

**COMMITTEE ON THE CARCINOGENICITY OF CHEMICALS IN FOOD,
CONSUMER PRODUCTS AND THE ENVIRONMENT**

**OECD GUIDANCE DOCUMENT FOR THE PERFORMANCE OF
CHRONIC TOXICITY AND CARCINOGENICITY STUDIES,**

**Chapter 3.6 Investigations (including Histopathological
Guidance)**

The Organisation for Economic Cooperation and Development (OECD) is currently developing a Guidance document to accompany the revised Test Guidelines for carcinogenicity studies and chronic toxicity studies. The chapters anticipated for inclusion in the Guidance were discussed by the COC at the April 2008 meeting. The contents of the Guidance are provided for information at Annex 2. Members considered that, although much guidance already exists on histopathology, it would be important to bring it into an OECD context, and recommended that the UK should propose leading on this chapter.

The UK agreed to the OECD Secretariat request to expand the contents of the Chapter to include all investigations. At its meeting in April 2009, the COC discussed the first draft outline of the UK Chapter based on existing OECD Guidance and the Society of Toxicological Pathologists Guidance. COC members made helpful suggestions for additional areas to be included in the Chapter. Concurrently, the meeting of the Working Group of National Coordinators of the OECD Test Guideline Programme (WNT) agreed that the new guidance should not incorporate and replace existing OECD Guidance Document "Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies" (GD35). The decision of the WNT entailed considerable revision of the UK draft.

The Chapter has now been revised taking into account the comments and contributions of COC members and deleting text taken from GD35. The revised draft of the Chapter is now provided at Annex 1. The advice of the UK GLP authority is also being sought. The final document needs to be acceptable to all OECD member

countries document and therefore should encourage best practice but allow flexibility. The final Guidance Document will be an OECD, not a COC document.

Members are requested to provide any additional comments on the current draft, to be revised and submitted to the OECD in the summer of 2009.

COC Secretariat

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**OECD GUIDANCE DOCUMENT FOR THE PERFORMANCE OF
CHRONIC TOXICITY AND CARCINOGENICITY STUDIES,
SUPPORTING TG 451, 452 AND 453**

DRAFT CHAPTER 3.6 INVESTIGATIONS (INCLUDING HISTOPATHOLOGICAL GUIDANCE)

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3.6.1 Introduction

1. This Chapter on investigations includes guidance on the design and conduct of pathological, histopathological and ophthalmoscopic investigations together with more general advice on the avoidance of bias during investigations. It complements existing Guidance contained in OECD Guidance Document No. 35 on the analysis and evaluation of chronic toxicity and carcinogenicity studies. Guidance Document 35 should be consulted for detailed guidance on mortality, clinical observations, body weight changes, food and water consumption, absolute and relative organ weights, haematological, clinical and urinary measurements, post mortem observations and analysis of toxicokinetic and metabolism data.
2. Ophthalmoscopy is an extremely useful clinical technique that allows examination of the back part of the eyeball (fundus), including the retina, optic disc, choroid, and blood vessels in the eye. Ophthalmoscopy can help to detect diseases of the eye, to diagnose other conditions or diseases that damage the eye, and can detect other diseases such as some brain tumours. In life ophthalmoscopy allows the progression of the disease to be followed in time and can indicate that additional sampling at necropsy would be informative.
3. Histopathology evaluation is an important part of the assessment of the adverse effects of chemicals on the whole organism. Conventional histological, histochemical and special staining techniques, together with electron microscopy can be used both to define the identity and morphology of tissue, cellular and subcellular structures and to indicate the chemical characteristics of their constituents. In addition to conventional special staining techniques and electron microscopy, new techniques involving immunohistochemistry, molecular biology and novel visualisation procedures can be applied to provide additional more objective methods to obtain better functional and morphological characterisation of induced alterations in tissues of the body, when needed.

3.6.2 Ophthalmoscopy

4. In chronic toxicity studies, ophthalmoscopic evaluation should be conducted by a suitably trained and experienced ophthalmologist, preferably using indirect fundoscopic examination *and* a slit-lamp evaluation. A topical mydriatic agent will normally be employed to facilitate ophthalmoscopic examination.
5. Animals should be examined pre-treatment, at least once during the course of the study and pre-terminally. Dependent upon the nature of any abnormalities observed, additional ophthalmoscopic examination may be warranted. Ocular abnormalities may require additional tissue sampling at postmortem to enable histological examination of the ocular adnexa. When ocular abnormalities are detected by ophthalmoscopy, histopathological examination of the eye should attempt to identify

the morphological correlate of the abnormality. If ocular abnormalities are focal, it may be appropriate to perform histological examination on additional sections.

6. The study report should contain an integrated interpretation of all ocular findings (ophthalmoscopic, macroscopic and microscopic examinations), along with pertinent individual animal data.

3.6.3 Pathology and Histopathology

3.6.3.1 General Considerations.

7. Pathology has an important role in toxicology since it provides information on the differences in tissue and organ morphology that establish the presence or absence of lesions and whether or not there are dose–effect relationships. Pathology data can facilitate the interpretation of other data, such as organ weight changes, clinical biochemistry or haematology findings (*e.g.* Krinke *et al.*, 1991), and evaluators should always make it clear whether there are any associations between pathological abnormalities and other findings of physiological significance. Nevertheless, not all changes in tissue morphology are accompanied by abnormalities in other parameters, and perturbation in organ biochemistry will not necessarily be accompanied by changes in the histological appearance of the affected organ(s). An overview of physiological and environmental factors that can complicate the interpretation of findings in a toxicity study may be found in the *Handbook of Toxicologic Pathology* (Bucci, 1991).

3.6.3.2 Sampling

8. The pathologist should ensure that standard sections of all appropriate tissues and organs are present on the slides to be evaluated. The use of standard sections helps to ensure comparable samples across all animals, thus reducing inconsistency. If flawed or incomplete specimens (*eg.* missing medulla of adrenal, pars distalis of pituitary, mucosa of intestine), impair the pathologist’s ability to detect, or evaluate treatment-related effects, it is the pathologist’s responsibility to obtain recuts, to the extent possible, of those missing or inadequate tissues. Any irretrievable omissions should be taken into account in the interpretation of the data and discussed in the pathology narrative.
9. Procedures for tissue sampling and trimming to ensure optimal fixation, vary between laboratories. However, more standardised approaches to the selection of blocks, orientation of tissues and number of slides examined for rat and mouse organs and tissues in regulatory type toxicity studies are now available (Ruel-Fehlert *et al.*, 2003; Kittel *et al.*, 2004 and Morawietz *et al.*, 2004) and are recommended. These publications are based on the experience of the European Registry of Industrial Toxicology Animal-Data (RITA) and American North American Control Animal Database (NACAD). They are an extended revision of the trimming guidelines published by Bahnemann *et al.* (1995). The articles describe in detail the optimum localisation for tissue preparation, the sample size, the direction of sectioning and the number of sections to be prepared, organ by organ. However, existing information on the substances tested and gross findings at autopsy may indicate that samples from non standard locations should also be taken. If a test substance is administered by the

dermal route or by inhalation route for example, samples should be taken from the site of application. In these instances, samples from the site of administration should be taken in addition to the standard specimens.

3.6.3.3 Histopathology specimen processing and quality

10. High quality tissue specimens are necessary for histopathologic evaluation. For routine purposes, fixation in a suitable concentration of a formalin solution followed by processing and embedding in paraffin wax, and cutting of a suitable thickness of histological section is adequate for most tissues. Exceptions are the eyes and testes for which formalin is generally regarded as an inadequate fixative. In these instances, Bouin's or Davidson's fixatives are preferable. Bouin's fluid is generally considered the best fixative for testis although there has been a move away from this because of the safety concerns of its picric acid content (Latendresse et al., 2002). Formalin, when used under stringent conditions is usually also a good fixative for many immunohistochemical and molecular biological techniques on tissue sections. Frozen sections are however usually needed for studies of enzyme activity or those requiring intact RNA. Tissues should be processed in a manner that reduces the potential for variation to be introduced among groups, as indicated in Section 3.6.5.
11. Special stains should be used where appropriate. Haematoxylin and eosin (H&E) remains the most widely used stain, supplemented, where appropriate by a Romanovsky stain for haemopoietic cells, Periodic-acid Schiff (PAS) stain for hepatic glycogen, glomerular basement membrane and the acrosome on testicular germ cells, trichrome and elastic stains for the myocardium, and blood vessels and oil red O applied to frozen sections for neutral lipids. While still not standard, immunohistochemical techniques are now widely used in special cases and the advantages and pitfalls of the techniques are discussed in detail by Greaves (2009). All specially stained tissues should be accompanied by a positive control specimen that confirms the proper functioning of the technique.
12. The use of larger semi-thin (1 to 3 μm thick) plastic or resin embedded sections is a technically demanding but cost effective compromise between electron microscopy and conventional light microscopy. It requires specialist equipment frequently not present in routine histology laboratories. Sometimes termed 'high resolution light microscopy', light microscopic evaluation of semi-thin sections provides a means of avoiding extensive use of the electron microscope because it can locate cytoplasmic organelles in a way sometimes not possible in a paraffin wax embedded material.
13. All slides evaluated should be identified with the study number, animal number, and slide (block) number clearly identifying the tissue[s] present on the slide.
14. In view of the hazard to health of formalin, used for fixation, and xylene, used during processing, an increasing number of laboratories are adopting technologies which are free of both formalin and xylene. Nassiri et al. (2008) describes the utilization of a formalin-free fixation and processing system for tissue detection of two important biomarkers in breast cancer at the RNA and protein levels. Falkeholm et al. (2001) demonstrated that xylene-free histological sections are qualitatively on a par with conventional paraffin sections for routine diagnostic work. Overall, these techniques appear to have good safety profiles, provide excellent histology quality and are suitable for further analysis by immunohistochemistry and nucleic acid extraction.

15. In addition to histopathology and immunohistochemistry, tissue is often required for molecular genomic or transcriptomic analysis of biomarkers. This should be borne in mind when collecting and studying tissue. It is important therefore to be prepared to extract RNA, DNA and even protein from paraffin embedded material. This can be achieved using both formalin-containing and formalin-free fixed tissues. Hewitt et al. (2008) summarizes the current state-of-the-art of preanalytic factors in tissue handling and processing as they impact the quality of RNA obtainable from formalin fixed paraffin-embedded tissue.
16. Artefacts may be produced at each of the following stages in the processing of tissue sections: before death, at postmortem or necropsy, during the fixation of tissues, during processing, paraffin embedding and microtomy, during the mounting of tissue sections onto glass slides, staining procedures and coverslipping. Some artefacts are easily distinguishable from normal or diseased tissue components but these render the task of the pathologist more difficult and great care should be taken to avoid their introduction. Some artefacts are difficult to distinguish from pathological changes and are thus of particular concern. McInnes (2005) sets out some of the more common artefacts that are most frequently encountered as a result of inadequacies in the preparation of microscopic tissue sections.

3.6.3.4 Histopathologic evaluation

17. Details of the nature of the test substance, results of any previous toxicity studies and known activities of this class of compounds should be made available to the pathologist before evaluation of the tissue slides begins. Knowledge of target organs and tissues and the types of changes previously encountered, even in different species, facilitates the evaluation of tissues and provides for the consistent use of terminology. Previous knowledge of target tissues should be utilized during the protocol development to determine whether special pathology procedures should be used in obtaining, fixing, processing, or staining of sections.
18. The study pathologist should have access to in-life clinical observations, organ and body weight data, haematology and clinical biochemistry data, and macroscopic findings of the postmortem examination for each animal in addition to complete information about the experimental design, signalment and husbandry of the study population. These include, but are not necessarily limited to: study protocol, including amendments and relevant deviations, species, strain, and age of animals, route, doses, and duration of dosing.
19. Metabolic, pharmacokinetic, or toxicokinetic information may be necessary for understanding patterns of change and interpreting differences in species responses.
20. In-life data (i.e., clinical signs, body weight changes, food consumption, ophthalmoscope findings etc.) from animals may help greatly in the identification of target organs and in understanding mechanisms of toxicity. Haematology, clinical chemistry, and urinalysis results also aid the identification of target organs and may contribute to an understanding of the mechanism of action. Results of special assays, such as hormone concentrations or enzyme induction, are equally important in locating morphologic changes and in the understanding of their significance.
21. Necropsy (gross) findings for individual animals must be available to the pathologist for lesion tracking and correlation with histopathology findings. The pathologist

should also be aware of organ weight changes. Often histomorphologic correlates of altered weights can be identified.

22. All of the above-mentioned data, if available, should be provided to the pathologist at the time of the initial slide evaluation. Provision of information on previous findings for the individual animals may render the treatment status of the animal obvious. The pathologist may wish to examine samples from animals of the same treatment group for the same histopathological changes. This may improve the efficiency of the process but comes at the price of inevitably introducing the potential for bias into the process. Careful consideration needs to be given to the balance between ensuring that subtle or rare changes are detected and the possible introduction of bias. This is discussed further in section 3.6.5.
23. Tissues may be evaluated animal by animal or organ by organ as the preference of the pathologist dictates. The animal by animal technique affords an encompassing overview of an animal's complete health status. The organ by organ technique allows more focused attention to changes and aids in the consistent grading of changes in a particular organ. Where concise terminology is inadequate to convey lesion complexity, detailed free text descriptions should be used to define the diagnostic term used for tabulation.
24. For common lesions in a species or strain of animal, it is important to know if the experimental treatment alters severity. The pathologist should use a severity grading system that allows for an appropriate severity classification, as treatment may affect the incidence (number of animals showing the pathology) or the severity of a particular pathology lesion. Toxicological lesions are frequently found in a continuous spectrum of severity. Therefore, severity grading systems should be: 1) definable, 2) reproducible, and 3) meaningful. A description of each of the various grades should be included in the narrative for target lesions where severity is critical to interpretation of the data. Photomicrographs may be helpful in conveying the severity differences for the grading system used. Well-defined severity grading systems greatly aid the pathology peer-review process. For carcinogenicity studies, it is the pathologist's responsibility to distinguish between hyperplasia, dysplasia, neoplasia and to classify tumours, where applicable, as 1) benign or malignant, and 2) primary or metastatic.
25. Computerised systems of recording findings help to organise the process of evaluation and ensure that all animals and all tissues have been examined, and that gross and microscopical correlation is followed. They also allow rapid, and reproducible, formation of the pathology tables needed to ensure appropriate interpretation of treatment-related effects.

3.6.3.5 Procedures to enhance the accuracy and consistency of histopathology

26. Histopathology is a descriptive and interpretive science and therefore includes an element of subjectivity. However, the pathologist should evaluate tissues as consistently as possible to avoid the introduction of artificial differences or between-group bias. Evaluation of all tissues within a study by one pathologist with consistent standards for detecting, naming, and grading tissue changes, facilitates the detection of differences induced by treatment. However, two or more pathologists are on rare occasions involved in evaluation of a study. In such circumstances, the utmost care must be taken to ensure that nomenclature and severity grading systems used by the

contributing pathologists are harmonized to limit variation and that steps are taken to avoid bias (see section 3.6.5). For example, the situation where all of the samples in one dose group are evaluated by one pathologist and those in another dose group by another pathologist should be avoided. Standardized criteria and consistent terminology should be agreed upon for grading systems of common spontaneous and treatment-related findings. The use of a shared computer system that can define a study-specific lexicon for capturing data facilitates this process. Guidance on standard nomenclature is available in OECD Guidance No. 35.

27. Pathologists are aware of the phenomenon of “diagnostic drift.” Drift refers to a gradual change in nomenclature or severity grading of lesions within a single study. Diagnostic drift usually develops from the increased awareness of a lesion by the pathologist and it is more of a problem in large studies with many animals and tissues requiring evaluation over a prolonged period of time. It is a source of inconsistency that can negatively impact detection of treatment-related lesions or artefactually introduce apparent treatment-related effects where none exist and can erroneously affect the determination of no-effect-levels. When a pathologist becomes aware of drift in his/her selection of terminology or severity grading, he/she must re-evaluate the tissue(s) involved. Any suspected treatment-related effects should normally be re-evaluated through the use of a “blinding” or “masking” technique, where appropriate.

3.6.3.6. Image Capture

28. It is useful to capture images of key features, both as an aide memoire but also as a means of sharing with other pathologists, thus reducing problems of diagnostic drift. Rather than relying solely upon subjective opinions consideration should be given to use of quantitative morphometry for morphological features, where possible, for example in metabolic bone studies, and the accurate quantitation of protein expression detected by immunofluorescence.

3.6.3.7 Peer Review of histopathology

29. Peer review increases confidence in the accuracy of the histopathology findings from a study. Peer review by an independent pathologist is essential to ensure consistency of the histopathological findings in any studies but in particular those evaluated by more than one pathologist. The objectives of a formal histopathology peer review are several: 1) determine accuracy and consistency of nomenclature i.e., survey for the presence of incorrectly diagnosed or inaccurately described treatment-related lesions, 2) determine completeness; i.e., survey for the presence of undiagnosed treatment-related lesions, 3) determine the appropriateness of the NOEL, NOAEL or other point of departure, e.g. BMDLx, by reviewing all target tissues and organs, and 4) review the correctness of the textual interpretations derived from those data. The methods employed may vary depending on the purpose of the peer review. For a routine peer review, tissues from a sufficient number of treated animals need to be evaluated to assure that significant lesions were not missed and that a “no effect level” can be verified.
30. A number of procedures can be used for peer review (Eighmy, 1996; Peters, 1996; The Society of Toxicologic Pathologists, 1991 and 1997). Peer reviews are generally included in the study protocol and are conducted prior to the issuance of the study report (Ward et al., 1995). It is important that the original plan for the review process

include a joint review by the initial and reviewing pathologist of the two sets of results to explain or resolve apparent differences, should they occur. The results of the peer review should be documented and archived and any differences of opinion resolved through consensus. This procedure is then part of the process that leads to finalizing diagnoses and interpretations.

31. If any differences cannot be resolved through a joint review of the data, then arbitration through a third pathologist should take place or the problem referred to a “pathology working group” (PWG) or to a panel of expert consultants for resolution.. A Pathology Working Group (PWG) may be formed to review, revise and/or interpret diagnoses (Peters, 1996; The Society of Toxicologic Pathologists, 1991) and this can be done prior to finalising the report or as a retrospective review after a report has been finalised. The PWG should be composed of individuals having expertise both in the pathology of the test species and with the specific lesion in question. The PWG may include both the original and the reviewing pathologist but the diagnoses of both the original and the reviewing pathologist should not be known to the other PWG members. Prior to beginning work, a PWG chairman is appointed who directs the focus of the PWG and orchestrates agreement of the criteria used to make the diagnoses of the lesions in question. This process should be clearly defined and documented. The PWG may request additional sections from tissues to aid with the diagnostic process of the peer review. Following slide examination by the PWG in a blinded fashion and tabulation of the results; the findings of the PWG are compared to those of the study pathologist. When the consensus of the PWG is clearly different from that of the study pathologist, the diagnosis for an individual lesion should be changed. When there is a close split on a vote of a diagnosis, however, the diagnosis of the study pathologist should be allowed to stand. The records of the PWG should indicate the final diagnoses for each lesion and the degree of certainty of the diagnoses.
32. Regardless of its exact form, the peer review process should encourage direct interaction between the original and the reviewing pathologist and should result in the production of a single, scientifically robust pathology report that appropriately and accurately summaries the results and any uncertainties. The process should be constructive, meet the needs and objectives of the review and have an inbuilt into it a procedure for resolving differences, should they arise.
33. Retrospective peer reviews by a single pathologist or a PWG may also be undertaken after the completion of the study report. Any changes required by a retrospective peer-review will result in the generation of a report amendment, and as such, must be documented as required by Good Laboratory Practices.

3.6.3.8 Early Death Investigations

34. Guidance on the interpretation of early deaths is provided in Guidance document 35. In this chapter further guidance on investigations that are valuable is provided.
35. In accordance with OECD Guidance (GD 19), the earliest possible endpoints that are indicators of distress, severe pain, or impending death should be used as indications for humanely killing the animals prior to them reaching a moribund state or dying. GD19 was developed largely for animal welfare reasons. However, this practice also has the advantage of reducing the number of animals that are found dead with the accompanying risk of loss of tissues from autolysis or cannibalism. It also avoids

confusing direct effects of the test substance with secondary effects resulting from post-mortem or moribund changes.

36. Reasonable efforts should be made to determine the cause, or likely cause, of individual deaths or severe toxicity leading to euthanasia. It is important to identify causes unrelated to exposure to the test agent (e.g. acute or chronic infections, age or disease-related degenerative processes, anatomical abnormalities, negligent handling or accident) from toxicity-induced effects. All in-life data, as well as the results of the post mortem, of euthanased or dead animals in a study, should be used in an attempt to make this distinction.
37. Assignment of the cause of death or of severe toxicity, leading to euthanasia is a component of the assessment of carcinogenicity studies in rodents, which is widely practised to aid statistical evaluation (Kodell et al., 1995). The US National Centre for Toxicological Research (NCTR) requests routine assignment of cause of death for all dead and moribund animals examined histologically in carcinogenicity studies. A determination as to whether a particular tumour under consideration was fatal to an animal or was simply an incidental finding at necropsy is important for determining the type of information that an individual animal contributes to age-adjusted statistical tests for carcinogenic effects (Peto et al., 1995).
38. Organ weights from animals that die or are euthanized prior to scheduled necropsy are of little value in most cases to the overall study because of differences in nutritional status and tissue congestion and oedema secondary to the health status of the animal. The absence of matched concurrent control data further hinders interpretation of organ weights from animals that die or are euthanased prior to termination of the study. They can however be useful in indicating a potential cause of death in the individual animal and should therefore still be taken at necropsy.
39. An experienced pathologist should, under appropriate conditions, be able to distinguish peri- and post-mortem changes in tissues from treatment-related effects on tissues. While cannibalism can inhibit accurate pathology evaluation it is almost always useful to fix and block tissues from all animals, even those found dead, since different tissues show different degrees of autolytic change in death and conclusions can often be made even when some time has elapsed between death and tissue sampling.

3.6.3.9 Pathology Final Conclusions

40. A final pathology/histopathology conclusion is reached when the following have been achieved: 1) histopathologic diagnosis and description of all findings for each animal on the study, 2) the diagnoses and descriptions reflecting the consensus of a peer review, if a peer review was done prospectively, 3) tabular summary data providing an accurate representation of the findings, 4) a NOEL, NOAEL, or other point of departure, e.g. BMDL_x, if appropriate. Final conclusions and diagnoses may often be the result of an iterative process in which the diagnosis is continually refined and modified until a final conclusion can be reached. This process may include taking additional samples for evaluation. Details of areas of disagreement and/or uncertainty that arose during the study and how these were resolved should be documented. The final pathology report must accurately and completely present the data and their interpretation. Benign and malignant neoplasms of the same cell type are sometimes combined because one is seen as the progression of the other. However, this may

result in a loss of sensitivity if the substance tested accelerates the progression to malignancy, since the increase in malignant neoplasms is balanced by a decrease in benign neoplasms. Therefore, whilst combined data may eventually be useful, the pathology narrative should be full and precise to prevent misinterpretation or doubts over the interpretation. The narrative of the final pathology report should address all the significant pathology findings and any group differences. There should be precise descriptions of any treatment-related findings and their likely importance. Clear reasons for concluding that any observations are unrelated to treatment or are not biologically or toxicologically significant should also be provided (Morton et al., 2006).

3.6.4 Supplementary Investigations

41. Previous knowledge of the effects of the substance may indicate that non-standard investigations would be of value in the interpretation of the results. Samples for such investigations should be taken and stored appropriately, provided that this does not jeopardise the basic requirements of the study. Such supplementary sampling would not thus invalidate the study as an OECD-compliant study according to the test guideline. Findings in the initial standard investigations may raise further questions that may be addressed by supplementary studies if appropriate stored samples are available.

3.6.5 Avoiding bias

42. Bias can arise if samples or observations in one group differ from those in another group for any reason other than treatment with the test substance. Therefore it is important that steps are taken to prevent the introduction of bias throughout the whole study and not restricted to the randomisation of animals to the treatment groups at the beginning of the study. Bias can be introduced whenever the operator or observer, equipment used or the timing of investigations are confounded with treatment group. The study design should minimise such effects by use of randomisation or a balanced design.
43. Randomisation should be done formally, not semi-subjectively. There are several methods for performing the randomisation process. The three most commonly used are card assignment, use of a random number table and the use of a computerised algorithm. Randomisation is normally used for the sequence of necropsy and can also be used for the sequence of other investigations. Similarly, if different operators or observers and equipment is used for the same investigation, randomisation should be used to prevent confounding with the treatment group. A balanced study design can be used instead of randomisation for investigations.
44. Re-randomisation of samples is not necessary at each stage of the study and an initial randomisation or balanced study design can be re-used throughout the study investigations. Slides are not normally randomised for histopathologic evaluation; slides from the control and highest dose groups are usually evaluated before other groups. The benefits and drawbacks of blinding or masking samples for histopathological evaluation are discussed below.

45. Subjective evaluations, such as clinical signs and histopathology are susceptible to bias if the evaluator is aware of the treatment group to which the individual animal or slide belongs. Blinded or masked evaluation is carried out without prior knowledge of treatment group, which could include untreated or other control groups as a mechanism to minimize the introduction of observational bias. Blinded/masked evaluation is a more rigorous scientific approach to assessment.
46. For the evaluation of clinical signs, the absolute masking of the treatment group for individual animals is practically difficult as animals within one cage are usually from the same treatment group. However, blinding of the evaluator should be maintained to the extent possible without jeopardising other aspects of the study, particularly by increasing the risk of errors in dosing or animal identification.
47. Appropriate blinding procedures should be considered by the pathologist before, during, and after the microscopic evaluation of tissues. Blinding need not prevent the pathologist being provided with detailed information of other observations in the study, including animal-specific findings (see Section 3.6.3.4). The foremost objection to blinded slide evaluation in the initial histopathological examination is loss of knowledge of the range of normal that exists in known controls. This may be particularly pertinent for studies on new chemical entities or in new species, where little is known about potential treatment-related effects and their background frequency in control animals. Without this baseline, subtle differences between treated and control groups may be difficult to detect. Knowledge of the treatment group also allows the pathologist to assess the spectrum of related morphological changes and determine the most appropriate diagnostic terminology, including combining related diagnoses where indicated. However, these advantages must be weighed against the inevitable bias introduced by non-blinded evaluations. If an initial non-blinded examination is carried out to allow the recognition of treatment-related findings, subsequent masked evaluations of target tissues can be very helpful because it ensures an unbiased determination of a NOEL (no-observable-effect-level), or other point of departure.
48. Randomisation, balanced design and blinding or masking procedures employed to minimise bias in investigations should be described in the protocol and study report.

3.6.6 Data Archives

49. Raw data in non-clinical studies is defined under the GLP regulations as the original data collected and the GLP-specified retained specimens, such as tissue blocks and glass slides). Other records, such as laboratory worksheets, notes, images and the pathologists interim notes as the diagnosis is refined are also useful and should be clearly kept and identified as appropriate.
50. In the GLP-compliant histopathology data collection computer systems used today, a variety of security measures have been incorporated to both ensure data integrity throughout the process of histopathology data collection and reporting and to ensure that any changes made after the pathology contribution has been locked are recorded, and that an audit trail for the changes, including who made them and when, is kept. These measures are set out in various sector-specific documents such as the US FDA 21 CFR Part 11 (Electronic Records/Signatures) and the EU Pharmaceutical Inspection Cooperation Scheme (PIC/S) PI 011. These security controls include:

limited user access, single and/or multiple password requirements, procedural controls and technical controls built into the systems. A critical requirement of an audit trail is a record of any change to the data.

51. Archiving of documents and specimens generated during a non-clinical laboratory study is a basic Good Laboratory Practice (GLP) requirement. The records and material that should be archived as well as the characteristics and the organisation of archive facilities are addressed in the OECD series on Principles of Good Laboratory Practice No. 1 (1997). Each kind of material has its own individual requirements for proper storage and may have different retention times. Materials should be retained for the period specified by the appropriate authorities.

References

Abdo KM, Kari FW (1997). The sensitivity of the NTP bioassay for carcinogen hazard evaluation can be modulated by dietary restriction. *Exp Toxic Pathol* 48: 129–137.

Bahnemann R, Jacobs M, Karbe E, Kaufmann W, Morawietz G, Nolte T, Rittinghausen S (1995) RITA – Registry of Industrial Toxicology Animal-data – Guides for organ sampling and trimming procedures in rats. *Exp Toxic Pathol* 47: 247–266.

Boorman GA & Eustis SL (1986) The pathology working group as a means for assuring pathology quality in toxicological studies. In: Hoover BK, Baldwin JK, Uelner AF, Whitmire CE, Davies CL & Bristol DW (Eds) *Managing conduct and data quality of toxicology studies*, pp. 271–275. Princeton Scientific Publishing Co., Princeton, N.J.

Bucci TJ (1991) Evaluation of altered morphology. In: Hascheck WM & Rousseaux CG (Eds) *Handbook of Toxicologic Pathology*, ch. 3, pp. 23–25. Academic Press, San Diego, Cal.

Cammermeyer J (1978). Is the solitary dark neuron a manifestation of postmortem trauma to the brain inadequately fixed by perfusion? *Histochemistry* 56: 97-115.

Chhabra RS & Fouts JR (1974) Sex differences in the metabolism of xenobiotics by extrahepatic tissues in rats. *Drug Metab Dispos* 6: 375–379.

Christian MS, Hoberman AM, Johnson MD, Brown WR & Bucci TJ (1998) Effect of dietary optimization on growth, survival, tumor incidences and clinical pathology parameters in CD Sprague–Dawley and Fischer 344 rats: a 104-week study. *Drug Chem Toxicol* 21: 97–117.

Darke PGG, Donagura JD, Kelly DF (1996) *Color atlas of veterinary cardiology*. Mosby, St Louis

Dayton PG & Sanders JE (1983) Dose-dependent pharmacokinetics: Emphasis on phase I metabolism. *Drug Metab Rev* 14: 347–405.

Eastin WC & Tennant RW (1998) In: Kitchin KT (Ed) *Carcinogenicity: Testing Predicting & Interpreting Chemical Effects*, pp. 395–410. Marcel Dekker Inc., New York, N.Y.

Eastin WC (1998) The U.S. National Toxicology Program evaluation of transgenic mice as predictive models for identifying carcinogens. *Environ Health Perspect* 106(Sup. 1): 81–84.

- Eighmy, J. J. (1996). "Study Pathologist Perspective of Pathology Peer Review." *Toxicol Pathol* **24**(5): 647–649.
- Falkeholm L, Grant CA, Magnusson A, Möller E. Xylene-free method for histological preparation: a multicentre evaluation. *Lab Invest*. 2001; 81:1213-21.
- Fix, AS & Garman, RH (2000). Practical Aspects of Neuropathology: A Technical Guide for Working with the Nervous System. *Toxicol Pathol* 2000; 28; 122
- Goodman, D. G. (1988). "Factors Affecting Histopathologic Interpretation of Toxicity-Carcinogenicity Studies." *Carcinogenicity: The Design, Analysis, and Interpretation of Long-Term Animal Studies*. ILSI Monographs, Springer-Verlag, New York.
- Greaves P (2009). General and Applied Toxicology, 3rd Edition, Wiley Press, Editors Ballantyne, B, Marrs, T, Syversen, T. (ISBN: 978-0-470-72327-2)
- Hart RW, Newmann DA, Robertson RT (eds.) (1995). Dietary Restriction: Implications for the Design and Interpretation of Toxicity and Carcinogenicity Studies. ILSI Press, Was, DC.
- Hausmann R, Bock H, Biermann T, et al (2004) Influence of lung fixation technique on the state of alveolar expansion—a histomorphometrical study. *Leg Med (Tokyo)* 6:61–65
- Hayward JJ, Shane BS, Tindall KR & Cunningham ML (1995) Differential in vivo mutagenicity of the carcinogen–noncarcinogen pair 2,4- and 2,6-diaminotoluene. *Carcinogenesis* 16: 2429–2433.
- Hewitt SM, Lewis FA, Cao Y, Conrad RC, Cronin M, Danenberg KD, Goralski TJ, Langmore JP, Raja RG, Williams PM, Palma JF, Warrington JA. Tissue handling and specimen preparation in surgical pathology: issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue. *Arch Pathol Lab Med*. 2008; 132:1929-35.
- House, D. E., E. Berman, J. C. Seely, and J. E. Simmons (1992). "Comparison of Open and Blind Histopathologic Evaluation of Hepatic Lesions." *Toxicol Lett* **63**: 127–133.
- IARC (1992–1997) International Classification of Rodent Tumours, Part 1. The Rat (set of 10 fascicles). Mohr U (Ed). IARC Scientific Publications 122, Lyon.
- Iatropoulos, M. J. (1984). "Editorial." *Toxicol Pathol* **12**(4): 305–306.
- Keenan, KP, Ballam GC, Soper KA, Laroque P, Coleman JB, Dixit R (1999). Diet, caloric restriction, and the rodent bioassay. *Toxicol Sci* 52 (Suppl): 24–34.
- Keenan, KP, Coleman JB, McCoy CL, Hoe C-M, Soper KA, Laroque P (2000). Chronic nephropathy in ad libitum overfed Sprague-Dawley rats and its early attenuation by increasing degrees of dietary (caloric) restriction to control growth. *Toxicol Pathol* 28: 788–798.
- Keenan, KP, Laroque P, Ballam GC, Soper KA, Dixit R, Mattson BA, Adams SP, Coleman JB (1996). The effects of diet, ad libitum overfeeding, and moderate dietary restriction on rodent bioassay: The uncontrolled variable in safety assessment. *Toxicol Pathol* 24: 757–768.

Kemi, M, Keenan KP, McCoy C, Hoe C-M, Soper KA, Ballam GC, vanZwieten MJ (2000). The relative protective effects of moderate dietary restriction versus dietary modification on spontaneous cardiomyopathy in male Sprague-Dawley rats. *Toxicol Pathol* 28: 285–296.

Kittel, B., Ruehl-Fehlert, C., Morawietz, G., Klapwijk, J., Elwell, M. R., Lenz, B., O'sullivan, M. G., Roth, D. R. & Wadsworth, P. F. (2004) Revised guides for organ sampling and trimming in rats and mice - Part 2 - A joint publication of the RITA and NACAD groups. *Experimental and Toxicologic Pathology*, 55, 413-431.

Kodell, R. L., Blackwell, B.-N., Bucci, T. J. & Greenman, D. L. (1995) Cause-of-death assignment at the National Center for Toxicological Research. *Toxicologic Pathology*, 23, 241-247.

Krinke GJ, Perrin LPA & Hess R (1991) Assessment of toxicopathological effects in ageing laboratory rodents. *Arch Toxicol (Sup. 14)* 43–49.

Latendresse, J.R., Warbritton, A.R., Jonassen, H. & Creasy, D.M. Fixation of testes and eyes using a modified Davidson's fluid: Comparison with Bouin's fluid and conventional Davidson's fluid. *Toxicologic Pathology* 30, 524-533 (2002).

Levy G (1968) Dose dependent effects in pharmacokinetics. In: Important Fundamental Principles in Drug Evaluation, pp. 141–172.

Mann, P. C. (1996). "Pathology Peer Review from the Perspective of an External Peer Review Pathologist." *Toxicol Pathol* 24(5): 650–653.

McInnes, E. (2005). Artefacts in histopathology. *Comp Clin Path* 13: 100–108.

Mohr U & Morawietz G (1995) Recent progress in the field of toxicologic pathology in Europe and the need for standardisation. *J Toxicol Pathol* 8: 219–226.

Mohr U, (1999) RITA – Registry of Industrial Toxicology Animal-data: Optimization of carcinogenicity bioassays. *Exp Toxic Pathol* 51: 461–475 (editorial).

Morawietz G, Rittinghausen S, Mohr U (1992) RITA – Registry of Industrial Toxicology Animal-data – Progress of the working group. *Exp Toxic Pathol* 44: 301–309.

Morawietz, G., Ruehl-Fehlert, C., Kittel, B., Bube, A., Keane, K., Halm, S., Heuser, A. & Hellmann, J. (2004) Revised guides for organ sampling and trimming in rats and mice - Part 3 - A joint publication of the RITA and NACAD groups. *Experimental and Toxicologic Pathology*, 55, 433-449.

Morton, D., Kemp, R.K., Francke-Carroll, S., Jensen, K., McCartney, J., Monticello, T.M., Perry, R., Pulido, O., Roome, N., Schafer, K., Sellers, R. & Snyder, P.W. Best practices for reporting pathology interpretations within GLP toxicology studies. *Toxicologic Pathology* 34, 806-809 (2006).

Munro IC (1977) Considerations in chronic toxicity testing: The chemical, the dose, the design. *J Environ Path Toxicol* 1: 183–197.

Nassiri M, Ramos S, Zohourian H, Vincek V, Morales AR, Nadji M. Preservation of biomolecules in breast cancer tissue by a formalin-free histology system. *BMC Clinical Pathology* 2008, 8:1doi:10.1186/1472-6890-8-1

National Academy of Sciences/National Research Council (1983) Risk Assessment in the Federal Government: Managing the Process. National Academy Press, Washington, D.C.

National Academy of Sciences/National Research Council (1994) Science and Judgement in Risk Assessment. National Academy Press, Washington, D.C.

Newberne, P. M. and F. A. de la Iglesia (1985). "Editorial: Philosophy of Blind Slide Reading." *Toxicol Pathol* **13**(4): 255.

OECD Environment, Health and Safety Publications (2002). Series on Testing and Assessment No. 35 and Series on Pesticides No. 14 Guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies

OECD (1981a) Guideline 451. Carcinogenicity studies (17 pages; adopted 12 May 1981). In: OECD Guidelines for the Testing of Chemicals (1993) Section 4: Health effects. Vol. 2. Organisation for Economic Cooperation & Development, Paris.

OECD (1998e) Guideline 424. Neurotoxicity study in rodents (15 pages; adopted 21 July 1997).

OECD (2000) Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Organisation for Economic Cooperation & Development, Paris. <http://www.oecd.org/ehs/test/monoweb19.doc>

Pearson GR, Logan EF (1978) The rate of development of post-mortem artefact in the small intestine of neonatal calves. *Br J Exp Pathol* **59**:178–182

Peters, T. S. (1996). "Pathology Peer Review—A Concept for Consideration." *Toxicol Pathol* **24**(5): 654–656.

Peto R, Pike MC, Day NE, Gray RG, Lee PN, Parish S, Peto J, Richards S, and Wahrendorf J (1980). Guidelines for simple, sensitive significance tests for carcinogenic effects in long-term animal experiments. In: Long-Term and Short-Term Screening Assays for Carcinogens: A Critical Appraisal (IARC Monographs, Supplement 2), R Montesano, H Bertsch, and L Tomatis (eds). International Agency for Research on Cancer, Lyon, pp. 311-426.

Position of the Society of Toxicologic Pathologists: Documentation of Pathology Peer Review. (1997). *Toxicol Pathol* **25**(6): 655.

Prasse, K., P. Hildebrandt, and D. Dodd (1986). "Letter to the Editor." *Vet Pathol* **23**: 540–541.

Rao, GN (1997). New nonpurified diet (NTP-2000) for rodents in the National Toxicology Program's toxicology and carcinogenesis studies. *J Nutr* **127**: 842S–846S.

Rao, GN, Morris RW, Seely JC (2001). Beneficial effects at NTP-2000 diet on growth, survival and kidney and heart diseases of Fischer 344 rats in chronic studies. *Toxicol sci*

Ruehl-Fehlert, C., Kittel, B., Morawietz, G., Deslex, P., Keenan, C., Mahrt, C. R., Nolte, T., Robinson, M., Stuart, B. P. & Deschl, U. (2003) Revised guides for organ sampling and trimming in rats and mice - Part 1. *Experimental and Toxicologic Pathology*, 55, 91-106.

Sharma RP & Coulombe RA, Jr (1996) Pharmacokinetics and risk assessment. In: Fan AM & Chang LW (Eds) *Toxicology and Risk Assessment*, ch. 7, pp. 81–99. Marcel Dekker Inc., New York, N.Y.

“Society of Toxicologic Pathologists’ Recommendations on Statistical Analysis of Rodent Carcinogenicity Studies (2002). *Toxicol Pathol* 30(3), 415–418.

“Society of Toxicologic Pathologists’ Position Paper on Blinded Slide Reading.” *Toxicologic Pathology* 14(4): 493–494.

Standardized System of Nomenclature and Diagnostic Criteria. *Guides For Toxicologic Pathology*. STP/ARP/AFIP. Washington, DC.

Summers BA, Cummings JF, De Lahunta A (1995) *Veterinary neuropathology*. Mosby Year Book, pp 34–35

Thompson SW, Luna LG (1978) *An atlas of artifacts encountered in the preparation of microscopic tissue sections*. Charles Louis Davis DVM Foundation, Gurnee

Travlos GS, Morris RW, Elwell MR, Duke A, Rosenblum S & Thompson MB (1996) Frequency and relationships of clinical chemistry and liver and kidney histopathology findings in 13-week toxicity studies in rats. *Toxicol* 107: 17–29.

WHO (1987) *IPCS Environmental Health Criteria 70: Principles for the safety assessment of food additives and contaminants in food*. World Health Organisation, Geneva.

OECD GUIDANCE DOCUMENT ON THE DESIGN AND CONDUCT OF CHRONIC TOXICITY AND CARCINOGENICITY STUDIES, SUPPORTING TG 451, 452 AND 453

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This draft was developed in parallel with the revision of Test Guidelines 451, 452 and 453 on carcinogenicity, chronic toxicity and combined carcinogenicity/chronic toxicity studies. Additional chapters will be developed and the Guidance Document will be updated.

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