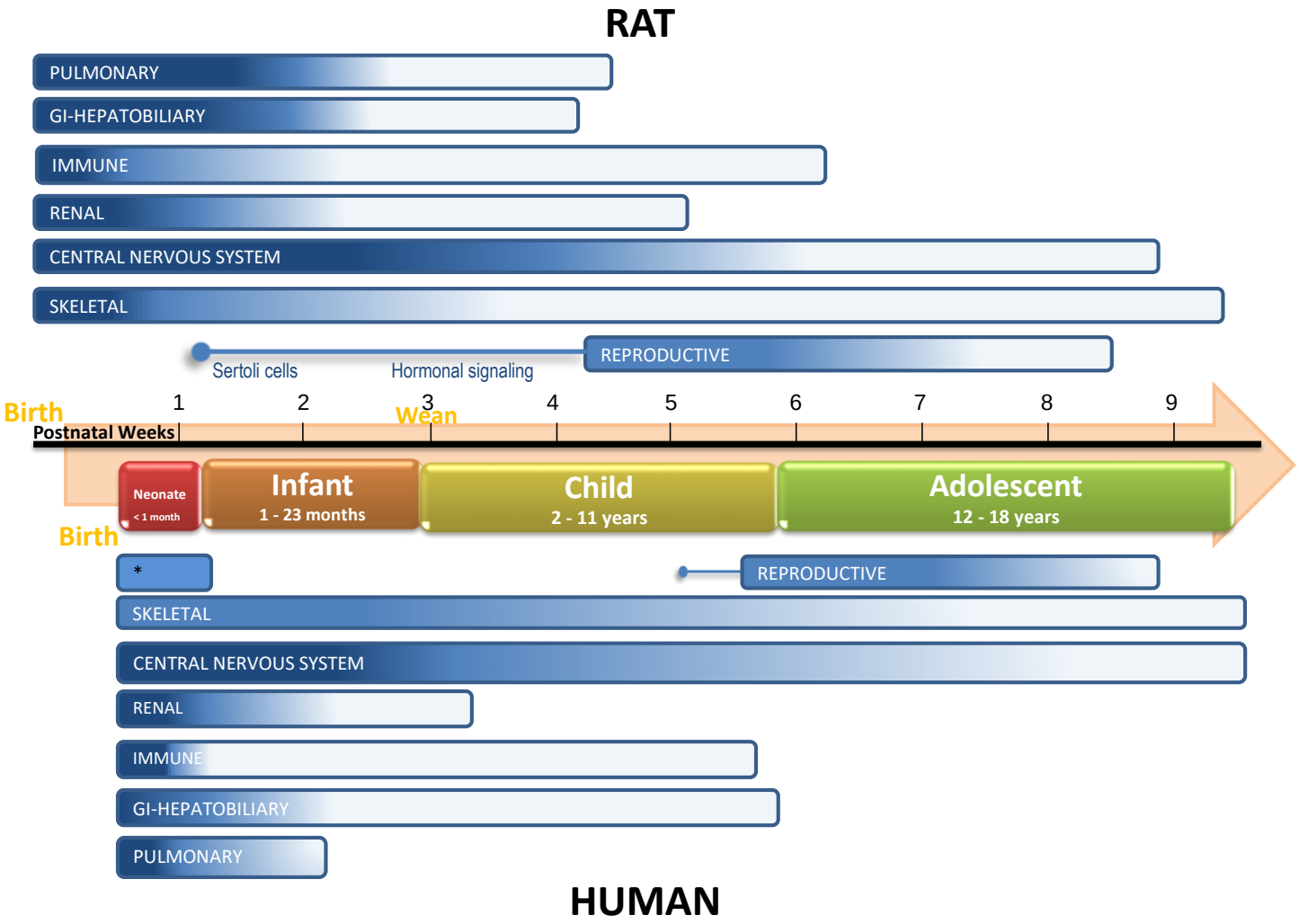
Table A4: age-dependent development of Göttingen minipig organ systems

System	General Considerations	Neonate (< 2 weeks)	1st Solid Food (~ 2-3 weeks)	Weaning (~ 4-6 weeks)	Puberty (~ 4-6 months)	Adulthood (> ~ 6 months)
Cardiovascular	• Critical neonatal physiologic transitions (pulmonary and systemic vascular resistance) • Adaptive myocardial and vascular changes and growth					
Endocrine	• Endocrine tissue development and initial hormone production occurs in utero • Endocrine functions are critical for growth/development and progression through puberty					
Eye	• Eyes morphologically well-developed at birth; eyes open within 1st 3 days postnatal • Full eyelid retraction by 3 weeks allowing ophthalmic examination					
Gastrointestinal	• Highly comparable to human postnatal GI development; critical neonatal absorption capacity • Postnatal acidification, microbiome, motility; adaptive digestive/transport functions achieved by weaning					
Hepatobiliary	• Structurally and functionally immature at birth; structurally similar to adult by 4 weeks • Progressive increase in metabolic functionality, especially over first 3 to 4 months					
Immune	• Minimal innate and passive immunity at birth; piglets require colostrum within ~ 2-4 hours postnatal • Anatomically fully developed by ~ 4 weeks of age • Progressive population of secondary immune tissues and development of memory as a function of time and environment					
Integument	• Critical neonatal function (barrier, conductance, sensation) • Poor thermoregulation from birth through 2-3 weeks					
Nervous	• Sexually dimorphic postnatal brain growth rapid through weaning and continuing until puberty • Myelin, glia and most cortical neurons present at birth • Neuromuscular system is more functionally mature at birth than in human					
Pulmonary	• Lungs are well developed at birth, with completion of alveolization over first 2 weeks postnatal • Continued growth and refinement of function through puberty					
Renal	• Nephron formation up to ~ 3 weeks after birth • Functionally mature at approximately 3 months of age					
Reproductive	• Structurally well developed and testes descended at birth • Sexual maturity in males by 4-5 months of age and in females by 5 to 6 months of age					
Skeletal	• Able to stand at birth with increased mobility in first few days • Rapid postnatal growth with closure of the epiphyseal growth plates at 18 months of age					

Table A5: age-dependent development of cynomolgus monkey organ systems

System	General Considerations	Neonate (< 1 month)	1st Solid Food (~ 3 months)	Weaning (~ 6 months)	Puberty (~ 3-4 years)	Adulthood (~ 4 years)
Cardiovascular	• Critical neonatal physiologic transitions (pulmonary and systemic vascular resistance) • Adaptive myocardial and vascular changes • Myocardiocyte expansion through 3 months of age, then progressive growth					
Endocrine	• Most glands are well developed at birth and critical for growth • Zona reticularis of adrenal cortex expands at 3 to 6 months of age (adrenarche) • Endocrine function of gonads expands at puberty					
Eye	• Structurally well-developed and eyes open at birth • Postnatal maturation of fovea and lens					
Gastrointestinal	• Functional at birth and comparable to human postnatal GI development • Postnatal acidification, microbiome, motility • Adaptive digestive/transport functions achieved by weaning to accommodate shift in diet/complexity and expand microbiome					
Hepatobiliary	• Structurally well developed at birth • Progressive increase in metabolic functionality, especially over first 3 to 6 months					
Immune	• Neonatal structural expansion of immune tissues • Progressive population of secondary immune tissues and development of memory as a function of time and environment					
Integument	• Functional (barrier, water and thermoregulation, conductance, sensation) with hair and adnexa present at birth					
Nervous	• Defined sequential and progressive development into adulthood • Postnatal apoptosis, pruning and migration most prominent before weaning • Myelin and glia present at birth, with further expansion postnatally • Neurotransmitter and conduction systems mature at variable rates (i.e.: GABA, serotonin, dopamine and noradrenalin all differ)					
Pulmonary	• Structurally mature at birth with progressive growth					
Renal	• Nephrogenesis complete at term birth • Progressive increase in GFR and renal function over first 6 months of age					
Reproductive	• Testes descended at birth, populated by germ cells, Sertoli cells and Leydig cells • Follicular development and atresia begins at 3 to 6 months • Subsequent reproductive changes (menarche and spermarche) occur at onset of puberty and continue until adulthood					
Skeletal	• Infant able to cling to dam from birth • Growth plates present at birth • Most rapid postnatal growth occurs prior to weaning, followed by slower growth until growth plates close during adulthood					

Figure A1 comparison of rat and human organ system development



* Human neonatal “mini puberty”, (See Table A1), also note leading dot with line to reproductive box denotes early pubertal hormonal signaling

Table A6. principal advantages and disadvantages of various mammalian species for use in juvenile animal studies

Species	Advantages	Disadvantages
Rat	• Well-studied species in juvenile animal studies with extensive historical control data • Several consistent developmental milestones (general growth, preputial separation/vaginal opening, puberty) • Often used for (adult) general and reproductive toxicology • Body size allows most manipulations/administrations starting early preweaning • Litter size allows balanced sex distribution and allocation of pups to different endpoints and dedicated cohorts of pups • Compressed development (~10 weeks) allows for inclusion of wide range of endpoints • Compressed development allows for inclusion of additional endpoints which are difficult to perform using large animals (such as developmental neurotoxicity, immunotoxicity, fertility/breeding) • Small body weight requiring low amount of test material • Relatively easy transportation, housing and management • Pups and dams are amenable to fostering • Easy to obtain many pups with the same postnatal	• Small body size, high metabolic rate and rapid growth can lead to fast decline in general condition and death • Several organ systems are less developed at birth relative to man (particularly CNS, lung, kidney, GI tract and immune system; eyes do not open until PND 12-14) • ADME characteristics of oral pharmaceuticals given in the preweaning phase often translate poorly to humans due to immaturity of the GI tract • Compressed development can make it difficult to identify distinct windows of vulnerability • Conventional blood samples are often terminal collections, particularly preweaning • Can easily become very large studies as most endpoints or collections require dedicated cohorts of pups • Less sensitive than humans to fertility perturbations • Can have more limited application for foreign proteins • Often not pharmacologically relevant for highly targeted therapies • Potential impact of immunogenicity

Species	Advantages	Disadvantages
	stage • Passive immunity present at birth	
Mouse	• Advantages are generally similar to those of the rat, but postnatal development occurs slightly faster • Broad CYP enzymes; metabolism can be more relevant than rat for some compounds • Mice have a gall bladder (unlike rats) • Detailed literature available, especially for development and characterisation of the CNS and immune system • Many genetic modification models available including some models that increase pharmacological relevance for highly targeted therapies	Similar to rat, additionally: • Small pup size allows fewer manipulations /administrations than rat from early on • Requires dedicated cohorts of pups for each endpoint or collection and can require sample pooling • Less historical background information than the rat

Species	Advantages	Disadvantages
Dog	• Often used in general (adult) toxicology • Relatively large at birth • Relatively easy to handle • Litter size allows allocation of pups to different endpoints • Puppies can be separated from dams for a few hours • Breeding can be planned in advance • Postnatal development of several organ systems reasonably comparable to that of human infants (cardiovascular, pulmonary, immune system) • CNS maturation relatively well characterised, with defined critical window for learning/cognitive development	• Protracted development (~5-12 months to sexual maturity, 12-18 months to skeletal maturity) with interindividual variability in growth and developmental milestones • Altricial at birth (i.e., eyes do not open and cannot bear weight until ~ 2 weeks postnatally) • Require colostrum for passive transfer of maternal Ig in immediate perinatal period • Variable litter sizes and sex distribution can make it difficult to populate study with minimal bias (genetic/litter, sex distribution) across groups • Limited historical background data, especially for nonstandard endpoints • Seasonal breeder (supply & study start over weeks or months) • Not amenable to fostering • Large body size requires comparably large amounts of test compound compared to rodents
Minipig /Pig	• Many similar developmental milestones as humans • Relatively large at birth • Relatively easy to handle • Breeding can be planned in advance • Litter size allows allocation of piglets to different endpoints • Amenable to fostering	• Less well-established historical control data than dog or NHP toxicology species • Require colostrum for passive transfer of maternal Ig in immediate perinatal period • Some organ systems relatively mature at birth compared to human infants (e.g., lungs, musculoskeletal) • Large body size requires comparably large amounts of test compound compared to rodents

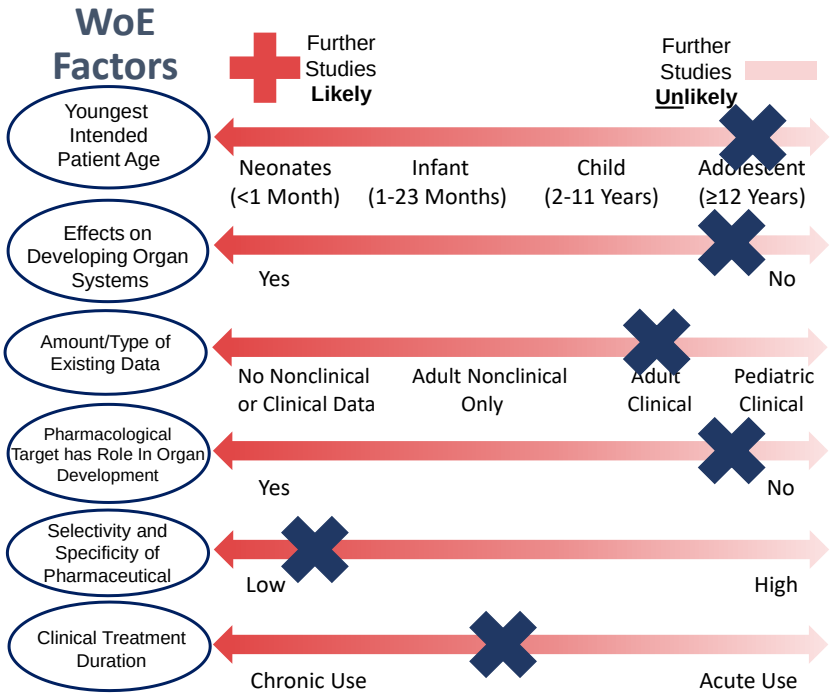
Species	Advantages	Disadvantages
	• Relatively large litters usually allow balanced sex distribution • Neonatal GI tract similar to human for orally administered drugs • All routes of administration feasible (except inhalation); best model for dermal studies (low density of adnexa and hair follicles, similar thickness of epidermis) • Short development (~6-9 months), relatively easy transport and housing compared to other large non-rodents	• IV and gavage administration can be challenging in very young piglets
NHP	• Usually cynomolgus but rhesus and marmosets also feasible • Many similar developmental milestones as humans • Neonates/infants similar to human for GI tract, immune system, cardiovascular, renal and special sense (eye, ear) development • Macaque infants are relatively large at birth • Extensive reference and historical background data from birth available • Often used for (adult) general and reproductive toxicology (e.g., ePPND), especially for biopharmaceuticals • Maternal transfer of immunoglobulin is similar to humans, so infants are born with passive immunity	• Protracted development (~3-6 years for sexual maturity, ~5-8 years for skeletal maturity in macaques) makes an extensive juvenile study to cover all developmental phases impractical • Single offspring for macaques with high inter-individual variability in growth and development • Marmosets typically have twins and require both maternal and paternal care in preweaning phase; offspring are relatively small • Offspring highly dependent on maternal care over first month (minimal procedural intervention recommended; preweaning manipulation & dosing feasible with risk of maternal rejection), and are cohoused with dam for first 3-6 months; with shipping and quarantine requirements it is rarely feasible to initiate studies in juvenile monkeys < 9 months of age • Neonatal NHP are precocious relative to human neonates in terms

Species	Advantages	Disadvantages
	(serum IgG) • Often the most pharmacologically relevant animal model for highly targeted therapies	of musculoskeletal, CNS, endocrine and respiratory system • Cannot synchronise breeding (supply & study start over weeks or months for seasonal breeders such as rhesus) • Ethical reservations (strong rationale to justify use of juvenile NHP for toxicity testing)
Rabbit	• Compressed development (~5-6 months) and small body size requiring comparably low amount of test material • Relatively easy to handle • Often used for reproductive toxicology; can also be used for ocular administration, evaluation of bone growth • Litter size allows balanced sex distribution and allocation of kits to different endpoints • Relatively easy transport and housing • Passive immunity present at birth	• Developmental milestones less well established than other non-rodent species • Not routinely used/well accepted in (adult) general toxicology • Less well-established historical control data than dog or NHP toxicology species • Handling young offspring can provoke cannibalism or rejection by the mother • Limitations similar to rat for foreign proteins, and immunogenicity can lead to acute hypersensitivity reactions • Sensitive to gastrointestinal disturbances • General physical condition difficult to monitor using clinical signs
Other Species	• Other species could be considered for cause when pharmacologically and toxicologically relevant. Examples of alternative mammalian test systems include the hamster, guinea pig, tree shrew, ferret, cat, sheep and goat. • Advantages tend to be species and program specific, but often reflect use of that species in genetic or disease models, or when there are data supporting interpretation and translatability of specific endpoints. Disadvantages include:	

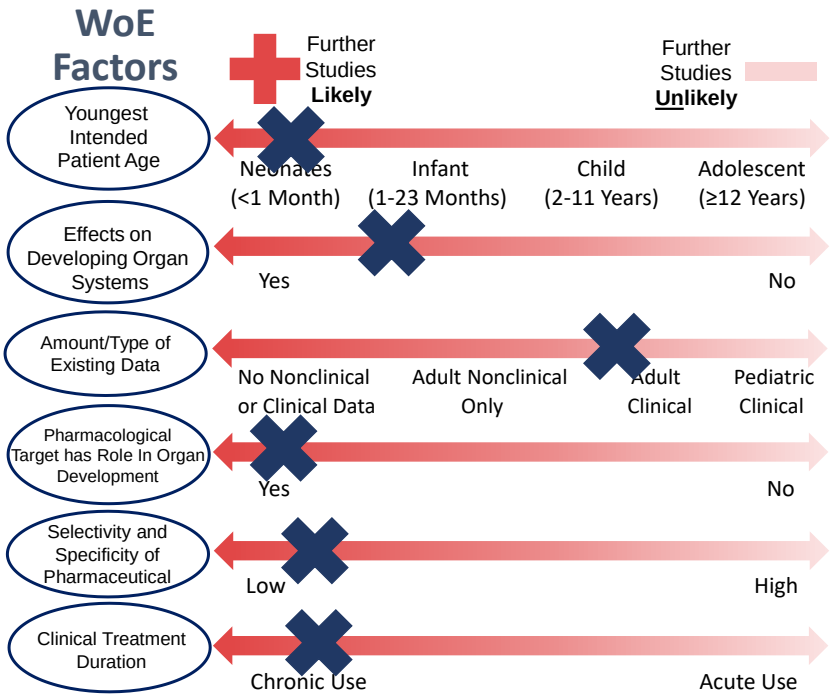
Species	Advantages	Disadvantages
	• Developmental milestones less well established than in rat, mouse, dog, minipig/pig and NHP • Not routinely used / well accepted in (adult) general toxicology • Limited historical control toxicology data • Limited use (model in special indications such as heart failure) • Many require colostrum for passive transfer of maternal Ig in perinatal period • Limited availability of purpose-bred animals and suitable laboratory housing	

Appendix B: case studies applying the weight of evidence approach

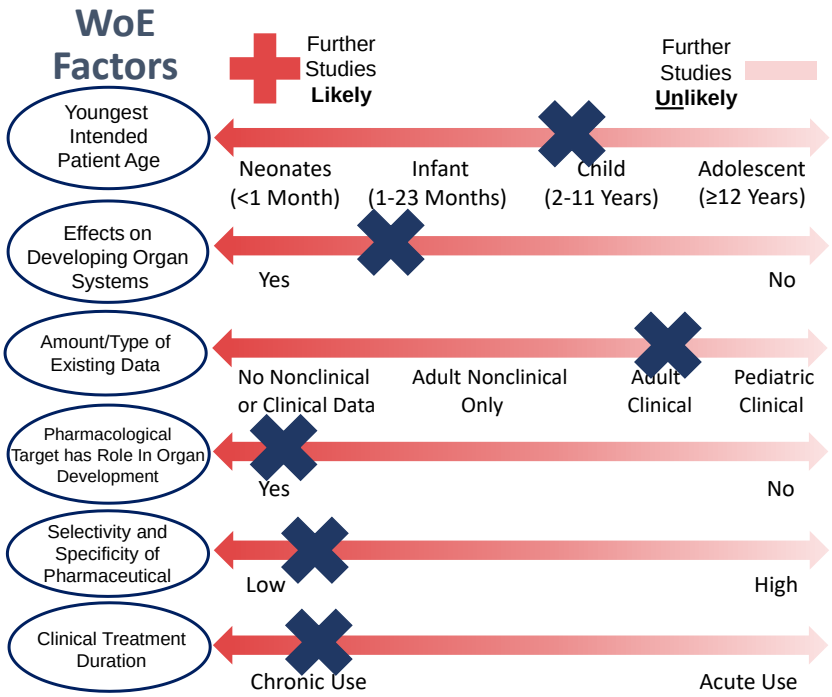
A. A small molecule with known pharmacology has available adult clinical and nonclinical data including repeated-dose toxicity data. None of these data suggest a safety concern in a developing organ for the intended paediatric population of adolescents (12 years and above) for a one-month duration of clinical treatment. The WoE analysis indicates that additional nonclinical investigations will not contribute useful information.



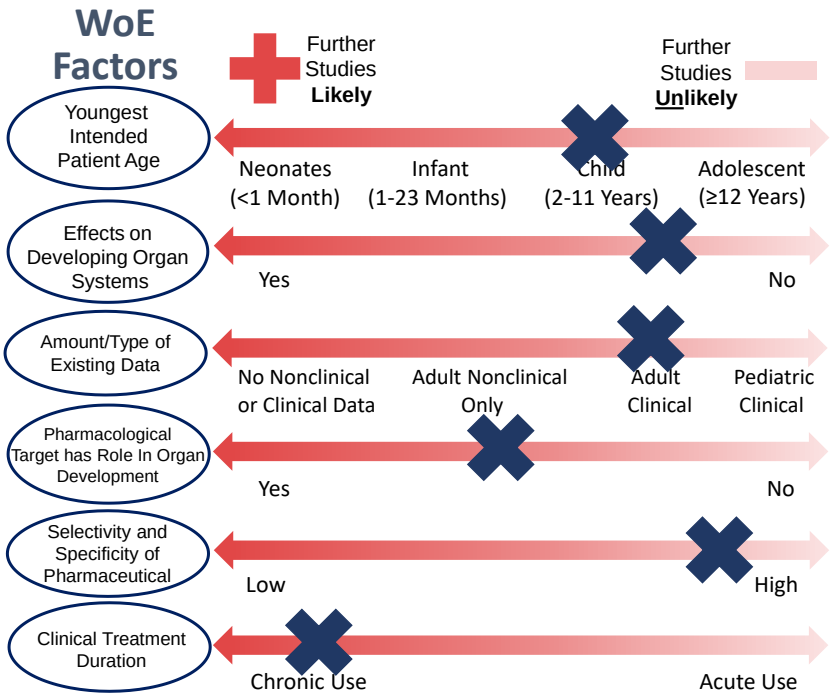
B. A small molecule with a novel mode of action intended for chronic use starting in neonates or infants has limited Phase 1 clinical and nonclinical safety data with no significant safety concerns identified. There are potential effects on developing organ systems based on the pharmacology. The WoE analysis indicates further nonclinical investigation, such as a JAS with additional endpoints based on the targeted developing organ systems, would be useful.



C. A small molecule with a pharmacological target that has an established critical role in CNS development is intended for chronic use in children (6 years and above). Nonclinical and adult clinical data are available. The concern for a potential effect on the developing CNS cannot be addressed clinically by monitoring and management. Existing data adequately address other developing systems. The WoE analysis warrants a postweaning JAS design that includes core endpoints and additional CNS endpoints to address the specific concern.



D. A monoclonal antibody targets a soluble cytokine and is intended for chronic paediatric use in rheumatologic and allergic diseases (>2 years old). The only findings are reversible decreased serum Ig and occasional injection site reactions (in both animals and adult patients). In a monkey ePPND study, offspring exposure was comparable to dams through PND 28 and decreased thereafter. T-cell-dependent antibody response (TDAR) results were similar to controls (between 3-6 months postnatally). The WoE analysis does not warrant a JAS.



Appendix C: Approaches to Preweaning Litter Allocation in the Rodent

Initiation of dosing rat pups during the preweaning phase of a JAS presents a unique situation in which the study should be designed to reduce potential confounders related to maternal care, litter size and other factors such as genetics. This can be achieved by how the study litters are constructed and standardised in combination with how the study litters are assigned to dose groups and how the individual pups are assigned to endpoint subsets. See also Section 3.9.1.

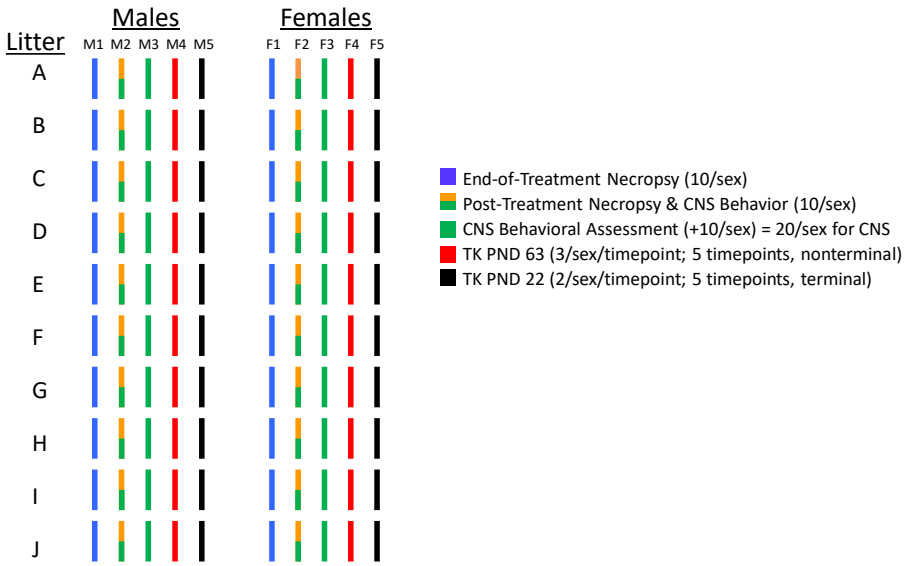
One approach to achieve the goal of an even distribution of potential confounders is to

- standardise the litter using a minimal fostering technique in which the majority of pups remain with their natural mother (i.e., those pups in good health) and, when necessary, a small number of pups are fostered into the litter to achieve the desired litter size and sex ratio
- assign each standardised whole litter to a single dose group
- assign individual pups within the study litter to endpoint subsets

In the following example, the definitive rat JAS design has a dosing interval from PND 14 through 63 and includes assessments of both core endpoints (with TK assessments on PND 14, 22, and 63) and study-specific additional endpoints (post-treatment necropsy and CNS assessments). Each dose group contains 10 minimally fostered rat litters (identified as A, B, C for each dam with a litter, etc.). The litters are standardised to 10 pups per litter (5 male and 5 female pups when possible) with each colored line in the figure representing an individual pup. The genetic offspring are assigned arbitrarily (F1, F2, etc. for the first and second female, respectively). Pups that were fostered are re-assigned the 'last' positions in the pup identification scheme (e.g., F5 for the fifth female). The dam identifiers for both their genetic (if available) and foster mothers are recorded in the study data. The whole litter is then assigned to the same dose group so all 10 pups in a litter will be treated with the same dose. Generally, the number of litters will depend on the total number of pups for the selected endpoints. Microsampling can minimise the number of animals for TK assessment, and thus is always encouraged.

In this example, the pups are assigned to core and study-specific additional endpoints across the 10 litters in an inter-litter fashion (i.e., one or two males and/or females from each litter to the specific endpoint subset). The end-of-treatment necropsy and other key endpoints are assigned to pups with low identification numbers (i.e., M1 or M2) so that fostered pups are more likely assigned to endpoints less affected by confounders. Depending on the specific study endpoints, there are different ways that the subsets can be allocated to accomplish the same objectives.

Figure Represents One Dose Group of 10 litters with 5 pups/sex



In this example, one pup/sex/litter is allocated for the end-of-treatment necropsy (M1/F1 blue lines; for a total of 10 pups/sex/dose group). The 10 pups/sex/dose group of the second set (M2/F2 half yellow and green lines) are assigned to two assessments, CNS behavioural assessments and post-treatment necropsy. These 10 pups/sex/dose group along with the 10 pups/sex/dose group of the third set (M3/F3 green lines) are combined for a total subset of 20 pups/sex/dose group for the CNS behaviour assessment. This CNS assessment includes detailed clinical observations, behaviour tests, and learning and memory tests, which can be conducted during the post-treatment period with clinical observations also conducted during treatment. Both of the necropsy subsets (end-of-treatment and post-treatment) include an expanded neuropathology assessment. The fourth set (M4/F4 red lines) is assigned to the PND 63 TK subset (serial sampling; non-terminal serial sampling is possible in post-weaning rats of this age, therefore only a portion of this subset is needed for TK and could be used for other assessments). Because maternal and litter confounders would not be relevant for a single dose TK assessment, the TK blood samples after the first dose on PND 14 are collected from spare litters (not shown) that would not continue on study after the blood collection (approximately 3-4 pups/timepoint/dose group). Lastly, 1 pup/sex/litter (M5/F5 black lines) is assigned to the PND 22 TK subset (an age that typically requires terminal blood collection unless microsampling is available).

The pups in the end-of-treatment necropsy (M1/F1) and the post-treatment necropsy subsets (M2/F2) also have core assessments for post-weaning food consumption, sexual development, clinical pathology, and long bone length. The core endpoints of mortality, clinical observations, and body weights are assessed for all pups.

In addition to the example above, there are other options that can decrease the number of pups assigned to the study. For instance, one set of 10 pups/sex/dose group could be split to serial sampling TK (4 pups/sex/dose group) and post-treatment necropsy (6 pups/sex/dose group). Alternatively, in the example above, the fourth set of 10 pups/sex/dose group (M4/F4) could be split into 4 pups/sex/dose group for the PND 63 TK assessment (an age when non-terminal serial blood collections are generally feasible) and 6 pups/sex/dose group for the CNS assessment. The third set of 10 pups/sex/dose group (M3/F3 green lines) could also be assigned to the CNS subset to achieve a total of 16 pups/sex/dose group. Both of these options obviate the need for extra animals for the CNS

assessments. Thoughtful study designs which include appropriate endpoints and animal allocation can minimise the number of litters and total animals used.