Etablierung von standardisierten Probennahmeplänen für Organe und Gewebe porziner Tiermodelle in der biomedizinischen Forschung

von

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1 Einleitung

Tiermodelle spielen in der biomedizinischen Forschung seit jeher eine wichtige Rolle. In vielen Bereichen der Wissenschaft werden vor allem „klassische“ murine Tiermodelle genutzt und sind seit langem bestens etabliert (Aigner *et al.*, 2010). Daneben finden aber auch weitere Tiermodellspezies, wie etwa das Schwein, eine immer größere Beachtung. Aufgrund der dem Menschen sehr ähnlichen Physiologie und Körperkondition stellt das Schwein ein ideales Tiermodell für die Grundlagenforschung, die Erforschung der Pathogenese verschiedener Krankheiten sowie für die Entwicklung und Etablierung neuer Therapien und Medikamente dar (Aigner *et al.*, 2010). Fortschritte auf dem Gebiet gentechnischer Methoden ermöglichten die Erstellung vielversprechender Schweinemodelle unter anderem für monogenetische Erbkrankheiten, metabolische und neurodegenerative Erkrankungen, aber auch für die Krebs- und Xenotransplantationsforschung (Aigner *et al.*, 2010, Gun and Kues, 2014, Klymiuk *et al.*, 2010, Prather *et al.*, 2013, Whitelaw *et al.*, 2016).

Die vorliegende Arbeit befasst sich mit der Entwicklung von standardisierten Probennahmeplänen zur Gewinnung von Organ- und Gewebeproben von Schweinen, um der wachsenden Bedeutung porziner Tiermodelle in der biomedizinischen Forschung und der damit verbundenen Anforderung an Repräsentativität und Reproduzierbarkeit bei der Probengewinnung gerecht zu werden. Im Gegensatz zu Nagetiermodellen wurden für das Schwein bisher keine entsprechenden, standardisierten Probennahmeprotokolle etabliert. Um die Probennahme beim Schwein, vergleichbar mit den bereits in der Toxikopathologie vorhandenen Protokollen zur Probennahme bei Mäusen oder Ratten (Kittel *et al.*, 2004, Morawietz *et al.*, 2004, Ruehl-Fehlert *et al.*, 2003) zu standardisieren und

durch systematische Probennahme repräsentatives und vergleichbares Probenmaterial zu generieren, wurden detaillierte Protokolle zur Probengewinnung eines weit gefassten Spektrums verschiedener Organe, Gewebe und Körperflüssigkeiten erstellt und getestet. Da bei porzinen Tiermodellen im Vergleich zu Nagermodellen oft nur geringere Tierzahlen zur Verfügung stehen und Schweine nicht so einfach, schnell und kostengünstig wie Mäuse oder Ratten für zusätzliche Analysen nachgezüchtet werden können, sind die einzelnen Tiere eines Schweinemodelles und die von ihnen stammenden, beziehungsweise die von ihnen zu generierenden Gewebe- und Organproben, besonders wertvoll. Dies ist insbesondere bei solchen Studien der Fall, in denen Tiere fortgeschrittenen Alters, etwa bei der Erforschung prolongierter Krankheitsverläufe, untersucht werden. Bei der Erstellung der Probennahmeprotokolle wurde daher besonderer Wert auf die optimale Ausnutzung des gesamten Spektrums des in einem einzelnen Schwein vorhandenen und damit potentiell nutzbaren Probenmaterials gelegt. Die Probennahmeprotokolle umfassen die Erstellung von Probenmaterial für ein breites Untersuchungsspektrum, welches sowohl für morphologische als auch für molekularbiologische und biochemische Analysen geeignet ist, und berücksichtigen auch die „vorausschauende“ Gewinnung von Probenmaterial für zunächst nicht geplante, aber eventuell im Laufe der Studie notwendig werdende Analyseverfahren und Untersuchungen. Für jedes Organ und Gewebe wurden unterschiedlich umfangreiche Probennahmeprotokolle (Typ-I, Typ-II und Typ-III) erstellt, welche bezüglich der Auswahl, Anzahl und Prozessierung der zu generierenden Proben an die Anforderungen eines jeweiligen Forschungszweckes angepasst werden können. Zusätzlich werden in den vorliegenden Probennahmeprotokollen für jedes Organ und Gewebe relevante anatomische Grundlagen und Präparationstechniken,

grundlegende Untersuchungsparameter sowie der einzukalkulierende Zeit- und Personalaufwand dargelegt.

Die jüngst im Fachjournal *Toxicologic Pathology* veröffentlichten Probennahmepläne (Publikation siehe Kapitel 3) wurden im Zusammenhang mit der Erstellung der „Munich-MIDY-Pig-Biobank“, einer umfangreichen Gewebe- und Organprobensammlung eines gentechnisch modifizierten diabetischen Schweinemodells, am Institut für Tierpathologie der Ludwig-Maximilians-Universität München entwickelt sowie praktisch getestet und umgesetzt (Abbott, 2015).

2 Wissenschaftlicher Hintergrund

2.1 Schweine als Tiermodelle in der biomedizinischen Forschung

2.1.1 Geschichtlicher Überblick

Aufgrund der Ähnlichkeit zum Menschen in Anatomie, Physiologie und Metabolismus spielen Schweine als Tiermodelle in der biomedizinischen Forschung bereits seit einigen Jahrzehnten eine wichtige und stetig bedeutsamer werdende Rolle (Aigner *et al.*, 2010, Lunney, 2007, Swindle *et al.*, 1994). In der Grundlagenforschung, auch als Alternative für den Einsatz von anderen Großtiermodellen wie zum Beispiel Hunde und Primaten, werden Schweine bereits seit etwa Mitte des letzten Jahrhunderts vor allem dazu genutzt, die physiologischen Funktionen einzelner Organsysteme und die Pathogenese verschiedener Erkrankungen beim Menschen besser verstehen zu können (Prather *et al.*, 2013, Swindle *et al.*, 1994). Ein Beispiel hierfür stellen die sogenannten „Göttingen Minipigs“ dar, welche an der Universität Göttingen in den 1960er Jahren speziell zu Versuchszwecken gezüchtet wurden und bis heute vielfältig in der Forschung genutzt werden (Jeppesen and Skydsgaard, 2015). Auch in der chirurgischen Forschung und in der Ausbildung von medizinischem Fachpersonal werden Schweine bereits seit mehreren Jahrzehnten eingesetzt (Lunney, 2007, Swindle *et al.*, 2012, Swindle *et al.*, 1994). Fortschritte auf dem Gebiet der Gentechnik wurden seit etwa Mitte der 80er Jahre, bei Schweinen hauptsächlich in der Landwirtschaft, beispielsweise zur Optimierung der Fleischproduktion oder im Zusammenhang mit der Widerstandsfähigkeit gegen bestimmte Krankheiten, genutzt (Gun and Kues, 2014, Hammer *et al.*, 1985, Whyte and Prather, 2011). Die Weiterentwicklung der gentechnischen Methoden in den letzten dreißig Jahren und die Sequenzierung des gesamten Schweinegenoms durch Groenen *et al.* im Jahr 2011 (Groenen *et al.*, 2012) ermöglichten die Erstellung von

verschiedenen gentechnisch modifizierten Schweinemodellen und ihre Verwendung in der translationalen medizinischen Forschung (Gun and Kues, 2014, Klymiuk *et al.*, 2015). In Ergänzung zu Nagermodellen konnten in den letzten Jahren zahlreiche „maßgeschneiderte“ gentechnisch modifizierte Schweinemodelle für diverse humane Erkrankungen entwickelt werden (siehe Kapitel 2.1.5.2) (Aigner *et al.*, 2010, Gun and Kues, 2014, Klymiuk *et al.*, 2010, Klymiuk *et al.*, 2013, Klymiuk *et al.*, 2012b, Kurome *et al.*, 2015, Lunney, 2007, Luo *et al.*, 2012, Prather *et al.*, 2013, Renner *et al.*, 2013, Renner *et al.*, 2010, Streckel *et al.*, 2015, Wolf *et al.*, 2014). Obwohl in der biomedizinischen Forschung bis heute hauptsächlich Nager als Tiermodell genutzt werden, etablieren sich porzine Tiermodelle stetig weiter. Diese Schweinemodelle und ihre Nutzung in der Erforschung humaner Erkrankungen sowie der damit verbundenen Entwicklung neuer Therapiemöglichkeiten, versprechen in Zukunft weitreichende Erkenntnisse und Fortschritte in der translationalen Medizin zu liefern (Aigner *et al.*, 2010, Luo *et al.*, 2012).

2.1.2 Vor- und Nachteile porziner Tiermodelle im Vergleich zu anderen Tiermodellspezies

Die vorhandene Ähnlichkeit des Schweines zum Menschen in Anatomie, Physiologie und Metabolismus stellt, im Vergleich zu Nagetieren oder anderen Spezies wie Hunde oder Katzen, den Hauptvorteil der porzinen Tiermodelle in der biomedizinischen Forschung dar. Durch die dem Menschen vergleichbare Größe des Schweines, lassen sich in der Humanmedizin zu verwendende Untersuchungsprozeduren und Behandlungstechniken entwickeln und optimieren und machen so das Schwein zu einem geeigneten Tiermodell in der Chirurgie, bei der Entwicklung bildgebender Verfahren und in zahlreichen weiteren Bereichen (Aigner *et al.*, 2010, Swindle *et al.*, 1994). Die Anatomie und Funktion des

Gastrointestinaltraktes des monogastrisch omnivoren Schweines, die Morphologie des Pankreas, des Herzkreislaufsystems, der Nieren, der Haut und weiterer Organsysteme und Gewebe, sowie die Organentwicklung haben große Ähnlichkeit mit dem Menschen (Lunney, 2007, Swindle *et al.*, 2012). Schweine werden vor allem in Gebieten eingesetzt, in denen der Einsatz muriner Tiermodelle aufgrund ihrer geringen Körpergröße, der kurzen Lebensspanne oder der nicht ausreichenden Widerspiegelung von beim Menschen vorkommenden Krankheitsphänotypen nur mit Einschränkungen möglich ist (Aigner *et al.*, 2010, Luo *et al.*, 2012). Schweine weisen bei bestimmten Erkrankungen dem Menschen sehr ähnliche phänotypische Merkmale und molekulare Pathogenesemechanismen auf, welche beispielsweise für die Erforschung und Entwicklung neuer Medikamente und Therapien von Bedeutung sind (Lunney, 2007, Luo *et al.*, 2012). Ein weiterer, nicht zu vernachlässigender Vorteil porziner Tiermodelle sind die weitaus geringeren ethischen Bedenken bei der Verwendung von Schweinen als Versuchstiere, im Gegensatz zu beispielsweise Primaten oder Hunden (Jeppesen and Skydsgaard, 2015, Kemter and Wolf, 2015). Die im Vergleich zu Primaten oder Hunden früh einsetzende sexuelle Reife, das kurze Generationsintervall, hohe Wurfzahlen und die asaisonale Fortpflanzung erleichtern die effiziente Erstellung und Nutzung von porzinen Tiermodellen nicht nur in reproduktionsmedizinischen Studien (Aigner *et al.*, 2010, Lunney, 2007). Ebenso ist die Infrastruktur der Haltung, Fütterung und des Hygienemanagementes in der Schweinehaltung sehr gut etabliert. Die gentechnischen Modifikationstechniken wurden in den letzten Jahren beim Schwein, im Gegensatz zu Primaten und Hunden, intensiv weiterentwickelt und erfolgreich eingesetzt (Aigner *et al.*, 2010, Lunney, 2007). Die große Ähnlichkeit des porzinen Genoms zu dem des Menschen stellt eine gute Voraussetzung für die Erforschung genetisch bedingter Erkrankungen des

Menschen im Tiermodell Schwein dar (Groenen *et al.*, 2012, Whyte and Prather, 2011).

Die Nachteile der Verwendung von Schweinen als Tiermodelle gegenüber der von Nagern sind vor allem die höheren Kosten und der höhere Arbeitsaufwand, die bei der Entwicklung sowie bei der Aufzucht und Haltung der Tiere entstehen (Aigner *et al.*, 2010). Während Nager auf vergleichbar geringem Raum in Käfigen gehalten werden können, werden für die Haltung von Schweinen größere Stallungen oder Einrichtungen benötigt, welche zudem auch mit höherem Arbeits-, Zeit- und Kostenaufwand verbunden sind (Swindle *et al.*, 1994) (TierSchG[1], SchHaltHygV[2], TierSchNutztV[3], TierSchVersV[4]).

Die im Vergleich zu Nagern höhere Lebenserwartung der Schweine birgt sowohl Vor- als auch Nachteile. Nager haben zwar nur eine relativ kurze Lebensdauer von wenigen Jahren, sind aber leichter zu halten, zu handhaben und schneller nachzuzüchten. Bestimmte Krankheitserscheinungen lassen sich aber erst mit Zunahme des Alters der Tiere leichter mit denen des Menschen vergleichen und ermöglichen beispielsweise die Untersuchung von Spätfolgen langsam progredient verlaufender Erkrankungen im Schweinemodell (Holm *et al.*, 2016).

[1]TierSchG: Tierschutzgesetz in der Fassung der Bekanntmachung vom 18. Mai 2006 (BGBl. I S. 1206, 1313), das zuletzt durch Artikel 8 Absatz 13 des Gesetzes vom 3. Dezember 2015 (BGBl. I S. 2178) geändert worden ist. Ausfertigungsdatum: 24.07.1972

[2]SchHaltHygV: Schweinehaltungshygieneverordnung in der Fassung der Bekanntmachung vom 2. April 2014 (BGBl. I S. 326), die zuletzt durch Artikel 5 der Verordnung vom 29. Dezember 2014 (BGBl. I S. 2481) geändert worden ist.

[3]TierSchNutztV: Tierschutz-Nutztierhaltungsverordnung in der Fassung der Bekanntmachung vom 22. August 2006 (BGBl. I S. 2043), die zuletzt durch Artikel 1 der Verordnung vom 5. Februar 2014 (BGBl. I S. 94) geändert worden ist.

[4]TierSchVersV: Tierschutz-Nutztierhaltungsverordnung in der Fassung der Bekanntmachung vom 22. August 2006 (BGBl. I S. 2043), die zuletzt durch Artikel 1 der Verordnung vom 5. Februar 2014 (BGBl. I S. 94) geändert worden ist.

Die höhere Lebenserwartung von Schweinen (bis zu 15 Jahre) bringt aber im Gegenzug auch erhöhte Haltungskosten mit sich und macht die einzelnen Tiere und das von ihnen gewonnene Probenmaterial sehr wertvoll (Holm *et al.*, 2016). Die Aufzucht und Produktion weiterer Tiere für bestimmte Studienzwecke ist sehr zeitaufwendig und nachgezüchtete Tiere sind nicht wie bei Nagern, schon nach kurzer Zeit wieder verfügbar (Lunney, 2007). Ein weiterer Nachteil von Schweinemodellen im Vergleich zu Nagermodellen stellt zudem der bislang meist nicht genau definierte genetische Hintergrund der verwendeten Schweine-Zuchtlinien dar (siehe auch Kapitel 2.1.3) (Aigner *et al.*, 2010).

2.1.3 Verwendung von Hausschweinen und Minipigs in der biomedizinischen Forschung

2.1.3.1 In der biomedizinischen Forschung verwendete Hausschweinrassen

Phylogenetisch gehören alle Hausschweinrassen, mit inbegriffen die sogenannten Minipigs, als domestizierte Form des Wildschweins zur Unterart *sus scrofa domestica (Linnaeus, Systema Naturae 1758)*. Die wichtigsten Hausschweinrassen, welche in der biomedizinischen Forschung eingesetzt werden sind Pietrain, Duroc, Hampshire und Deutsche Landrasse sowie Hybriden und Kreuzungen dieser Rassen (Smith and Swindle, 2006). Im Gegensatz zu „Labormäusen“ ist der genetische Hintergrund von in der biomedizinischen Forschung genutzten Hausschweinen meist nicht genau definiert (Aigner *et al.*, 2010).

2.1.3.2 In der biomedizinischen Forschung verwendete Minipigs

Minipigs (auch Miniaturschweine genannt) sind speziell auf Kleinwüchsigkeit gezüchtete Hausschweine. Sie werden neben den „normalen“ Hausschweinrassen in der Forschung vielseitig genutzt. Vor allem in der Toxikopathologie werden Minipigs häufiger verwendet als normale Schweinerassen (Aigner *et al.*, 2010, Glerup *et al.*,

2013, Luo *et al.*, 2012). Minipigs weisen gegenüber den großen, schweren Schweinerassen den Vorteil auf langsamer zu wachsen und ein Endgewicht von lediglich 45-100 Kilogramm zu erreichen, was die Sicherheit und den Umgang mit den Tieren und deren Haltung erleichtert und die entstehenden Haltungskosten reduziert (Glerup *et al.*, 2013, Swindle *et al.*, 2012, Swindle *et al.*, 1994). Die Unterschiede zwischen Minipigs und den in der biomedizinischen Forschung verwendeten Hausschweinrassen liegen vor allem in der geringeren Wachstumsrate und der geringeren Größe zu Beginn der sexuellen Reife, nicht jedoch in morphologisch-anatomischen oder physiologischen Unterschieden (Swindle *et al.*, 2012). Minipigs verschiedener Altersstufen und mit definiertem genetischen Hintergrund werden von Firmen kommerziell angeboten und können für verschiedene Studien und Forschungszwecke erworben werden. Hierbei sind auch spezifisch pathogen frei (SPF) gehaltene Minipigs verfügbar (Aigner *et al.*, 2010, Swindle *et al.*, 2012). Die hauptsächlich genutzten Minipig-Rassen sind Yucatan, Hanford, Sinclair und Göttingen Minipigs (Swindle *et al.*, 2012, Swindle *et al.*, 1994). Letztere wurden an der Universität Göttingen in den 1960er Jahren speziell zu Versuchszwecken gezüchtet und verfügen deshalb über einen sehr gut definierten genetischen Hintergrund (Aigner *et al.*, 2010, Glerup *et al.*, 2013, Jeppesen and Skydsgaard, 2015). Minipigs können für Versuchszwecke ähnlich wie Hunde gehalten werden, wachsen aber schneller als diese und eignen sich, aufgrund der frühen Geschlechtsreife im Alter von 4-6 Monaten, sehr gut für Studien, in denen reproduktionstechnische Aspekte im Vordergrund stehen (Swindle *et al.*, 2012). Sie werden aber auch für pharmakologische und toxikologische Forschungszwecke vielseitig eingesetzt (Aigner *et al.*, 2010, Glerup *et al.*, 2013, Swindle *et al.*, 2012). In diesen Studien werden Minipigs, im Gegensatz zu den großen Schweinerassen, unter anderem aufgrund des einfacheren Umgangs, auch in Bezug auf die

verschiedenen Verabreichungswege von Medikamenten, eingesetzt (Glerup *et al.*, 2013, Gun and Kues, 2014). Im Gegensatz zu den Hausschweinen ist die benötigte Wirkstoffmenge bei Medikamententests durch das viel geringere Körpergewicht der Tiere erheblich reduziert, wodurch viele Versuche deutlich kostengünstiger durchzuführen sind (Glerup *et al.*, 2013, Swindle *et al.*, 2012). Von internationalen Zulassungsbehörden wie der European Medicines Agency (EMA), der US Food and Drug Administration (FDA), der International Organisation for Standardisation (ISO), und der Organisation for Economic Co-operation and Development (OECD) sind Minipigs für den Einsatz in toxikologischen und pharmakologischen Studien zugelassen (Aigner *et al.*, 2010, Glerup *et al.*, 2013). Minipigs werden in toxikopathologischen Studien unter anderem für dermale, orale, intravenöse, subkutane, embryofetale und juvenile Toxizitätstests sowie in Wundheilungsstudien verwendet (Glerup *et al.*, 2013). Ein weiteres Beispiel ist das Münchner Miniaturschwein Troll, welches bereits seit mehreren Jahrzehnten in der Melanomforschung genutzt wird. Bei diesen Schweinen konnte eine genetische Komponente bei der Entwicklung von kutanen Pigmentzellanomalien nachgewiesen werden (Müller *et al.*, 1995, Wanke, 2013).

2.1.4 Gentechnische Modifikationstechniken beim Schwein

In den letzten Jahrzehnten haben neue Methoden in der Gentechnik die Entwicklung von „maßgeschneiderten“ Schweinemodellen für die Erforschung humaner Erkrankungen ermöglicht und vorangetrieben (siehe Kapitel 2.1.5.2) (Aigner *et al.*, 2010, Gun and Kues, 2014, Klymiuk *et al.*, 2010, Klymiuk *et al.*, 2012a, Klymiuk *et al.*, 2015, Lunney, 2007, Luo *et al.*, 2012, Renner *et al.*, 2013, Wolf *et al.*, 2014). Die folgenden Abschnitte gewähren einen kurzgefassten allgemeinen Überblick über etablierte Methoden zur Erstellung gentechnisch modifizierter Schweinemodelle. Hierzu zählen die DNA-Mikroinjektion, der spermienvermittelte Gentransfer, der lentivirale Gentransfer, der somatische Zellkerntransfer (sog. „Klonen“) und moderne Verfahren des sogenannten „Genome Editing“. Eine umfassende Übersicht gentechnisch modifizierter Schweinemodelle für verschiedene Erkrankungen sowie zu den gentechnischen Modifikationstechniken, mit welchen diese Modelle erstellt wurden, findet sich in der von Luo *et al.* 2012 veröffentlichten Übersichtsarbeit “Genetically modified pigs for biomedical research“ (Luo *et al.*, 2012).

2.1.4.1 DNA-Mikroinjektion

Die ersten Versuche gentechnischer Modifikation an Säugetieren wurden mithilfe pronukleärer DNA-Mikroinjektion in den 80er Jahren des 20. Jahrhunderts erfolgreich an Mäusen durchgeführt (Aigner *et al.*, 2010, Hammer *et al.*, 1985). Die Erstellung des ersten transgenen Schweines gelang bereits kurze Zeit später mittels Mikroinjektion eines DNA-Konstruktes (Transgenes) in den Vorkern (Pronukleus) einer befruchteten Eizelle (Brem *et al.*, 1985, Hammer *et al.*, 1985, Luo *et al.*, 2012). Bei der Mikroinjektion werden Transgene mithilfe einer dünnen Glaskapillare extrakorporal in den Pronukleus einer befruchteten Eizelle (Zygote) eines hormonell stimulierten, superovulierten Spendertieres übertragen. Im zweiten Schritt werden die

Zygoten dann in den Eileiter synchronisierter Empfängertiere übertragen (Aigner *et al.*, 2010, Luo *et al.*, 2012). Obwohl diese Technik eine relativ geringe Effizienz besitzt, wurden mit ihrer Hilfe viele verschiedene transgene Schweinemodelle entwickelt (Aigner *et al.*, 2010, Luo *et al.*, 2012). Nachteile der Methode sind vor allem eine vergleichsweise geringe Anzahl an transgenen Ferkeln, da das Transgen nicht immer in das Genom integriert wird und die Integration des Transgens bei den transgenen Nachkommen an zufälligen Orten im Genom erfolgt. Ebenso kann es bei den Nachkommen zu einer Mosaikbildung, das heißt zur Integration des Transgens in nur einige Zellen des Organismus, kommen. Bei der Etablierung von Zuchtlinien des gentechnisch modifizierten Tiermodells werden jedoch keimbahntransgene Tiere, also Tiere, welche das Transgen in Ihren Keimzellen (Spermien und Eizellen) tragen und das Transgen an ihre Nachkommenschaft weiter vererben, benötigt (Aigner *et al.*, 2010, Luo *et al.*, 2012, Whyte and Prather, 2011).

2.1.4.2 Spermienvermittelter Gentransfer

Der spermienvermittelte Gentransfer beruht auf der Eigenschaft von Spermien, exogene DNA-Moleküle zu binden und diese so bei der Befruchtung in die Eizelle zu übertragen. Spermien werden mit exogenen DNA-Molekülen „geladen“ und dann mittels künstlicher Besamung in den Uterus von geeigneten Empfängertieren übertragen (Aigner *et al.*, 2010, Luo *et al.*, 2012). Ebenso ist eine intrazytoplasmatische Injektion der Spermien (intracytoplasmic sperm injection, ICSI) in die Eizelle (in vitro) möglich (Kurome *et al.*, 2006). Vorteil des spermienvermittelten Gentransfers ist die relativ kostengünstige Durchführung sowie der Verzicht auf den Umgang mit Embryonen (Luo *et al.*, 2012). Die Nachteile sind mit denen der DNA-Mikroinjektion (siehe Kapitel 2.1.4.1) vergleichbar.

2.1.4.3 Lentiviraler Gentransfer

Beim lentiviralen Gentransfer werden Transgene mithilfe von Lentiviren, aus der Familie der Retroviren, in die Zielzellen eingeschleust (Aigner *et al.*, 2010). In den transfizierten Zellen wird die lentivirale RNA revers in DNA transkribiert und dann in das Genom der Zelle integriert. Beim Schwein wird diese Methode häufig eingesetzt und führt zu einer relativ hohen Anzahl an transgenen Ferkeln in einem Wurf (Aigner *et al.*, 2010). Lentiviren können ihr virales Genom auch in nicht teilungsfähige Zellen integrieren, wodurch sich ein breites Anwendungsgebiet ergibt und die Mosaikbildung reduziert wird (Aigner *et al.*, 2010). Die Einschränkungen, die DNA-Mikroinjektion, spermienvermittelter sowie lentiviraler Gentransfer gemeinsam haben, sind vor allem die fehlende Möglichkeit einer Voruntersuchung auf eine erfolgte Integration des Transgens in das Genom und die daraus resultierende relativ geringe Anzahl an transgenen Ferkeln in einem Wurf sowie die zufällige Integration des Transgens an unterschiedlichen Stellen des Genoms und in unterschiedlicher Anzahl (Aigner *et al.*, 2010, Luo *et al.*, 2012, Whyte and Prather, 2011).

2.1.4.4 Somatischer Zellkerntransfer

Somatischer Zellkerntransfer (Klonen) ist eine Form der asexuellen Generierung von genetisch identischen Organismen (Kurome *et al.*, 2015, Luo *et al.*, 2012). Die Technik des somatischen Zellkerntransfer in Verbindung mit dem Ziel einer gentechnischen Modifikation in den erzeugten Nachkommen umfasst sechs Schritte: (1) Entkernung (Enukleation) einer Oozyte durch Mikromanipulation, (2) gentechnische Modifizierung von somatischen Spenderzellen (z.B. Fibroblasten), (3) Transfer des Zellkernes der Spenderzelle durch Mikroinjektion in die enukleierte Oozyte, (4) Elektrofusion und Aktivierung der Oozyten *in vitro*, (5) *in vitro* Kultivierung der rekonstruierten Embryonen, (6) Embryotransfer in synchronisierte

Empfängertiere (Aigner *et al.*, 2010, Luo *et al.*, 2012). Somatischer Zellkerntransfer in Verbindung mit der Nutzung von gentechnisch modifizierten Spenderzellen ist die momentan wichtigste Methode um transgene Schweinmodelle für die biomedizinische Forschung zu erstellen (Kurome *et al.*, 2015, Luo *et al.*, 2012). Die Kerne der Spenderzellen werden durch gezielte Veränderungen im Genom, wie zum Beispiel durch das Einfügen von Transgenen, oder das gezielte „Ausschalten" (knock-out) von bestimmten Genen, gentechnisch modifiziert (Aigner *et al.*, 2010, Prather *et al.*, 2013). Ein großer Vorteil dieser Methode ist es, dass alle generierten Nachkommen/Klone eines gentechnisch modifizierten Schweines dieselbe Genmutation tragen, was durch eine Voruntersuchung der Spenderzellen sichergestellt werden kann. Zusätzlich kommt es beim Klonen nicht zur Mosaikbildung in den Nachkommen (Aigner *et al.*, 2010, Kurome *et al.*, 2015). Das „Klonen" als asexuelle Vermehrungsmethode von gentechnisch modifizierten Schweinen ist zusätzlich bei nicht auf „natürlichem Wege" vermehrungsfähigen Tieren, oder bei Tieren, welche vor Erreichen der Fortpflanzungsreife versterben, von großer Bedeutung (Klymiuk *et al.*, 2015).

2.1.4.5 Moderne Verfahren des sogenannten „Genome Editing"

„Genome Editing" bezeichnet gentechnische Modifikationstechniken, bei welchen Restriktionsenzyme (Endonukleasen) zum Einsatz kommen, welche die DNA der Zielzelle an einer vorher bestimmten Zielsequenz schneiden, um so das Genom an dieser Stelle zu verändern (Whitelaw *et al.*, 2016). Die beim „Genome Editing" verwendeten Systeme bestehen aus einer Domäne, die an einer bestimmten Sequenz der genomischen DNA der Zielzelle bindet, sowie einem Restriktionsenzym, welches die DNA an eben dieser Stelle schneidet. Die Genorte beziehungsweise DNA-Zielsequenzen an denen die Systeme binden, werden dabei im Vorhinein

festgelegt. Die DNA-Doppelstrangbrüche werden mithilfe von zwei natürlichen Reparaturmechanismen, der nicht homologen Endverknüpfung (= non-homologous end joining) und der homologen Rekombination (= homology-directed repair), wieder zusammengefügt. Diese Reparaturmechanismen können dazu genutzt werden, durch gezieltes Ausschalten oder Hinzufügen von DNA-Sequenzen Veränderungen am Schweinegenom vorzunehmen (Klymiuk *et al.*, 2015, Luo *et al.*, 2012, Whitelaw *et al.*, 2016). Die am meisten genutzten „Genome Editing" Verfahren sind „Clustered regularly interspaced palindromic repeats and CRISPR associated 9 nuclease" (CRISPR/Cas9), „Transcription activator-like effector nukleases" (TALENs), und Zinkfingernukleasen (Klymiuk *et al.*, 2015, Luo *et al.*, 2012, Whitelaw *et al.*, 2016). Die CRISPR/Cas9-System stammt aus Bakterien, welche dieses zur Abwehr von Viren nutzen (Klymiuk *et al.*, 2015). Das System besteht aus der CRISPR-Komponente, welche mithilfe einer individuell erstellten Primersequenz (vorderer Teil der RNA, der die DNA-Zielsequenz erkennt) an die DNA-Zielsequenz bindet, und aus der Cas9-Endonuklease, welche den DNA-Doppelstrang an der gewünschten Zielsequenz schneidet (Klymiuk *et al.*, 2015, Whitelaw *et al.*, 2016). Während Zinkfinger und TALENs sehr aufwendige Methoden sind, ist die CRISPR/Cas9-Methode relativ einfach, schnell und kostengünstig durchzuführen und stellt das zurzeit am häufigsten genutzte „Genome Editing" Verfahren dar (Klymiuk *et al.*, 2015, Whitelaw *et al.*, 2016). Mit CRISPR/Cas9 können auch mehrere Mutationen im Genom gleichzeitig und gezielt durchgeführt werden (Klymiuk *et al.*, 2015, Whitelaw *et al.*, 2016).

2.1.5 Einsatzgebiete porziner Tiermodelle in der biomedizinischen Forschung

2.1.5.1 Konventionelle Schweinemodelle

2.1.5.1.1 Schweinemodelle in der Grundlagenforschung

Die anatomische und physiologische Ähnlichkeit des Schweines zum Menschen (siehe Kapitel 2.1.2) stellt den relevantesten Vorteil für den Einsatz porziner Tiermodelle in der Grundlagenforschung dar. Gentechnisch nicht modifizierte Schweine spielen in der Erforschung grundlegender physiologischer, aber auch pathologischer Mechanismen eine wichtige Rolle, aus welchen Erkenntnisse für die Entwicklung neuer Therapien und Medikamente sowie auch für die Entwicklung weiterer transgener Schweinemodelle gewonnen werden können (Lunney, 2007, Swindle *et al.*, 2012). Schweine werden beispielsweise in der Erforschung kardiovaskulärer Erkrankungen wie Atherosklerose, Herzkranzgefäßstörungen, sowie auch in Wundheilungsstudien, der Ophthalmologie, der Reproduktionsmedizin, in der Biomechanik, in toxikologischen und pharmakologischen Studien und im Bereich der Krebs- und Diabetesforschung und auch in ernährungswissenschaftlichen Studien genutzt (Lunney, 2007, Prather *et al.*, 2013, Swindle *et al.*, 2012, Swindle *et al.*, 1994).

2.1.5.1.2 Schweinemodelle in der chirurgischen Forschung

Schweine werden bereits seit Jahrzehnten in der chirurgischen Forschung sowie in der chirurgischen Ausbildung eingesetzt (Lunney, 2007, Swindle *et al.*, 2012, Swindle *et al.*, 1994). Hierbei werden Schweine als Modell sowohl in der Diagnostik, zu Ausbildungszwecken in den grundlegenden Operationstechniken, als auch in der Entwicklung neuer Operationsmethoden genutzt (Swindle *et al.*, 2012, Wolf *et al.*, 2014). Weitere chirurgische Gebiete, in denen Schweine eingesetzt werden, sind Wundheilungsstudien, die plastische oder rekonstruktive Chirurgie und die

Transplantationsmedizin (Swindle *et al.*, 1994). In der Herzkreislauf-Chirurgie werden Schweine aufgrund der dem Menschen sehr ähnlichen Anatomie, häufig eingesetzt und dienen hier vor allem zur Optimierung von Verfahren wie Bypass-, Stent- und Katheteroperationen, aber auch als Modell zur Erprobung von Notfallmaßnahmen (Lunney, 2007, Swindle *et al.*, 1994). Ebenso werden Schweine vielfach als Modelltiere in der Knochen- und Gelenkchirurgie sowie in den damit verbundenen bildgebenden Verfahren eingesetzt (Lunney, 2007, Luo *et al.*, 2012).

2.1.5.2 Gentechnisch modifizierte Schweinemodelle

2.1.5.2.1 Gentechnisch modifizierte Schweinemodelle in der Diabetesforschung

Als Zuckerkrankheit oder Diabetes mellitus wird eine Reihe von Stoffwechselkrankheiten bezeichnet, die durch eine Störung der Regulation des Blutglukosestoffwechsels gekennzeichnet sind (American Diabetes Association, 2014, Gun and Kues, 2014, Wolf *et al.*, 2014). Diese Erkrankungen werden von der American Diabetes Association (ADA) in vier verschiedene Klassen mit weiteren Unterteilungen spezieller Formen gegliedert (American Diabetes Association, 2014, Wolf *et al.*, 2014). Die ersten beiden Klassen werden als Typ-1 und Typ-2 Diabetes mellitus bezeichnet. Typ-1 Diabetes des Menschen führt durch autoimmune oder idiopathische Zerrstörung der insulinproduzierenden Betazellen des endokrinen Pankreas zu einem absoluten Insulinmangel. Typ-2 Diabetes ist gekennzeichnet durch eine Insulinresistenz und einen damit verbundenen relativen Insulinmangel (American Diabetes Association, 2014, Luo *et al.*, 2012, Wolf *et al.*, 2014). Klasse 3 bezeichnet weitere spezielle Typen, unter anderem Erkrankungen auf genetischer Grundlage und als Klasse 4 wird der Schwangerschaftsdiabetes (Gestationsdiabetes) bezeichnet (American Diabetes Association, 2014, Wolf *et al.*, 2014). Diabetes mellitus hat sich in den letzten Jahrzehnten in der Humanmedizin zu

einem Gesundheitsproblem mit bereits heute epidemischen Dimensionen entwickelt, dessen Prävalenz weltweit immer weiter steigt. Herzkreislauferkrankungen, diabetische Retinopathie, diabetische Nephropathie und diabetische Neuropathie gehören zu den gefürchtetsten Langzeitkomplikationen des Diabetes mellitus (American Diabetes Association, 2014, Wolf *et al.*, 2014).

Das Pankreas des Schweines mit seinen exokrinen und endokrinen Anteilen ähnelt dem des Menschen in Größe, Form, Lage und Blutversorgung (Murakami *et al.*, 1997). Porzines Insulin unterscheidet sich in nur einer Aminosäure vom humanen Insulin und wurde deshalb lange Zeit zur Behandlung von Patienten mit Diabetes mellitus eingesetzt (Bromberg and LeRoith, 2006, Wolf *et al.*, 2014). Das Schwein stellt durch diese physiologische und anatomische Ähnlichkeit einen Modellorganismus dar, der in der Lage ist, die Lücke zwischen den bereits etablierten transgenen Mäusemodellen und dem humanen Patienten zu schließen (Wolf *et al.*, 2014). Für diese Forschungszwecke wurden bereits mehrere transgene Schweinemodelle mit unterschiedlichen Formen des Diabetes mellitus entwickelt und charakterisiert, wie beispielsweise INS^{C94Y} transgene Schweine (Renner *et al.*, 2013, Wolf *et al.*, 2014). Diese entwickeln durch eine Mutation im Insulingen einen permanenten neonatalen Diabetes, der beim Menschen als MIDY (mutant INS gene-induced diabetes of the youth) bezeichnet wird (Liu *et al.*, 2010). Das genannte Modell und weitere diabetische Schweinemodelle wurden zum Teil bereits erfolgreich in Therapiestudien verwendet und stellen vielversprechende Modelle in der Diabetesforschung dar (Aigner *et al.*, 2010, Renner *et al.*, 2016, Renner *et al.*, 2013, Streckel *et al.*, 2015, Wolf *et al.*, 2014).

2.1.5.2.2 Gentechnisch modifizierte Schweinemodelle neurodegenerativer Erkrankungen

Neurodegenerative Erkrankungen zeichnen sich durch einen langsam fortschreitenden Krankheitsverlauf mit dem Verlust von Nervenzellen in meist anatomisch zusammenhängenden, funktionellen Gebieten des Nervensystems aus. Dies führt zu neurologischen Symptomen, welche sich meist in motorischen und kognitiven Defiziten äußern (Holm *et al.*, 2016). Die anatomische und physiologische Ähnlichkeit des Schweines zum Menschen findet sich auch im Bereich des Nervensystems wieder (Holm *et al.*, 2016, Luo *et al.*, 2012). Für einige neurodegenerative Erkrankungen wurden bereits murine Tiermodelle erstellt, wobei diese die Pathogenitätsmechanismen und Krankheitsphänotypen beim Menschen meist nur ansatzweise widerspiegeln (Holm *et al.*, 2016). Die Lücke zwischen den Nagermodellen und dem Menschen soll mithilfe von Schweinemodellen geschlossen werden, um die Erforschung der Pathogenese, die Diagnostik, die Prävention und die Weiterentwicklung von Therapiemöglichkeiten dieser Erkrankungen weiter voran zu bringen (Holm *et al.*, 2016). Für Krankheiten wie Morbus Alzheimer, Chorea Huntington, Morbus Parkinson und spinale Muskelatrophie wurden bereits transgene Schweinemodelle etabliert (Holm *et al.*, 2016, Luo *et al.*, 2012). Diese sollen die oft multifaktoriellen humanen Erkrankungen bestmöglich rekapitulieren, das heißt sowohl die Symptome als auch die Läsionen des Nervensystems sollen denen des Menschen weitest möglich entsprechen (Holm *et al.*, 2016). Die relativ große Lebensspanne von Schweinen, im Unterschied zu Nagern, kann hierbei eine wichtige Rolle spielen, da sich Erkrankungen wie Morbus Alzheimer und Morbus Parkinson meist erst im fortgeschrittenen Alter entwickeln. Ziel der Forschung ist es, Schweinemodelle mit multiplen gentechnischen Modifikationen und ausreichend

großer Lebensspanne zu entwickeln, um die Durchführung der notwendigen Untersuchungen über längere Zeiträume hinweg zu ermöglichen (Holm *et al.*, 2016).

2.1.5.2.3 Gentechnisch modifizierte Schweinemodelle in der Krebsforschung

Krebserkrankungen umfassen eine Gruppe von mehr als 100 verschiedenen Erkrankungen, die durch das unkontrollierte Wachstum von Zellen charakterisiert sind (Gun and Kues, 2014, Prather *et al.*, 2013). Ihre frühzeitige Diagnose und Therapie, spielen aufgrund ihrer hohen Morbidität und Mortalität in der Humanmedizin eine herausragende Rolle (Flisikowska *et al.*, 2014, Prather *et al.*, 2013). Während in der Grundlagenforschung Mäuse als Tiermodell einen wichtigen Beitrag leisten und dazu beitragen die zugrundeliegenden molekularen Mechanismen dieser neoplastischen Erkrankungen zu verstehen, ist es nun Ziel der Forschung mithilfe von Schweinemodellen die vorklinischen Studien und deren Erkenntnisse mit der klinisch angewandten Onkologie zu verbinden (Cheon and Orsulic, 2011). Hierbei können die Schweinemodelle vor allem zur Entwicklung und Validierung von Therapien und Medikamenten beitragen (Flisikowska *et al.*, 2013). Gentechnisch modifizierte porzine Modelle wurden bereits für Brustkrebs, für die familiäre adenomatöse Polyptosis, eine erblich bedingten Form des colorektalen Karzinoms, und für das ebenfalls erblich bedingte Li-Fraumeni-Syndrom entwickelt (Flisikowska *et al.*, 2014, Gun and Kues, 2014, Luo *et al.*, 2012).

2.1.5.2.4 Gentechnisch modifizierte Schweinemodelle für seltene, monogenetische Erbkrankheiten des Menschen

Fortschritte der gentechnischen Modifikationstechniken beim Schwein ermöglichten auch die Erstellung von „maßgeschneiderten“ porzinen Modellen für seltene, monogenetische Erbkrankheiten des Menschen, wie zum Beispiel für die zystische Fibrose oder die progressive Muskeldystrophie Duchenne.

Zystische Fibrose bezeichnet eine autosomal rezessiv vererbte Stoffwechselerkrankung, der ein durch Mutation bedingter Defekt von Chloridkanälen zugrunde liegt, was unter anderem zu einer veränderten Zusammensetzung von Körpersekreten führt (Gun and Kues, 2014, Klymiuk *et al.*, 2015, Prather *et al.*, 2013). Muköse Sekrete werden zähflüssig und führen so zu Funktionsstörungen der betroffenen Organe (Whyte and Prather, 2011). Bei der zystischen Fibrose handelt es sich um eine multisystemische Erkrankung, die sowohl die Atemwege, den Gastrointestinaltrakt, den Pankreas und die Leber sowie auch den Reproduktionstrakt betreffen kann (Klymiuk *et al.*, 2015). Chronische Infektionen und Entzündungen, vor allem in der Lunge, sind der Hauptgrund für die hohe Sterblichkeitsrate der betroffenen Patienten (Klymiuk *et al.*, 2015, Whyte and Prather, 2011). Im Gegensatz zu den transgenen Mäusen, die als Tiermodell für die zystische Fibrose entwickelt wurden, jedoch keine typischen Symptome zeigen, entwickeln gentechnisch modifizierte Schweine ein dem Menschen in einigen Aspekten sehr ähnliches Krankheitsbild bereits kurz nach der Geburt (Gun and Kues, 2014, Klymiuk *et al.*, 2015, Prather *et al.*, 2013). Aufgrund dieser Ähnlichkeiten zum Menschen, kann das Schwein als Tiermodell der zystischen Fibrose die Erforschung der Pathogenese sowie die damit verbundene Entwicklung von Therapiemöglichkeiten weiter vorantreiben (Aigner *et al.*, 2010, Klymiuk *et al.*, 2015, Prather *et al.*, 2013).

Die progressive Muskeldystrophie Duchenne (DMD) ist eine rezessive X-chromosomale Erbkrankheit, die durch eine Genmutation zu einem Mangel des Muskelproteins Dystrophin führt (Klymiuk *et al.*, 2015). Die betroffenen Patienten leiden unter fortschreitender Muskelschwäche und Muskelschwund. Die Krankheit endet meist im jungen Erwachsenenalter aufgrund des Versagens von Herz- und Atemmuskulatur tödlich (Klymiuk *et al.*, 2015). Um die Erkrankung am Tiermodell weiter zu untersuchen, sind sowohl gentechnisch modifizierte Maus- als auch Schweinemodelle für die Muskeldystrophie Duchenne etabliert worden. Während die etablierten Mausmodelle mit Ausnahme des Zwerchfells keinen mit dem Menschen vergleichbaren Muskelschwund entwickeln und eine fast normale Lebenserwartung besitzen, zeigen gentechnisch modifizierte Schweine, die eine bei humanen Duchenne-Patienten häufig vorkommende Veränderung des DMD Genes tragen, welche zur Ausbildung eines funktionslosen DMD Proteins führt, eine durch Dystrophinmangel bedingte Skeletmuskeldystrophie, welcher mit progredient eingeschränkter Bewegungsfähigkeit und Schwäche einhergeht (Klymiuk *et al.*, 2015). Die pathologischen und histopathologischen Veränderungen entsprechen der humanen Erkrankung, zeigen im Schweinemodell jedoch einen deutlich schnelleren Verlauf (Klymiuk *et al.*, 2015). Das Potential des porzinen DMD-Tiermodells liegt auch hier in der Entwicklung und Erprobung neuer Therapieansätze, wie beispielsweise dem sogenannten Exon skipping, einer posttranskriptionalen Modifikation der defekten DMD-mRNA durch alternatives Splicing, durch Verwendung mutationsspezifischer antisense Oligonukleotide, mit dem Ziel, den Leserahmen des Transkriptes so zu verändern, dass ein verkürztes, aber nicht vollständig funktionsloses Dystrophinprotein gebildet wird (Fairclough *et al.*, 2013, Jirka *et al.*, 2015).

2.1.5.2.5 Gentechnisch modifizierte Schweine in der Xenotransplantationsforschung

Der in der Transplantationsmedizin immer gravierender werdende Mangel an geeigneten Spenderorganen und die Zunahme der Anzahl an Patienten, die ein solches Spenderorgan dringend benötigen, könnte mithilfe der sogenannten Xenotransplantation von Schweineorganen in den Menschen reduziert oder sogar behoben werden (Cooper *et al.*, 2016, Gun and Kues, 2014, Klymiuk *et al.*, 2010, Luo *et al.*, 2012, Prather *et al.*, 2013). Minipigs scheinen aufgrund ihrer dem Menschen ähnlichen Organgröße hierbei am besten geeignet zu sein (Klymiuk *et al.*, 2010).

Transplantatabstoßungsreaktionen bei immunologisch vom Empfängerorganismus als fremd erkannten Organen stellen die größte Hürde für die Verwendung von vom Schwein stammenden Xenotransplantaten dar (Cooper *et al.*, 2016, Klymiuk *et al.*, 2010). Um das Problem der Abstoßungsreaktionen zu überwinden, wurden bereits verschiedene gentechnisch veränderte Schweine mit Modifikationen von diversen Faktoren (Antigene, Gerinnungsfaktoren und Rezeptormoleküle), die für die Abstoßungsreaktionen eine wesentliche Rolle spielen, entwickelt (Cooper *et al.*, 2016, Klymiuk *et al.*, 2010, Wünsch *et al.*, 2014). Die Organe gentechnisch modifizierter Schweine, welche beispielsweise Faktoren wie CD46/CD55/CD59 oder hTM überexprimieren beziehungsweise zum Beispiel GGTA1 defizient sind, werden bisher vor allem bei Primaten wie Pavianen eingesetzt und erprobt (Cooper *et al.*, 2016, Klymiuk *et al.*, 2010, Wünsch *et al.*, 2014). Mittlerweile können auch multiple gentechnische Modifikationen in einem einzigen Schweinemodell umgesetzt werden (Klymiuk *et al.*, 2010, Wünsch *et al.*, 2014). Die Herausforderung dabei ist es, die wichtigsten und effizientesten Modifikationen so zu kombinieren, dass überlebensfähige, geeignete Tiermodelle für die klinischen

Xenotransplantationsforschung entstehen (Cooper *et al.*, 2016, Klymiuk *et al.*, 2010). Die Überlebensdauer von Primaten mit transplantierten Schweineorganen konnte dabei bereits von anfangs wenigen Minuten auf bis zu mehrere Monate gesteigert werden (Cooper *et al.*, 2016). Neben der immunologisch bedingten Transplantatabstoßungsreaktion stellen die noch nicht vollständig sichergestellte normale physiologische Funktion des porzinen Xenotransplantates im Menschen und die Sicherheit bezüglich vom Schwein auf den Menschen übertragbarer Erkrankungen wie zum Beispiel das PER-Virus (= porcine endogenous retrovirus) weitere Hürden in der Entwicklung funktionierender Xenotransplantate vom Schwein dar (Cooper *et al.*, 2016, Klymiuk *et al.*, 2010, Luo *et al.*, 2012).

2.2 Organ- und Gewebeprobennahme bei Tiermodellen in der biomedizinischen Forschung

2.2.1 Grundlegende Überlegungen zur Organ- und Gewebeprobennahme: Untersuchungsziele, Untersuchungsumfang und Analyseverfahren

Die für eine Studie generierten Proben und damit auch die Probennahme selbst stellen in der Regel die Grundlage aller weiterführenden Untersuchungen dar. Bei der Probennahme müssen das Ziel der Studie (Untersuchungszweck), die geplanten Analyseverfahren, für welche die Proben geeignet sein sollen, und die damit verbundenen Anforderungen an das Probenmaterial sowie eine Reihe weiterer Faktoren berücksichtigt und miteinander in Einklang gebracht werden. Im Hinblick auf den Untersuchungszweck ist zu unterscheiden, ob Probenmaterial für orientierende Untersuchungen, wie beispielsweise zur Identifikation von qualitativen morphologischen Organalterationen oder zur Detektion der Expression verschiedener (Trans-)Genprodukte in Organen/Geweben generiert werden soll, oder ob weiterführende Untersuchungen mit speziellen Analyseverfahren an definiertem Probenmaterial durchgeführt werden sollen. Das Spektrum der prinzipiell möglichen Untersuchungen ist außerordentlich breit gefächert. Dieses umfasst unter anderem morphologische feingewebliche und ultrastrukturelle Analysen mit qualitativen und quantitativen Untersuchungsparametern, Expressionsuntersuchungen von DNA, RNA oder Proteinen mittels in-situ-Hybridisierung (ISH), Fluoreszenz-in-situ-Hybridisierung (FISH), Immunhistochemie (IHC), Immunfluoreszenz (IF), nasschemische Analysemethoden wie Western-, Southern-, und Northern-Blotting, holistische Genexpressionsanalysen (Proteom- und Transkriptomanalysen), Metabolomanalysen, Mikrobiomanalysen und epigenetische Untersuchungen (Davis *et al.*, 2013, McDorman *et al.*, 2013). Besondere Proben werden für das Anlegen von

Zell- und Gewebekulturen oder zur Gewinnung bestimmter Gewebestrukturen, wie beispielsweise isolierter Glomerula (Blutke *et al.*, 2011) oder Pankreasinseln (van Buerck *et al.*, 2012), sowie für mikrobiologische (bakteriologische, virologische und mykologische) Untersuchungen oder für bildgebende Verfahren benötigt. Entsprechend dem Untersuchungszweck müssen die Anforderungen, welche die geplanten Analyseverfahren an die zu generierenden Proben stellen, sowie die entsprechenden unterschiedlichen, vom Analyseverfahren abhängigen Prozessierungen der Proben bereits bei der Probennahme berücksichtigt werden. In einer Studie muss also eine Vielzahl von Einflussgrößen und Anforderungen bei der Erstellung von geeignetem Probenmaterial beachtet werden. Hierzu gehören die adäquate Probengröße, die benötigte Anzahl und die Orientierung der Proben sowie auch die Zeit-, Temperatur- und pH-Bedingungen vor, während und nach der Probennahme und die weitere Prozessierung der Proben (z.B. Konservierung durch Einfrieren oder Kühlen bei verschiedenen Temperaturen, Fixierung des Gewebes in verschiedenen Fixantien, Einbettung in verschiedenen Medien und das bei unterschiedlichen Einbettungsmedien auftretende unterschiedliche Ausmaß der Gewebeschrumpfung, etc.) (Adams and Crabbs, 2013, Schneider and Ochs, 2014). Des Weiteren ist der Zeitpunkt der Probengewinnung zu beachten, das heißt ob die Organ- oder Gewebeproben *post mortem* gewonnen werden können oder ob die Proben bereits *in vivo* gewonnen werden müssen (z.B. Blut, Serum oder Plasma) (Adams and Crabbs, 2013). Möglicherweise müssen in einem Probennahmedurchgang verschiedene Proben zur Untersuchung mehrerer unterschiedlicher Fragestellungen generiert werden. Hierbei ist die Kombinierbarkeit mehrerer unterschiedlicher Probennahmestrategien für verschiedene Versuche und Untersuchungen, insbesondere im Hinblick auf die Art und den Umfang der zu generierenden Proben sowie die Reihenfolge der Probennahmen von Bedeutung.

Auch der Zeit- und Arbeitsaufwand, der Personaleinsatz, die äußeren Anforderungen und Einflüsse sowie die Geschwindigkeit der Probennahme müssen mit den Untersuchungszielen einer Studie vereinbar sein (Adams and Crabbs, 2013).

2.2.2 Generelle Anforderungen an Probenmaterial und Probennahmeverfahren in der biomedizinischen Forschung: Repräsentativität, Reproduzierbarkeit und Effizienz

Repräsentativität, Reproduzierbarkeit und Effizienz stellen die wichtigsten Anforderungen, welche an Proben respektive Probennahmeverfahren gestellt werden, dar (Boyce *et al.*, 2010, Gundersen *et al.*, 2013, Howard and Reed, 2005). Während bei sehr kleinen Organen, wie beispielsweise der Nebenschilddrüse oder der Zirbeldrüse, oftmals das gesamte Organ als Probe verwendet wird, können bei großen Organen und Geweben (z.B. Leber, Milz, Skelettmuskulatur) lediglich Teile dieser Organe und Gewebe als Proben verwendet werden. Diese Proben repräsentieren somit eine Teilmenge der „Grundgesamtheit“ beziehungsweise eines Referenzkompartiments im Organ oder Gewebe.

Repräsentativität der Proben bedeutet, dass mit hinreichender Genauigkeit auf Eigenschaften des gesamten zu untersuchenden Organs oder Gewebes (Grundgesamtheit) geschlossen werden kann, aus dem die Proben gewonnen wurden (Boyce *et al.*, 2010, Howard and Reed, 2005). Zur Gewinnung repräsentativer Proben werden Probennahmestrategien angewandt, in denen (I) jede mögliche Probenahmelokalisation des gesamten zu untersuchenden Organs oder Gewebes die gleiche Wahrscheinlichkeit besitzt beprobt zu werden (zufällige Probennahme siehe Kapitel 2.2.4) und (II) eine ausreichend große Anzahl an Proben gewonnen wird (Boyce *et al.*, 2010). Die Probenanzahl ist unter anderem abhängig von der Streuung der Werte des untersuchten Parameters in unterschiedlichen

Kompartimenten des Organs, hängt aber auch von der Varianz der Werte unterschiedlicher Individuen ab. Letztere kann durch eine Erhöhung der Anzahl der zu untersuchenden Tiere reduziert werden (Howard and Reed, 2005). Bei der Gewinnung von repräsentativen Proben gilt es etwaige Unterschiede in der Verteilung der Zielstrukturen in einem Organ zu berücksichtigen. Ein Beispiel hierfür bildet die Dichte der Inseln im Pankreas. Die Dichte der Inseln im Pankreas und die Verteilung der Betazellen innerhalb der Inseln variieren in den verschiedenen Anteilen des Pankreas (Steiner *et al.*, 2010). Würde lediglich eine einzige Lokalisation innerhalb des Pankreas beprobt werden, wäre die Zusammensetzung der Probe nicht repräsentativ für das gesamte Organ. Um ein repräsentatives Ergebnis zu erhalten ist es deshalb notwendig, eine ausreichend große Probenanzahl aus dem gesamten Pankreasvolumen (Referenzkompartiment) zu gewinnen, um die Verteilung und Dichte im gesamten Referenzkompartiment ausreichend widerzuspiegeln. Ebenso sind weitere Eigenschaften der Zielstrukturen wie Polydispersität, Polymorphie und die Vorzugsrichtung (Anisotropie) im jeweiligen Referenzkompartiment bei der Auswahl der Entnahmestellen der Proben und der Orientierung der Proben zu berücksichtigen (siehe Kapitel 2.2.8).

Die **Reproduzierbarkeit** im Zusammenhang mit der Generierung von Probenmaterial bezieht sich auf die Wiederholbarkeit von Ergebnissen bestimmter Parameter unter gleichen vorgegebenen Probennahmebedingungen. Ziel der Probennahme ist dabei mit gleichen Versuchsaufbauten oder -strategien, beispielsweise in unterschiedlichen Laboren oder mit verschiedenen Probennehmern, annähernd gleiche Ergebnisse zu generieren (Howard and Reed, 2005, Rousseaux and Gad, 2013). Die Reproduzierbarkeit von Ergebnissen im Rahmen einer Probennahme stellt die Vergleichbarkeit der Untersuchungen und Ergebnisse zwischen und innerhalb von Studien sicher. Ein gewisses Maß an

Abweichung in Bezug auf die Reproduzierbarkeit einer Untersuchung muss immer mit berücksichtigt werden und lässt sich nicht ausschließen (Rousseaux and Gad, 2013).

Statistische Maße und Parameter im Rahmen einer Probennahmestrategie lassen sich mithilfe stochastischer Mittel berechnen (Gundersen *et al.*, 2013, Nyengaard, 1999, Rousseaux and Gad, 2013):

- Die **Standardabweichung SD** (= Standard Deviation) ist die durchschnittliche Abweichung der Einzelwerte einer Messreihe von ihrem Mittelwert ($\bar{x}$). Sie zeigt dabei die Streuung der einzelnen Werte an und gibt Aufschluss über die Repräsentativität des Mittelwerts der Verteilung. Die Standardabweichung besitzt immer die gleiche Einheit wie die zugrundeliegenden Daten, was eine Auswertung und Interpretation vereinfacht.
- Der **relative Standardfehler des Mittelwerts SEM** (= Standard Error of the Mean) gibt die theoretische Abweichung bzw. Streuung der Mittelwerte an.

  $$\mathbf{SEM = SD/\sqrt{n}}$$

  SD = Standardabweichung

  n = Anzahl der Proben
- Der **Fehlerkoeffizient CE** (= Coefficient of Error), auch relativer Standardfehler genannt, dient zur Bestimmung der Streuung beziehungsweise der Präzision der Ergebnisse in Bezug auf den Mittelwert der Population. Er bestimmt somit die Ungenauigkeit einer Probennahmestrategie. Der Fehlerkoeffizient kann durch Änderungen der Probennahmestrategie, (z.B. durch die Änderung der Anzahl der generierten Proben) verändert werden. (Boyce *et al.*, 2010, Gundersen *et al.*, 2013, Howard and Reed, 2005). Unter Berücksichtigung der jeweiligen Probennahmestrategie ist der CE und damit

verbunden die Effizienz einer Probennahme unterschiedlich zu berechnen (siehe Kapitel 2.2.4).

- Der beobachtete (= observed) Variationskoeffizient (**CV_{obs}**) stellt ein relatives Streuungsmaß, welches die Streuung der Messwerte in Bezug auf den Mittelwert angibt, dar (Boyce *et al.*, 2010, Gundersen *et al.*, 2013).

  $CV_{obs} = SD/\bar{x}$

  CV_{obs} = beobachteter Variationskoeffizient

  SD = Standardabweichung

  $\bar{x}$ = Mittelwert

  Der Variationskoeffizient CV_{obs} der Messwerte ergibt sich im Weiteren aus der biologischen Variation (CV_{biol}) und dem Fehlerkoeffizienten CE (Boyce *et al.*, 2010, Gundersen *et al.*, 2013, Nyengaard, 1999).

  $CV_{obs}^2 = CV_{biol}^2 + CE^2$

  CV_{obs} = beobachteter Variationskoeffizient

  CV_{biol} = biologische Variation

  CE = Fehlerkoeffizient

- Als **biologische Variation** eines Parameters wird die Streuung seiner Werte innerhalb einer Population bezeichnet. Diese ist in der Regel nicht veränderbar. Ein Beispiel für die biologische Variation ist die Schwankung des Körpergewichts innerhalb einer Versuchsgruppe mit Tieren gleichen Alters und Geschlechts. Je größer die biologische Variation zwischen den einzelnen Tieren, umso mehr Tiere werden für eine Untersuchung oder Studie benötigt, damit der beobachtete Variationskoeffizient CV_{obs} kleiner wird (Gundersen *et al.*, 2013, Howard and Reed, 2005). Umgekehrt kann ein hoher CV_{obs} bei

einem Parameter mit einer geringen biologischen Variation nicht durch eine Erhöhung der Anzahl der Proben verringert werden (Nyengaard, 1999).

Ziel einer effizienten Probennahmestrategie sollte die Reduzierung der Anzahl der Probenlokalisationen sein ohne dabei die Präzision des Experiments herabzusetzen (Boyce *et al.*, 2010, Gundersen *et al.*, 2013). In einer Studie sollte ein CV_{obs}-Wert von unter 0,2 (= 20%) erreicht werden (Boyce *et al.*, 2010, Ochs and Mühlfeld, 2013). Der Wert des Fehlerkoeffizient CE sollte dabei etwa die Hälfte des Variationskoeffizienten CV_{obs} betragen, damit die Effizienz der Probennahme und eine ausreichende Präzision sichergestellt werden kann (Boyce *et al.*, 2010, Ochs and Mühlfeld, 2013). Um mithilfe von CV_{biol} und CE die Anzahl der zu untersuchenden Individuen abschätzen zu können, kann vor der Durchführung einer Studie eine Pilotstudie durchgeführt werden, die eine Einschätzung der nicht beeinflussbaren biologischen Variation und dem in der Studie vorliegenden und beeinflussbaren Fehlerkoeffizienten erlaubt. Die Planung der eigentlichen Studie mit einer Anpassung der Probennahmestrategie in Bezug auf die Anzahl Tiere und Anzahl der Probenlokalisationen pro zu untersuchendem Organ erfolgt im Anschluss an die Pilotstudie (Boyce *et al.*, 2010, Gundersen *et al.*, 2013, Ochs and Mühlfeld, 2013).

2.2.3 Konsequenzen der Anforderungen an Proben für die praktische Durchführung einer Probennahme: Probenanzahl, Probengröße, Probenlokalisation, Reihenfolge der Probenentnahme

Für jede Probennahme sind bestimmte Vorgaben, wie beispielsweise die Anzahl und Größe der zu entnehmenden Proben und die Bestimmung der Probenentnahmestellen (Lokalisationen) aus einem Organ oder Gewebe wichtig. Diese Vorgaben sind bei der praktischen Durchführung zu berücksichtigen und ergeben sich aus den generellen Anforderungen an die Probennahme und das Probenmaterial (siehe Kapitel 2.2.2). Die notwendige Probenanzahl, die Probengröße und die Probenlokalisationen sind somit abhängig von den Eigenschaften des zu untersuchenden Organs oder Gewebes, wie zum Beispiel von seiner Größe und Zusammensetzung (Gewebekomposition), aber auch von den zu untersuchenden Parametern und den Anforderungen der Analyseverfahren, welche mit dem gewonnenen Material durchgeführt werden sollen.

Die Probengröße richtet sich grundsätzlich nach der Menge des zur Verfügung stehenden zu beprobenden Organs oder Gewebes und auch nach den Anforderungen des jeweiligen Analyseverfahrens an die Probengröße. Für Letzteres wird die Probengröße beispielsweise durch die unterschiedliche Eindringtiefe verschiedener Fixantien oder die eingeschränkte Kapazität von Einbettungskapseln in der Histologie limitiert. Andererseits kann für bestimmte Untersuchungsverfahren auch ein benötigter Mindestbedarf an Gewebematerial gegeben sein. Ein Beispiel hierfür sind molekularbiologische Analysen.

Die notwendige **Anzahl der zu generierenden Proben**, als ein wichtiges Beurteilungskriterium für die Genauigkeit der Ergebnisse einer Untersuchung, ist abhängig von der Effizienz der Probennahmestrategie und der Repräsentativität des generierten Probenmaterials für das zu untersuchende Organ oder Gewebe (siehe

Kapitel 2.2.2 und Kapitel 2.2.4). Die Anzahl der zu generierenden Proben, um eine aussagekräftige Untersuchung zu gewährleisten, differiert bei unterschiedlichen Probennahmeverfahren. Dabei müssen morphologische Eigenschaften des zu beprobenden Organs oder Gewebes und auch die biologische Variation der untersuchten Parameter innerhalb der zu beprobenden Population berücksichtigt werden.

Bei der Bestimmung der **Probenlokalisationen** sind morphologische Eigenschaften des zu untersuchenden Organs oder Gewebes (Referenzkompartiments) zu berücksichtigen. Hierzu gehören die Form, die Größe, die Vorzugsrichtung (Anisotropie), sowie die Zusammensetzung und Verteilung unterschiedlicher Strukturelemente innerhalb eines Organs oder Gewebes. Etwaige Unterschiede in der Zusammensetzung einer Gewebeart in Abhängigkeit zur Lokalisation im Tierkörper müssen mit berücksichtigt werden. Beispiele hierfür sind die unterschiedliche Zusammensetzung von viszeralem und subkutanem Fettgewebe in den Fettdepots des Körpers oder auch die variierende Hautdicke an verschiedenen Lokalisationen der Körperoberfläche (Ouchi *et al.*, 2011, Turner *et al.*, 2015). Organtropismen und Verabreichungswege bestimmter Substanzen beispielsweise in der Toxikopathologie, aber auch die Prädilektionsstellen bestimmter Erkrankungen grenzen die zu untersuchenden Probenlokalisationen von vorneherein näher ein oder legen diese genau fest (Ruehl-Fehlert *et al.*, 2003).

Die **Reihenfolge der Entnahme** von Probenmaterial ist von mehreren Faktoren abhängig. Hierzu zählen die Analyseverfahren, die mit dem jeweiligen Probenmaterial durchgeführt werden sollen und deren Anforderungen. Dabei muss beispielsweise berücksichtigt werden, ob die Proben sofort auf Trockeneis verbracht und tiefgefroren werden sollen oder ob die Proben in frisch entnommenen Zustand zur weiteren Untersuchung oder Prozessierung benötigt werden. Auch organtypische

Eigenschaften, wie etwa die Geschwindigkeit des Einsetzens postmortaler Alterationen (z.B. die schnell eintretende Autolyse des Pankreas im Vergleich zu beispielsweise Knochen) müssen in diesem Zusammenhang berücksichtigt werden. Organisatorische Notwendigkeiten und Belange im Ablauf einer Sektion, wie etwa die routinemäßige Reihenfolge der Sektion und Organentnahme oder bestimmte spezielle durchzuführende Sektionsmethoden (z.B. die Nierenperfusion, siehe Abschnitt 2.10.6 der Supplemente der Publikation) müssen zusätzlich berücksichtigt werden (Adams and Crabbs, 2013).

2.2.4 Unterschiedliche Probennahmeverfahren

Die Generierung von Organ- oder Gewebeproben kann durch unterschiedliche Probennahmeverfahren erfolgen. Prinzipiell werden nicht-zufällige Probennahmeverfahren von zufälligen Probennahmeverfahren unterschieden (Howard and Reed, 2005, Rousseaux and Gad, 2013). Die Repräsentativität der Proben steht bei der Gewinnung von Probenmaterial dabei immer im Vordergrund. Die generierten Stichproben werden als eine unter bestimmten Gesichtspunkten ausgewählte Teilmenge einer Grundgesamtheit definiert. Hierbei werden im Weiteren Zufallsstichproben, systematische Stichproben und systematische Stichproben mit zufälligem Ausgangspunkt unterschieden (Howard and Reed, 2005, Rousseaux and Gad, 2013). In einer optimalen Probennahmestrategie bei zufälligen Probennahmen besitzt jede mögliche Probenahmelokalisation innerhalb eines bestimmten Referenzkompartiments (Organ oder Gewebe) die gleich große zufällige Chance beprobt zu werden (Gundersen *et al.*, 2013, Nyengaard, 1999). Grundlegendes Ziel bei der Generierung von Probenmaterial sollte dabei immer die Reduzierung der Anzahl der Probenlokalisationen sein, ohne dabei die Präzision des Experiments herabzusetzen (Boyce *et al.*, 2010).

2.2.4.1 Nicht-zufällige Probennahme

Bei nicht-zufällig, willkürlich (= arbitrarily) gewonnenen Proben sind die Probenahmelokalisationen vom Probennehmer vorher festgelegt und/oder werden von Fall zu Fall neu bestimmt. Ein Beispiel für diese Art der Lokalisationsbestimmung in toxikologischen Studien bei murinen Tiermodellen stellen die “Revised guides for organ sampling and trimming in rats and mice – Part 1-3“ dar (Kittel *et al.*, 2004, Morawietz *et al.*, 2004, Ruehl-Fehlert *et al.*, 2003). Die Proben werden immer aus den gleichen vorher definierten Lokalisationen eines bestimmten Organs beziehungsweise Gewebes gewonnen (Ruehl-Fehlert *et al.*, 2003). Der Bestimmung dieser festgelegten Probenlokalisationen liegen vor allem anatomische und pathologische Kenntnisse sowie das Wissen über eventuelle Prädilektionsstellen oder Organtropismen bestimmter Krankheiten oder Stoffe zu Grunde. Hierbei wird sichergestellt, dass alle Proben einer bestimmten Studie oder eines bestimmten Probennahmeprotokolls aus ein und derselben anatomischen Lokalisation stammen, was die standardisierte Untersuchung und Auswertung der Proben vereinfacht (Gundersen *et al.*, 2013). In diesem Zusammenhang wird oftmals neben der Lokalisation auch die Orientierung, die Größe der Probe sowie die weitere Prozessierung definiert, um die Kohärenz und die Aussagekraft der Daten und Ergebnisse sicherzustellen (Ruehl-Fehlert *et al.*, 2003). Dies ist vor allem auch bei größeren Organen, Organen mit inhomogenem Aufbau oder Hohlorganen von Vorteil, da die Probenehmer davon ausgehen, dass die Proben aus einer definierten Lokalisation mit einer definierten Schnittrichtung eine vergleichbarere histologische Auswertung erlauben (Ruehl-Fehlert *et al.*, 2003). Vor allem in toxikologischen Studien ist die Definition bestimmter Probenlokalisationen eine Grundlage für den Vergleich innerhalb der Studie und auch zwischen verschiedenen Studien. Hierbei kann jedoch nicht grundsätzlich davon ausgegangen werden, dass auftretende

Veränderungen gleichmäßig im Organ oder Gewebe auftreten (Gundersen *et al.*, 2013). Ebenso können eventuelle Veränderungen in Volumen, Gewicht oder Größe des Organs, welche Einfluss auf die weitere Auswertung und die Ergebnisse nehmen, nicht ausgeschlossen werden (Gundersen *et al.*, 2013). Der Nachteil der nicht-zufälligen Probennahme ist dabei, dass das so gewonnene Probenmaterial nicht selbstverständlich als repräsentativ für das jeweilige gesamte zu untersuchende Organ oder Gewebe angesehen werden kann und im Weiteren nicht für sämtliche weiterführenden Analyse- und Untersuchungsverfahren geeignet ist (Adams and Crabbs, 2013).

2.2.4.2 Einfache zufällige Probennahme (= independent random sampling)

Im Rahmen der einfachen zufälligen, unabhängigen Probennahme (= independent random sampling), auch als einfache Zufallsstichprobe bezeichnet, werden Proben aus einem Organ oder Gewebe streng zufällig, aus beliebigen Lokalisationen, welche durch den jeweiligen Probennehmer, zum Beispiel mithilfe einer Zufallszahlentabelle festgelegt werden, generiert (Howard and Reed, 2005, Nyengaard and Gundersen, 2006, Rousseaux and Gad, 2013). Jede mögliche Lokalisation innerhalb eines bestimmten Referenzkompartiments, beispielsweise einem Organ oder Gewebe, hat hierbei die gleiche Wahrscheinlichkeit beprobt zu werden (Howard and Reed, 2005, Rousseaux and Gad, 2013). Ein einfaches Beispiel für eine einfache zufällige Probennahme ist die Ziehung der ersten Kugel im Lottospiel, hierbei besitzt jede der Lottokugeln die gleiche zufällige Wahrscheinlichkeit aus der Lottotrommel gezogen zu werden. Ein einfaches praktisches Beispiel aus der Pathologie wäre die für weiterführende Untersuchungen zufällige Auswahl eines Organs bei paarig angelegten Organen durch Münzwurf (o.ä.), wie beispielsweise Auge, Niere, Nebenniere oder Schilddrüse.

Die Berechnung der Effizienz dieser Probennahmestrategie erfolgt mittels Berechnung des Fehlerkoeffizienten CE. Die folgenden Formeln gelten hierbei nur für die einfache, unabhängige Zufallsstichprobennahme, nicht jedoch für systematisch zufällige Probennahmeverfahren. Bei unterschiedlichen systematisch zufälligen Probennahmeverfahren erfolgt die Berechnung des Fehlerkoeffizienten mithilfe komplexer Formeln (siehe Kapitel 2.2.4.3).

$$\mathbf{CE = SEM/\bar{x}}$$

SEM = relativer Standardfehler des Mittelwerts

$\bar{x}$ = Mittelwert

Bei einem einfachen, zufälligen und unabhängigen Probennahmeverfahren mit einer Probenanzahl n, ist der Fehlerkoeffizient CE proportional zum Kehrwert der Quadratwurzel aus der Probenanzahl (Boyce *et al.*, 2010):

$$\mathbf{CE \sim 1/\sqrt{n}}$$

Die **Effizienz** einer Probennahmestrategie wird als Maßstab der Präzision einer Probennahme definiert und wird durch die Streuung der Ergebnisse bestimmt. Die Effizienz einer Probennahmestrategie ist umgekehrt proportional zum Fehlerkoeffizienten CE:

$$\mathbf{Probennahmeeffizienz \sim 1/CE^2}$$

Im Vergleich zur einfachen zufälligen Probennahme ist die Effizienz bei der systematisch zufälligen Probennahme erheblich höher (siehe Kapitel 2.2.4.3) (Boyce *et al.*, 2010, Gundersen and Jensen, 1987).

2.2.4.3 Systematisch zufällige Probennahme (= systematic uniform random sampling)

Unter systematisch zufälliger Probennahme (= systematic uniform random sampling) versteht man eine Probennahmestrategie mit einem systematischen sowie einem zufälligen Anteil (Nyengaard, 1999, Ochs and Mühlfeld, 2013). Die systematisch zufällige Probennahme stellt die bevorzugteste Methode zur zufälligen Bestimmung der zu untersuchenden Lokalisationen für quantitative stereologische Untersuchungen dar (Gundersen *et al.*, 2013). Die erste zu beprobende Lokalisation wird zufällig ausgewählt (zufälliger Anteil der Probennahme) und darauf folgend werden mit konstantem Intervall die weiteren Probenlokalisationen bestimmt (systematischer Anteil der Probennahme) (Gundersen *et al.*, 2013, Nyengaard, 1999). Im Rahmen der systematisch zufälligen Probennahmestrategie besitzt jede mögliche Lokalisation die gleiche zufällige Chance beprobt zu werden (Gundersen *et al.*, 2013, Nyengaard, 1999). In quantitativ-stereologischen Studien werden die Probenahmelokalisationen sowie die Anzahl der zu generierenden Proben mit stochastischen Mitteln, sowie auf der Grundlage der anatomisch-morphologischen und der funktionellen Eigenschaften eines Organs oder Gewebes bestimmt. Die systematisch zufällige Probennahme ist hierbei auf allen Ebenen der Probengewinnung, von der Auswahl der Tiere, der Bestimmung der zu beprobenden Lokalisationen im Organ bis zur Bestimmung der in einem histologischen Schnittpräparat auszuwertenden Areale durchzuführen (Howard and Reed, 2005, Ochs and Mühlfeld, 2013). Die systematisch zufällige Probennahmestrategie vermeidet Fehler bereits bei der Probennahme, reduziert die technisch bedingte Streuung oder Variabilität und steigert effektiv die Präzision des gesamten Experiments (Howard and Reed, 2005).

Die Bestimmung der Präzision in unterschiedlichen systematischen Probennahmeverfahren erfolgt anhand komplexer Formeln (Gundersen and Jensen, 1987, Mattfeldt, 1990).

Bei systematisch zufälliger Probennahme ist der Fehlerkoeffizient CE proportional zum Kehrwert der Probenanzahl (Boyce *et al.*, 2010).

$$CE \sim 1/n$$

Die Effizienz ist damit deutlich höher als bei der einfachen zufälligen Probennahme (siehe Kapitel 2.2.4.2) (Boyce *et al.*, 2010, Gundersen and Jensen, 1987).

2.2.5 Verschiedene systematisch zufällige Probennahmeverfahren in der biomedizinischen Forschung

In der biomedizinischen Forschung wird Probenmaterial für viele unterschiedliche Zwecke und Untersuchungen gewonnen. Eine effiziente systematisch zufällige Probennahmestrategie mit der Gewinnung von repräsentativen Proben bereits im Rahmen der Sektion stellt die Voraussetzung für die Durchführbarkeit der daraus folgenden quantitativen stereologischen Analysen dar (Gundersen *et al.*, 2013, Howard and Reed, 2005, Nyengaard, 1999, Ochs and Mühlfeld, 2013). Hierbei werden mehrere systematisch zufällige Probennahmeverfahren unterschieden. Im Rahmen eines **volumengewichteten Probennahmeverfahrens** werden die Proben aus dem gesamten Volumen des zur Verfügung stehenden Organs oder Gewebes gewonnen. Das Volumen des zu beprobenden Organs oder Gewebes stellt dabei die Bezugsgröße (Referenzkompartiment) für die weiteren Untersuchungen dar. Ein solches volumengewichtetes Probennahmeverfahren kann relativ einfach durchgeführt werden. Das Organ wird entlang der Längsachse orthogonal planparallel äquidistant lamelliert und die Scheiben werden auf die immer gleiche Seite abgelegt. Es folgt eine systematisch zufällige Probennahme mithilfe eines

Punktraster auf der Oberfläche dieser Organscheiben (Howard and Reed, 2005). Durch die gleiche Scheibendicke kann eine volumengewichtete Probennahme sichergestellt werden. Eine detaillierte Darstellung der Durchführung dieses Probennahmeverfahrens mit Schemazeichnung und fotografischen Abbildungen findet sich in den Supplementen der Publikation in Fig. S2 und in Abschnitt 2.10.8 am Beispiel der Nebenniere.

Die sogenannte **Fractionator-Methode** und ihre Abwandlungen (z.B. „smooth fractionator“, „fast fractionator“ u.a.) stellen weitere, noch effizientere systematisch zufällige Probennahmeverfahren dar, welche insbesondere zur Schätzung der Gesamtzahl von Strukturelementen in einem Referenzkompartiment (Organ, Zelle u.a.) verwendet werden können. Das Grundprinzip stellt die systematisch zufällige Auswahl eines kleinen festgelegten Anteils (= fraction) des gesamt zu untersuchenden Gewebes dar, aus welchem dann in der weiteren Untersuchung Rückschlüsse auf Parameter im gesamten Organ oder Gewebe gewonnen werden (Gundersen *et al.*, 2013, Howard and Reed, 2005, Nyengaard, 1999). Vorteile dieser Methoden sind die einfache und schnelle Durchführung und die Unabhängigkeit von der Gewebeschrumpfung. Nachteil ist die in der Regel notwendige komplette Zerstückelung des Organs oder Gewebes, was andere zusätzliche Untersuchungen oftmals nicht mehr ermöglicht (Howard and Reed, 2005, Ochs and Mühlfeld, 2013).

2.2.6 Etablierte Probennahmepläne für Organ- und Gewebeproben in der biomedizinischen Forschung bei Nagetier- und Nicht-Nager-Versuchstierspezies

Grundvoraussetzung für die Interpretation und Validierung von wissenschaftlichen Ergebnissen sind unter anderem die Repräsentativität der Proben, die

Vergleichbarkeit und die Eignung des generierten Probenmaterials und die Standardisierung der Probennahme, sowohl bei der Generierung der Proben, als auch bei der weiteren Prozessierung und Auswertung des Probenmaterials (Ruehl-Fehlert *et al.*, 2003). Für die Probennahme bei murinen Tiermodellen existieren bereits unterschiedliche Vorgaben und Richtlinien von nationalen und internationalen Einrichtungen und Arbeitsgruppen, welche dazu dienen sollen, die Anforderungen an die Probennahme und das Probenmaterial in den einzelnen Studien, aber auch zwischen verschiedenen Studien festzulegen. Diese Vorgaben, die für toxikopathologische, pharmazeutische oder histopathologische Forschungs- und Studienzwecke entwickelt wurden, listen für die Gewinnung von Probenmaterial von Versuchstieren meist die jeweiligen Organe, welche beprobt werden sollen, auf. Welche Lokalisationen oder Kompartimente des Organs dabei untersucht werden sollen, ist in den Richtlinien nicht näher definiert. Eine Ausnahme stellen dabei die von den Arbeitsgruppen der RITA (Registry of Industrial Toxicology Animal-data) und der NACAD (North American Controll Animal Database) veröffentlichten “Revised guides for organ sampling and trimming in rats and mice – Part 1-3“ (Kittel *et al.*, 2004, Morawietz *et al.*, 2004, Ruehl-Fehlert *et al.*, 2003), dar (Ruehl-Fehlert *et al.*, 2003). Anatomische Kenntnisse bezüglich des Aufbaus und der Zusammensetzung verschiedener Organe sind für die Probennahme und eine standardisierte histopathologische Evaluation notwendig und wurden bei der Erstellung der etablierten Richtlinien für Nager beispielsweise für die Festlegung der Probenlokalisationen und der weiteren Prozessierung zugrunde gelegt (Ruehl-Fehlert *et al.*, 2003). Die Einführung dieser Probennahmeplänen für die standardisierte Probennahme muriner Tiermodelle hat erheblich dazu beigetragen, die Qualität und die Vergleichbarkeit der Ergebnisse in und zwischen verschiedenen Studien zu erhöhen (Kittel *et al.*, 2004, Morawietz *et al.*, 2004, Ruehl-Fehlert *et al.*,

2003). Diese Protokolle für Mäuse und Ratten stellen detaillierte und standardisierte Anweisungen dar, welche für die einzelnen Organe genaue Probenahmelokalisationen, Probenmenge, Probenanzahl, Orientierung und Prozessierung vorgeben und mit Schemazeichnungen und fotografischen Abbildungen die Probennahme vereinfachen und die Qualität des histologischen Materials steigern sollen (Ruehl-Fehlert *et al.*, 2003). Auch die Society of Toxicologic Pathology (STP) setzt sich in ihren Positionspapieren zu verschiedenen Organsystemen und in den INHAND (International Harmonization of Nomenclature and Diagnostic Criteria) Publikationen mit den Grundlagen der Probennahme in der Toxikopathologie in Bezug auf verschiedene Organsysteme und Studienziele auseinander. Das Positionspapier von Bregman *et al.* (2003) (Recommended tissue list for histopathologic examination in repeat-dose toxicity and carcinogenicity studies: a proposal of the Society of Toxicologic Pathology (STP)) legt eine Basisliste mit Organen und Geweben vor, die für alle Tierarten und toxikologische sowie auch kanzerogene Studienziele angewendet und modifiziert werden kann. Ziel ist es, Pathologen und anderen Wissenschaftlern eine Grundlage für die zu beprobenden Organe und die daraus resultierende Probennahme in der Toxikopathologie darzulegen (Bregman *et al.*, 2003). Auch staatliche Institutionen, wie die FDA (Food and Drug Administration), die EPA (United States Environmental Protection Agency) oder die EMA (European Medicines Agency) legen in ihren jeweiligen Richtlinien für Toxizitätsstudien eine Liste an Organen für die histopathologische Untersuchung vor. Tabelle 1 im Manuskript der vorliegenden Publikation (Kapitel 3 der Dissertation) verschafft einen Überblick über die jeweiligen zu beprobenden Organe in den Richtlinien der oben genannten Organisationen, im direkten Vergleich mit den entwickelten Probennahmeprotokollen für porzine Tiermodelle.

2.2.7 Quantitative Stereologie

Die Lehre der Stereologie (von griechisch *stereos* = fest, räumlich, körperlich) befasst sich mit der räumlichen Interpretation von Schnittbildern dreidimensionaler Objekte. Sie wird in verschiedenen Fachgebieten der Wissenschaft, beispielsweise in der Geologie bei Mineralienzusammensetzungen, aber auch bei morphologischen Analysen biologischer Proben eingesetzt. Die Bedeutung von stereologischen Untersuchungsansätzen nimmt in der biomedizinischen Forschung in den letzten Jahrzehnten immer weiter zu. Für morphologische Untersuchungen des gewonnenen Probenmaterials sind quantitativ-stereologische Methoden mittlerweile unverzichtbar geworden. Zahlreiche renommierte Fachjournale verlangen inzwischen bei der Publikation quantitativ-morphologischer Ergebnisse die Verwendung von adäquaten stereologischen Methoden (Madsen, 1999). In der Stereologie werden aus zweidimensionalen Abbildungen Rückschlüsse auf die dreidimensionale Struktur von Objekten gewonnen (Boyce *et al.*, 2010, Gundersen *et al.*, 2013, Nyengaard, 1999). In Abgrenzung zur Stereologie befasst sich die Morphometrie mit allen Verfahren zur quantitativen Erforschung morphologischer Strukturen (Mattfeldt, 1990). Stereologische Analysen stellen somit stets dreidimensionale, aber nicht notwendigerweise quantitative Analysen dar. Morphometrische Untersuchungen sind *per se* quantitativer, aber nicht zwingend räumlicher Natur. In der Histopathologie dient die quantitative Stereologie dazu aus den zweidimensionalen histologischen Schnitten quantitative Rückschlüsse auf die dreidimensionalen zugrunde liegenden Strukturen, wie etwa Organe oder Gewebe, zu ziehen (Gundersen *et al.*, 2013, Mattfeldt, 1990, Nyengaard, 1999). Hierbei stellt die quantitative Stereologie eine Kombination aus statistischen Probennahmeprinzipien und geometrischen Analysen der Mikrostruktur des Gewebes dar (Gundersen *et al.*, 2013). Die Stereologie gründet auf Prinzipien der Statistik. Die Anwendbarkeit stereologischer Methoden hängt

daher von der strikten Einhaltung zufälliger Probennahmestrategien ab (Howard and Reed, 2005). Grundvoraussetzung der stereologischen Methoden ist es, repräsentative Proben zu generieren, die die zu untersuchenden Eigenschaften der Gewebe, Organe oder Organkompartimente ausreichend widerspiegeln und berücksichtigen (Gundersen *et al.*, 2013, Howard and Reed, 2005). Quantitativ-stereologische Methoden können in der Histopathologie durch ihre hohe Sensitivität bereits kleinste morphologische Veränderungen, welche subjektiv nicht als solche vom Untersucher erkannt werden können, feststellen (Boyce *et al.*, 2010, Gundersen *et al.*, 2013, Hoeflich *et al.*, 2002, Nyengaard, 1999). Die Ergebnisse aller stereologischen Untersuchungen stellen dabei mathematische Schätzwerte der zu untersuchenden Parameter dar. Man unterscheidet neben den geometrischen Grundgrößen (Länge, Fläche, Oberfläche, Volumen, Anzahl) klassische stereologische Strukturparameter (z.B. Volumendichte) und Partikelparameter (z.B. mittleres Volumen von bestimmten Strukturelementen) (Gundersen *et al.*, 2013, Howard and Reed, 2005, Mattfeldt, 1990, Weibel, 1979).

2.2.8 Orientierung der Proben für quantitativ stereologische Untersuchungen: IUR und VUR Proben

Quantitative morphologische Merkmale können verlässlich und verzerrungsfrei (= unbiased), modellfrei (model independent, auch als design-based bezeichnet) mit quantitativen stereologischen Methoden bestimmt werden (Howard and Reed, 2005). Hierbei müssen die Eigenschaften der untersuchten Gewebe- oder Zellstrukturen, wie Polydispersität, Polymorphie, Inhomogenität und Anistropie berücksichtigt werden. Daher ist die räumliche Orientierung der Probe für die Bestimmbarkeit quantitativ-morphologischer Parameter, wie beispielsweise mittlere, relative und absolute Anzahl, Länge und Oberfläche verschiedener Zellen, von entscheidender

Bedeutung und beeinflusst die Durchführbarkeit einer quantitativ-stereologischen Analyse maßgeblich (Howard and Reed, 2005). Die Anforderungen an die Orientierung der Proben und die weitere Prozessierung (beispielsweise das Einbettungsmedium) sind deshalb je nach zu untersuchenden Parametern und Geweben unterschiedlich und müssen im Vorhinein festgelegt werden (Gundersen *et al.*, 2013, Howard and Reed, 2005).

In **IUR-Schnitten** (= isotropic uniform random) sind die Schnittebenen, die in einer stereologischen Analyse untersucht werden, in allen Ebenen des Raumes zufällig orientiert. Daher können IUR-Schnitte zur Bestimmung aller stereologischen Parameter verwendet werden. In einer Studie muss eine ausreichend große Anzahl an IUR-Proben quantitativ-stereologisch untersucht werden, damit die Eigenschaften (z.B. Form, Größe, Verteilung u.a.) der zu untersuchenden Strukturelemente ermittelt werden können. Ein Nachteil der Verwendung von IUR-Schnitten ist die oft nicht mehr mögliche Orientierung an der Morphologie innerhalb der Probe, durch die völlig zufällige Schnittrichtung (Gundersen *et al.*, 2013, Howard and Reed, 2005, Nyengaard, 1999). IUR-Schnitte kleiner Objekte oder Gewebeproben lassen sich beispielsweise mit der sogenannten Isektor-Methode anfertigen. Mit der als Orientator-Methode bezeichneten Technik können IUR-Schnitte größerer Objekte oder Gewebeproben erstellt werden. Die Herstellung von IUR-Schnitten mit der Isektor- und der Orientator-Methode wird im Folgenden beschrieben:

Die **Isektor-Methode** zur Herstellung von IUR-Schnitten kleiner Proben oder Gewebestücke beginnt mit der Herstellung einer sphärischen und isotropen Form durch Einbettung (z.B. mit Agar) einer kleinen Probe beziehungsweise eines kleinen Gewebestücks in eine kugelförmige Form. Bei der Isektor-Methode wird die Orientierung der Probe selbst randomisiert, indem die isotrope Kugel in einer zufälligen Position (z.B. zufällig erzeugt durch Rollen auf Unterlage) geschnitten wird.

Durch diese Randomisierung der Lage der Probe im Raum entsteht eine isotrope zufällige Schnittfläche (Nyengaard and Gundersen, 1992).

Die **Orientator-Methode** erweist sich bei anisotropen Strukturen als besonders effizient (Mattfeldt, 1990). Bei dieser Methode wird zur Herstellung von IUR-Schnitten von größeren Organ- oder Gewebeproben die gewonnene Probe zweimal in zufällig orientierter Richtung geschnitten, um eine IUR-Schnittebene zu erhalten. Hierbei wird im Gegensatz zum Isektor die Schnittebene der Probe randomisiert (Mattfeldt *et al.*, 1990). Der Orientator stellt eine einfache und schnelle Methode dar, für die kein spezielles Herstellungsmaterial benötigt wird (Nyengaard, 1999).

Ortrips (= orthogonal triplet) sind Schnitte aus drei zueinander rechtwinkligen Schnittflächen, bei der die erste Schnittfläche ein IUR-Schnitt ist. Zuerst wird eine IUR-Schnittfläche einer Gewebeprobe erstellt und dann werden zwei weitere Schnitte im rechten Winkel zueinander und zur ersten Schnittfläche generiert (Mattfeldt *et al.*, 1985).

Für **VUR-Proben** (= vertical uniform random) wird in der gewonnenen Probe eine vertikale Achse durch den Experimentator festgelegt, welche stets wiedererkennbar ist (Baddeley *et al.*, 1986). Dann wird die Probe um diese Achse rotiert und in einem zufälligen Winkel, parallel zu der vertikalen Achse eine Schnittfläche erzeugt. VUR-Proben werden oftmals aus Gewebeproben, welche eine natürliche flache und wiedererkennbare Oberfläche besitzen (z.B. Haut, Schleimhaut), generiert. Hierbei kann eine Achse vertikal zur natürlichen Oberfläche dann bereits als die festgelegte Achse fungieren. Abgesehen von Längendichten können in VUR-Proben alle auch in IUR-Proben bestimmbaren Parameter ermittelt werden. Vorteil dieser Methode ist die verbleibende bekannte Ebene in der Probe und die dadurch mögliche Orientierung anhand der Morphologie. VUR-Proben

eigenen sich insbesondere zur Bestimmung von Oberflächendichten (Baddeley *et al.*, 1986).

Für weiterführende anschauliche detaillierte Erklärungen oben genannter Methoden wird auf die in dieser Arbeit enthaltene Publikation (siehe Kapitel 3) und den in den zugehörigen Supplementen enthaltenen Abschnitt 1.6 verwiesen.

3 Publikation

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Tissue Sampling Guides for Porcine Biomedical Models

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Abstract

This article provides guidelines for organ and tissue sampling adapted to porcine animal models in translational medical research. Detailed protocols for the determination of sampling locations and numbers as well as recommendations on the orientation, size, and trimming direction of samples from ~50 different porcine organs and tissues are provided in the Supplementary Material. The proposed sampling protocols include the generation of samples suitable for subsequent qualitative and quantitative analyses, including cryohistology, paraffin, and plastic histology; immunohistochemistry; *in situ* hybridization; electron microscopy; and quantitative stereology as well as molecular analyses of DNA, RNA, proteins, metabolites, and electrolytes. With regard to the planned extent of sampling efforts, time, and personnel expenses, and dependent upon the scheduled analyses, different protocols are provided. These protocols are adjusted for (I) routine screenings, as used in general toxicity studies or in analyses of gene expression patterns or histopathological organ alterations, (II) advanced analyses of single organs/tissues, and (III) large-scale sampling procedures to be applied in biobank projects. Providing a robust reference for studies of porcine models, the described protocols will ensure the efficiency of sampling, the systematic recovery of high-quality samples representing the entire organ or tissue as well as the intra-/interstudy comparability and reproducibility of results.

Keywords

biobank, biomedical research, minipig, necropsy, organ/specimen collection, pig, systematic random sampling.

Introduction

Pigs are increasingly being used as disease models in translational medicine and as large animal model systems in surgery, transplantation research, and toxicologic pathology (Aigner et al. 2010; Gun and Kues 2014; Lunney 2007; Wuensch et al. 2014). The growing popularity of porcine models in biomedical research is due to several advantageous similarities between pigs and human beings that cannot be reproduced adequately in classical rodent models (Aigner et al. 2010). Due to the proximity to human anatomy, physiology and body dimensions, the comparably short generation interval (1 year), and high fertility rates, pigs are an ideal model organism for basic research and the study of disease mechanisms as well as model organisms for testing novel surgical and pharmacological therapeutic strategies (Aigner et al. 2010). Moreover, sound molecular biological methods for genetic modification of pigs are currently available, allowing for generation of tailored porcine large animal models for diverse human diseases (Aigner et al. 2010; Klymiuk et al. 2010; Klymiuk et al. 2012a; Kurome et al. 2015; Wu et al. 2013). Such genetically modified pig models have successfully been established for cystic fibrosis, diabetes mellitus, Duchenne muscular dystrophy, and other important human diseases (Aigner et al. 2010; Gun and Kues 2014; Klymiuk et al. 2013; Klymiuk et al. 2012b; Lunney 2007; Renner et al. 2013; Wolf et al. 2014). The potential of tailored pig models for testing targeted therapies is outlined by Klymiuk et al. in this issue of *Toxicologic Pathology* (Klymiuk et al. 2015).

Deriving optimal benefit from porcine animal models requires experimental study designs and examination protocols that warrant representative samples, reproducible results, and comparable analyses between different studies and

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investigators. Here, the applied mode of selection of biological samples, including the location, size, number, and orientation, is of great importance because it will affect the results of the subsequent investigations, ranging from histological examinations to molecular profiling analyses. In rodent models, the introduction and broad application of guidelines for the standardized generation of samples have greatly contributed to the quality as well as to the intra- and interstudy comparability of results (Kittel et al. 2004; Morawietz et al. 2004; Ruehl-Fehlert et al. 2003). Comparable sampling guidelines have not been established so far for porcine models.

However, rodent sampling protocols cannot be directly adapted to pig models because there are considerable differences in the anatomy and size of porcine and rodent organs/tissues. In addition to physical and anatomic features, several other important aspects have to be considered in the sampling strategies for porcine animal models. The considerably longer generation interval of pigs compared to rodents, as well as the significantly higher costs, time, and personnel efforts required for the generation of porcine models and for pig husbandry, limits the number of available animals.

Therefore, the individual animals of a respective porcine model and the samples generated from these pigs are particularly valuable, especially if genetically modified pigs and/or long-term experimental issues, such as prolonged disease courses, are to be examined. In the course of any study, additional experiments, which had not been scheduled at the beginning, might later turn out to be relevant. If suitable samples for such additional experiments are not available, they have to be generated from additional animals. Particularly, if aged pigs of genetically modified models are examined, the efforts that have to be deployed for the generation of additional animals are considerably higher than in corresponding rodent models.

In light of the steadily growing relevance of pig models in biomedical research and species-specific differences, the implementation of uniform and standardized protocols for sample generation from porcine organs and tissues applicable to a wide range of subsequent types of analyses is urgently needed to take full advantage of the translational value of porcine animal models. The proposed guidelines will allow the generation of comparable and reproducible high-quality specimens and might reduce the number of animals needed in a study by avoiding the unnecessary sacrifice of valuable animals for the repeated generation of samples (Tornqvist et al. 2014).

Sampling Guides for Porcine Organs and Tissues

In total, sampling protocols for ~50 porcine organs and tissues (see Supplementary Material), adjusted to the expenditures and scopes of the following 3 different study types, are provided:

Type I: Routine screenings for the detection of histopathological organ alterations in new porcine models, studies examining general gene expression patterns in organs/tissues, and general toxicity studies.

Type II: Advanced examinations of distinct organs/tissues, with the generation of a sufficient number of backup samples, suitable for a wide range of diverse analyses, including analyses not specified at the time point of sampling.

Type III: Biobank projects, requiring large-scale sampling procedures to generate high numbers of various different types of samples suitable for as many different types of analyses as possible, taken from a broad spectrum of different organs/tissues.

The respective protocols are designed to fit the demands of the industrial standards of the pharmaceutical industry and toxicologic pathology. They have been developed based on extensive experiences in pig toxicopathology, in pathomorphological characterization of numerous genetically modified pig models, and in porcine animal model biobanking (Abbott 2015; Aigner et al. 2010; Kemter et al. 2012; Klymiuk et al. 2013; Klymiuk et al. 2012a; Klymiuk et al. 2012b; Klymiuk et al. 2012c; Renner et al. 2010, 2012, 2013; Streckel et al. 2015; Wuensch et al. 2014). The proposed sampling protocols are intended as general guidelines but not as requirements for the sampling of tissues in any porcine model. The protocols can generally be applied to the organs/tissues of pigs weighing ~10 to ~400 kg and can be modified accordingly if smaller or younger animals are examined.

In studies of the first type (I) or in experiments that, in addition to a different main experimental task, a broad set of organs/tissues has to be examined in a routine, overview fashion by standard analyses methods, the applied sampling protocols allows for fast, uncomplicated and less elaborate sampling. Therefore, type I study sampling protocols include the collection of a limited number of samples per organ/tissue taken from deliberately defined locations, with uniform sample sizes and predefined orientations/cutting directions of a histological specimen. Type I sampling is considered adequate for the identification of qualitative histopathological changes and general organ-/tissue-specific gene expression patterns in routine studies. If organs/tissues display macroscopically evident pathological alterations, additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered sites, as appropriate.

The list of porcine organs and tissues scheduled for routine examination in type I studies and lists of organs/tissues recommended for pathohistological examination in routine toxicity studies in rodent and nonrodent species by the Society of Toxicologic Pathology (STP) and by different public institutions and regulatory authorities are shown in Table 1. Except for rodent-specific organs, all organs and tissues regularly evaluated in other species are also examined in porcine models. Additionally, the generation of samples for histopathology and molecular analyses is scheduled for some porcine organs/tissues, which are not regularly included in established sampling guidelines for routine toxicity studies. These include organs and anatomical structures that are sampled because they are characteristically well developed in pigs, such as the

Table 1. List of Organs and Tissues Scheduled for Routine Examination in Type I Studies of Porcine Models and Organ Lists Recommended for Histopathological Examination in Routine Toxicity Studies by the STP and Various Public Institutions and Regulatory Authorities.

Organ System	Organ/Tissue	STP	EMA	EPA	FDA	RITA/ NACAD	Porcine Type I Study	Organ System	Organ/Tissue	STP	EMA	EPA	FDA	RITA/ NACAD	Porcine Type I Study
Nervous	Brain	+	+	+	+	+	+	Gastrointestinal	Tongue	−	−	−	−	+	+
	Spinal cord	+	+	+	+	+	+		Pharynx	−	−	+	−	+	+
	Peripheral nerve	+	+	+	+	+	+		Salivary glands	+	+	+	+	+	+
Cardiovascular	Heart	+	+	+	+	+	+		Esophagus	+	+	+	+	+	+
	Aorta	+	+	+	+	+	+		Stomach	+	+	+	+	+	+
Respiratory	Nasal septum	+[a]	+[a]	+	+	+	+		Duodenum	+	+	+	+	+	+
	Conchae	+[a]	+[a]	+	+	+	+		Jejunum	+	+	+	+	+	+
	Olfactory mucosa	+[a]	+[a]	+	+	+	+		Ileum	+	+	+	+	+	+
	Larynx	+[a]	+[a]	+	−	+	+		Caecum	+	+	+	+	+	+
	Trachea	+[a]	+[a]	+	+	+	+		Colon	+	+	+	+	+	+
	Lungs	+	+	+	+	+	+		Rectum	−	−	+	+	+	+
Endocrine	Pituitary gland	+	+	+	+	+	+	Hepatobiliary and pancreatic	Pancreas	+	+	+	+	+	+
	Thyroid gland	+	+	+	+	+	+		Liver	+	+	+	+	+	+
	Parathyroid gland	+	+	+	+	+	+		Gall bladder	+	+	+	+	+	+
	Adrenal glands	+	+	+	+	+	+	Genital	Ovaries	+	+	+	+	+	+
Urinary	Kidney	+	+	+	+	+	+		Fallopian tube	−	−	−	+	+	+
	Ureter	−	−	−	−	+	+		Uterus	+	+	+	+	+	+
	Urinary bladder	+	+	+	+	+	+		Cervix	−	−	−	−	+	+
	Urethra	−	−	−	−	−[b]	+		Vagina	+	+	−	+	+	+
Immune and hematopoietic	Lymph nodes	+	+	+	+	+	+		Clitoral gland	−	−	−	−	+	−
	Thymus	+	+	+	+	+	+		Testes	+	+	+	+	+	+
	Bone marrow	+[c]	+[c]	+	+[d]	+[d]	+		Epididymis	+	+	+	+	+	+
	Spleen	+	+	+	+	+	+		Prostate	+	+	+	+	+	+
	Tonsil	−	−	−	−	−	+		Vesicular gland	+	+	+	+	+	+
Musculoskeletal	Skeletal muscle	+	+	−	+	+	+		Spermatic cord	−	−	−	−	−	+
	Bones	+[e]	+[e]	−	+[f]	+[g]	+		Bulbourethral gland	−	−	−	−	−	+
	Joints	+	+	−	−	+[g]	+		Coagulating gland	−	−	−	−	+	−
	Tendon	−	−	−	−	−	+		Penis	−	−	−	−	−	+
Integument	Skin	+	+	+	+	+	+		Prepuce	−	−	−	−	−	+
	Mammary gland	+[h]	+[h]	+[h]	+	+[i]	+[h]		Preputial gland	−	−	−	−	+	−
	Adipose tissue	−	−	−	−	−	+	Other	Harderian gland	+	+	+	+	+	+
Special senses	Eyes	+	+	+	+	+	+		Zymbal's gland	−	−	−	+	+	−
	Ear	−	−	−	−	−	+	Pathological lesions/masses		+	+	+	+	+	+

Note. STP = The Society of Toxicologic Pathology (STP) recommended tissue lists for histopathologic examination in repeat-dose toxicity and carcinogenicity studies (Bregman et al. 2003). EMA = the guidelines on repeated dose toxicity from the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) from March 18, 2010 (CPMP/SWP/1042/99 Rev 1). Both the STP and the EMA documents provide a minimum core list of organs/tissues to be examined by histopathology in all types of repeat-dose toxicity and carcinogenicity studies, regardless of the route of administration, species or strain of mammalian laboratory animal, the duration of study, or class of drug being tested. If appropriate, the addition of other tissues relevant to the route of administration is recommended. EPA = The Health Effects Test Guidelines (OPPTS 870.3150) for 90-day oral toxicity in nonrodents from the U.S. Environmental Protection Agency (EPA 712–C–98–200 August 1998). FDA = food and drug administration (FDA) of the U.S. Department of Health and Human Services. Organ list for microscopic examination in toxicity studies. In Redbook 2000: Toxicological Principles for the Safety Assessment of Food Ingredients (2003). General Guidelines for Designing and Conducting Toxicity Studies. RITA/NACAD = revised guides for organ sampling and trimming in rats and mice (parts 1–3) of the Registry of Industrial Toxicology Animal-data (RITA) and the North American Control Animal Database (NACAD) groups (Kittel et al. 2004; Morawietz et al. 2004; Ruehl-Fehlert et al. 2003).

[a]Inhalation studies.

[b]Urethral tissue is usually present in sections of the prostate gland.

[c]Nonrodents: bone marrow from either the rib or the sternum. Rodents: the femoral bone marrow.

[d]Sternum.

[e]Nonrodents: either the rib or the sternum. Rodents: femur with articular cartilage.

[f]Femur.

[g]Knee joint with distal femur and proximal tibia.

[h]Females only.

[i]Both sexes.

bulbourethral gland and the palatine tonsil, or because they represent routinely examined predilection sites for pathological alterations in certain porcine diseases, such as the ileal papilla in swine dysentery. Sampling of other tissues and organs included in the type I study sampling list, such as adipose tissue, tendons, middle and inner ear structures, the urethra, the spermatic cord, the penis, and the prepuce, may be skipped in routine toxicity studies if no gross lesions are present at necropsy and no clinical findings support a histopathological examination. However, when genetically modified, ''new'' porcine models are necropsied for an initial, overall pathological examination, the rare opportunity to collect and examine these ''uncommon'' tissues/structures should be used.

The sampling protocols designed for type II and III studies are particularly designed for the examination of genetically modified pig models and allow for the generation of samples that are quantitatively and qualitatively suitable for a large(r) range of possible subsequent analyses. Aside from the generation of samples for the analyses actually scheduled in the experimental design of a specific study, sampling protocols for type II and III studies also provide the opportunity to generate sufficient numbers of differentially processed backup samples for additional types of analyses in advance. Furthermore, these protocols allow for the provision of a comprehensive biobank collection of redundant, adequately processed samples from any organ or tissue of potential interest (Abbott 2015). The spectrum of possible downstream analyses may include descriptive and quantitative histopathological analyses, such as histological examinations of differentially fixed samples and samples embedded in different embedding media, including paraffin or plastic resin as well as frozen-section histology, immunohistochemistry, *in situ* hybridization, electron microscopy, and quantitative stereological analyses. Additionally, clinical laboratory diagnostic analyses as well as DNA, RNA, and protein analyses including holistic OMICS profiling of frozen, and of otherwise preserved, sample materials might be performed.

Wherever applicable, type II and III sampling protocols schedule volume-weighted systematic random sampling procedures (Gundersen and Jensen 1987) for several organs, including the liver, spleen, kidneys, adrenal and thyroid glands, pancreas, salivary glands, thymus, and lungs. In this instance, the sampling positions and numbers of samples to be taken are based on stochastic parameters and depend on anatomic–morphological and functional properties of the respective organ/tissue. These sampling regimes ensure sampling of representative specimens, avoid systematic sampling biases, reduce experimental variability, and efficiently increase the precision of the overall experiment (Howard and Reed 2005). From each of the systematically randomly determined sampling positions, multiple samples are harvested and differentially processed according to the respective scheduled subsequent analyses. This time- and labor-saving principle may easily be adjusted to the individual sample-number and sample-type demands of a specific study. An exception to the general systematic random sampling approaches, where the entire organ is sampled, was made in organs/tissues with numerous and/or complexly structured morphologic components, such as the central nervous system or the heart, where the necessary number of sampling sites determined by systematic random sampling over the total organ would be exceedingly disproportionately high and therefore impractical. Therefore, in these organs, samples are taken from defined locations, such as defined brain areas, or distinct cardiac structures that are of interest in a specific experiment. If appropriate, the excised tissue regions of interest are then subjected to a subsequent random sampling procedure to generate representative subsamples for different downstream analyses. In practice, the workload, the personnel requirements, and the time frame and temperature conditions of a distinct sampling procedure must be compatible with the requirements of the study design and the scheduled analyses. Thus, unless the primary scientific scope of a study necessarily requires systematic random sampling of the entire organ/tissue, taking samples from defined anatomical locations appears sufficient for most qualitative histopathological and molecular–biological analyses in tissues, such as the mammary glands, adipose tissue, skin, and skeletal musculature.

For selected organs, study type II and III sampling protocols additionally present appropriate methods for the determination of the total organ (i.e., the reference compartment) volumes by Cavalieri volumetry or via the determination of the specific density of the tissue (Howard and Reed 2005; Scherle 1970). Moreover, the generation of backup specimens suitable for quantitative histomorphological analyses requiring isotropic uniform random–sectionable and vertical uniform random–sectionable samples is routinely scheduled in type II and type III study sampling protocols for several organs/tissues. These samples enable the assessment of a wide range of quantitative stereological parameters that might yet emerge to be of interest in later courses of a study and could not be adequately determined without the respective specimen (Gundersen et al. 2013; Howard and Reed 2005). Further information on the practical application of systematic random sampling, volumetry, and sample processing for quantitative stereological analyses is provided in the Supplemental Material and in the pertinent literature cited there.

Sampling Protocol Instructions and Illustrations

The sampling protocols proposed in the Supplemental Material provide detailed descriptions of applicable sampling procedures (type I–III studies) for different organs/tissues and various different downstream analyses, as illustrated by schematic drawings, macroscopic images, and histological images. The initial section presents different sampling strategies applicable to porcine organs and tissues, the determination of the specific density of porcine tissues, organ volumetry, estimation of embedding-related tissue shrinkage, and the generation of samples for quantitative stereological analyses. The sampling guides for the different organs/tissues usually cover particular information on the following topics:

(1) *Relevant pig-specific anatomic features and practical recommendations regarding the preparation of different organs/tissues*.
(2) *Cutting directions and orientations of samples for histopathological examinations*. The symbols used to indicate different cutting directions, sample orientations, and section planes in schematic drawings and photo images are explained in Supplemental Figure S14.
(3) *Sample numbers/locations*. For routine screenings in type I studies, the anatomic location of the samples to be taken from the respective organs/tissues is indicated. The number of samples that has to be taken by systematic random sampling generally depends on the size of the organ/tissue, the size of the tissue sample pieces, the statistical properties of the investigated parameters, such as interindividual/biological and interspecimen variances, as well as the type and extent of the scheduled subsequent analyses. Therefore, the sample numbers indicated in the sampling guidelines merely represent recommended guidance levels, which should generally be sufficient for most analyzed parameters. Depending on the investigated parameter, the actual number of necessary sampling positions per organ/tissue might, thus, be lower for a specific experiment.
(4) *Individual sample sizes and specific tissue processing methods for different downstream analyses*. The maximal size of an individual sample is limited by different factors, including the size of the respective organ, the number of samples to be harvested, and the specific conditions of the subsequent processing of the sample, such as the maximal penetration depth of fixatives and the size of embedding cassettes and test tubes. The dimension of individual samples designated for molecular analyses is approximately 3 × 3 × 3 mm. These samples are frozen on dry ice and then stored at −80°C until further analysis. The size of samples to be processed for histological examination is usually up to 2 × 2 × 0.5 cm for paraffin-embedded specimens, ∼1 × 1 × 0.5 cm for plastic resin (glycol methacrylate/methylmethacrylate [GMA/MMA])-embedded specimens, ≤1 × 1 × 0.5 cm for cryohistology samples, and ≤2 × 2 × 2 mm for the glutaraldehyde-fixed specimen. The standard fixatives used in the present guidelines are 10% formalin (4% neutrally buffered formaldehyde solution), fixation for 24 hr at room temperature (RT); methacarn solution (60% absolute methanol, 30% chloroform, 10% glacial acetic acid v/v), fixation for ≥24 hr at RT, samples are then rinsed in 70% ethanol and tissue processing for paraffin embedding is started in 70% ethanol; and glutaraldehyde solution (2.5% or 6.25% in Sorensen's buffer, as indicated). For cryosectioning, tissue specimens are embedded in Tissue-Tek® O.C.T.™-blocking medium (Sakura Finetek Europe B.V., the Netherlands), frozen in liquid nitrogen-cooled isopentane and stored at −80°C until sectioning. Alternatively, the samples can be frozen in dry ice-cooled isopentane without a blocking medium to include methods such as the mass spectrometry imaging in the spectrum of possible downstream analyses (Goodwin et al. 2011). Formalin-fixed bone specimen are decalcified, using a slow-acting, acid-based decalcification solution (DC3; Labonord, Germany) for 3–30 days at RT. The embedding media for samples used for histological and quantitative histopathological analyses are paraffin, glycidyl ether (Epon) resin, and GMA/MMA (Hermanns, Liebig, and Schulz 1981). Nonstandard materials used for sampling/tissue processing are specified in the descriptions of the respective sampling protocols. For a simplified presentation, the different downstream analysis types are indicated by pictograms (Supplemental Figure S15).
(5) *A comparison of the study type I sampling protocols with established recommendations for histopathological examinations in routine toxicity studies in rodents*. For each organ/tissue, the similarities and differences between the type I study sampling protocols for porcine models, standard guidelines for organ sampling and trimming in rats and mice (Kittel et al. 2004; Morawietz et al. 2004; Ruehl-Fehlert et al. 2003), and applicable "Best Practices" guides of Working Groups of the STP are discussed (Bolon et al. 2013; Haley et al. 2005; Reagan et al. 2011).
(6) *The recommended cross grid sizes for systematic random sampling procedures*.
(7) *Schematic illustrations* are provided where appropriate and are drawn in gray scales to preserve the recognizability of image details in black and white printouts.
(8) *Histological images*. In the type I study sampling guidelines, HE-stained histological images of the indicated section planes are provided, with the relevant tissue structures indicated.
(9) *Estimates of the expected time and personnel efforts for sample collection in type II and III studies*.
(10) References to the most relevant literature.

Conclusions

Because of the increasing importance of porcine animal models in biomedical research, the application of consistent and appropriate sampling procedures for tissue evaluation will increase the quality of these studies. The sampling protocols provide a basis for the generation of representative, high-quality samples using standardized procedures, which will contribute to the validity of inter- and intrastudy comparisons in porcine studies. The proposed sampling procedures and indicated sample numbers and sizes are intended as guidelines for sampling organs/

tissues in pigs but not as requirements that must be met in any research project involving porcine animal models. The different sampling protocols for the type I, II, and III studies can be combined and individually adjusted to the protocol-defined objectives of a specific research project. The proposed ''forward-looking'' sampling strategies ensure that all necessary samples are correctly collected and processed for a given study so that they might contribute to a reduction in the number of animals needed in a study (Tornqvist et al. 2014).

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Author Contribution

Authors contributed to conception or design (BA, SH, CB, ES, SR, FS, EW, RW, AB); data acquisition, analysis, or interpretation (BA, SH, CB, ES, SR, FS, EW, RW, AB); and drafting the manuscript (BA, EW, RW, AB). All authors critically revised the manuscript, gave final approval, and agreed to be accountable for all aspects of work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved. AB crafted the illustrations.

Declaration of Conflicting Interests

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Supplemental Material

The online data supplements are available at http://tpx.sagepub.com/supplemental.

References

Abbott, A. (2015). Inside the first pig biobank. *Nature* **519**, 397–98.

Aigner, B., Renner, S., Kessler, B., Klymiuk, N., Kurome, M., Wunsch, A., and Wolf, E. (2010). Transgenic pigs as models for translational biomedical research. *J Mol Med (Berl)* **88**, 653–64.

Bolon, B., Garman, R. H., Pardo, I. D., Jensen, K., Sills, R. C., Roulois, A., Radovsky, A., Bradley, A., Andrews-Jones, L., Butt, M., and Gumprecht, L. (2013). STP position paper: Recommended practices for sampling and processing the nervous system (brain, spinal cord, nerve, and eye) during nonclinical general toxicity studies. *Toxicol Pathol* **41**, 1028–48.

Bregman, C. L., Adler, R. R., Morton, D. G., Regan, K. S., and Yano, B. L. (2003). Recommended tissue list for histopathologic examination in repeat-dose toxicity and carcinogenicity studies: a proposal of the Society of Toxicologic Pathology (STP). *Toxicol Pathol* **31**, 252–53.

Goodwin, R. J., Pitt, A. R., Harrison, D., Weidt, S. K., Langridge-Smith, P. R., Barrett, M. P., and Logan Mackay, C. (2011). Matrix-free mass spectrometric imaging using laser desorption ionisation Fourier transform ion cyclotron resonance mass spectrometry. *Rapid Commun Mass Spectrom* **25**, 969–72.

Gun, G., and Kues, W. A. (2014). Current progress of genetically engineered pig models for biomedical research. *Biores Open Access* **3**, 255–64.

Gundersen, H. J. G., and Jensen, E. B. (1987). The efficiency of systematic sampling in stereology and its prediction. *J Microsc* **147**, 229–63.

Gundersen, H. J. G., Mirabile, R., Brown, D., and Boyce, R. W. (2013). Stereological principles and sampling procedures for toxicologic pathologists. In *Haschek and Rousseaux's Handbook of Toxicologic Pathology* (W. Haschek, C. Rousseaux, and M. Wallig. eds.), 3rd ed., 215–86. London: Academic Press.

Haley, P., Perry, R., Ennulat, D., Frame, S., Johnson, C., Lapointe, J. M., Nyska, A., Snyder, P., Walker, D., and Walter, G. (2005). STP position paper: Best practice guideline for the routine pathology evaluation of the immune system. *Toxicol Pathol* **33**, 404–7, discussion 408.

Hermanns, W., Liebig, K., and Schulz, L. C. (1981). Postembedding immunohistochemical demonstration of antigen in experimental polyarthritis using plastic embedded whole joints. *Histochemistry* **73**, 439–46.

Howard, C. V., and Reed, M. G. (2005). *Unbiased stereology*. QTP Publications, Coleraine, UK.

Kemter, E., Lieke, T., Kessler, B., Kurome, M., Wuensch, A., Summerfield, A., Ayares, D., Nagashima, H., Baars, W., Schwinzer, R., and Wolf, E. (2012). Human TNF-related apoptosis-inducing ligand-expressing dendritic cells from transgenic pigs attenuate human xenogeneic T cell responses. *Xenotransplantation* **19**, 40–51.

Kittel, B., Ruehl-Fehlert, C., Morawietz, G., Klapwijk, J., Elwell, M. R., Lenz, B., O'Sullivan, M. G., Roth, D. R., Wadsworth, P. F., Group, R., and Group, N. (2004). Revised guides for organ sampling and trimming in rats and mice—Part 2. A joint publication of the RITA and NACAD groups. *Exp Toxicol Pathol* **55**, 413–31.

Klymiuk, N., Aigner, B., Brem, G., and Wolf, E. (2010). Genetic modification of pigs as organ donors for xenotransplantation. *Mol Reprod Dev* **77**, 209–21.

Klymiuk, N., Blutke, A., Graf, A., Krause, S., Burkhardt, K., Wuensch, A., Krebs, S., Kessler, B., Zakhartchenko, V., Kurome, M., Kemter, E., Nagashima, H., Schoser, B., Herbach, N., Blum, H., Wanke, R., Aartsma-Rus, A., Thirion, C., Lochmuller, H., Walter, M. C., and Wolf, E. (2013). Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle. *Hum Mol Genet* **22**, 4368–82.

Klymiuk, N., Bocker, W., Schonitzer, V., Bahr, A., Radic, T., Frohlich, T., Wunsch, A., Kessler, B., Kurome, M., Schilling, E., Herbach, N., Wanke, R., Nagashima, H., Mutschler, W., Arnold, G. J., Schwinzer, R., Schieker, M., and Wolf, E. (2012a). First inducible transgene expression in porcine large animal models. *FASEB J* **26**, 1086–99.

Klymiuk, N., Mundhenk, L., Kraehe, K., Wuensch, A., Plog, S., Emrich, D., Langenmayer, M. C., Stehr, M., Holzinger, A., Kroner, C., Richter, A., Kessler, B., Kurome, M., Eddicks, M., Nagashima, H., Heinritzi, K., Gruber, A. D., and Wolf, E. (2012b). Sequential targeting of CFTR by BAC vectors generates a novel pig model of cystic fibrosis. *J Mol Med (Berl)* **90**, 597–608.

Klymiuk, N., Seeliger, F., Bohlooly, Y. M., Blutke, A., Rudmann, D. G., and Wolf, E. Tailored pig models for preclinical efficacy and safety testing of targeted therapies. *Toxicol Pathol*. Published electronically October 27, 2015. doi:0192623315609688.

Klymiuk, N., van Buerck, L., Bahr, A., Offers, M., Kessler, B., Wuensch, A., Kurome, M., Thormann, M., Lochner, K., Nagashima, H., Herbach, N., Wanke, R., Seissler, J., and Wolf, E. (2012c). Xenografted islet cell clusters from INSLEA29Y transgenic pigs rescue diabetes and prevent immune rejection in humanized mice. *Diabetes* **61**, 1527–32.

Kurome, M., Kessler, B., Wuensch, A., Nagashima, H., and Wolf, E. (2015). Nuclear transfer and transgenesis in the pig. *Methods Mol Biol* **1222**, 37–59.

Lunney, J. K. (2007). Advances in swine biomedical model genomics. *Int J Biol Sci* **3**, 179–84.

Morawietz, G., Ruehl-Fehlert, C., Kittel, B., Bube, A., Keane, K., Halm, S., Heuser, A., Hellmann, J., Group, R., and Group, N. (2004). Revised guides for organ sampling and trimming in rats and mice–Part 3. A joint publication of the RITA and NACAD groups. *Exp Toxicol Pathol* **55**, 433–49.

Reagan, W. J., Irizarry-Rovira, A., Poitout-Belissent, F., Bolliger, A. P., Ramaiah, S. K., Travlos, G., Walker, D., Bounous, D., Walter, G., and Bone Marrow Working Group of, A. S. (2011). Best practices for evaluation of bone marrow in nonclinical toxicity studies. *Vet Clin Pathol* **40**, 119–34.

Renner, S., Braun-Reichhart, C., Blutke, A., Herbach, N., Emrich, D., Streckel, E., Wunsch, A., Kessler, B., Kurome, M., Bahr, A., Klymiuk, N., Krebs, S., Puk, O., Nagashima, H., Graw, J., Blum, H., Wanke, R., and Wolf, E. (2013). Permanent neonatal diabetes in INS(C94Y) transgenic pigs. *Diabetes* **62**, 1505–11.

Renner, S., Fehlings, C., Herbach, N., Hofmann, A., von Waldthausen, D. C., Kessler, B., Ulrichs, K., Chodnevskaja, I., Moskalenko, V., Amselgruber, W., Goke, B., Pfeifer, A., Wanke, R., and Wolf, E. (2010). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. *Diabetes* **59**, 1228–38.

Renner, S., Romisch-Margl, W., Prehn, C., Krebs, S., Adamski, J., Goke, B., Blum, H., Suhre, K., Roscher, A. A., and Wolf, E. (2012). Changing metabolic signatures of amino acids and lipids during the prediabetic period in a pig model with impaired incretin function and reduced beta-cell mass. *Diabetes* **61**, 2166–75.

Ruehl-Fehlert, C., Kittel, B., Morawietz, G., Deslex, P., Keenan, C., Mahrt, C. R., Nolte, T., Robinson, M., Stuart, B. P., Deschl, U., Group, R., and Group, N. (2003). Revised guides for organ sampling and trimming in rats and mice—Part 1. *Exp Toxicol Pathol* **55**, 91–106.

Scherle, W. (1970). A simple method for volumetry of organs in quantitative stereology. *Mikroskopie* **26**, 57–60.

Streckel, E., Braun-Reichhart, C., Herbach, N., Dahlhoff, M., Kessler, B., Blutke, A., Bahr, A., Ubel, N., Eddicks, M., Ritzmann, M., Krebs, S., Goke, B., Blum, H., Wanke, R., Wolf, E., and Renner, S. (2015). Effects of the glucagon-like peptide-1 receptor agonist liraglutide in juvenile transgenic pigs modeling a pre-diabetic condition. *J Transl Med* **13**, 73.

Tornqvist, E., Annas, A., Granath, B., Jalkesten, E., Cotgreave, I., and Oberg, M. (2014). Strategic focus on 3R principles reveals major reductions in the use of animals in pharmaceutical toxicity testing. *PLoS One* **9**, e101638.

Redbook. (2000). *IV.B.1 General Guidelines for Designing and Conducting Toxicity Studies*. U.S. Food and Drug Administration. Last modified November 2003. http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078315.htm.

Wolf, E., Braun-Reichhart, C., Streckel, E., and Renner, S. (2014). Genetically engineered pig models for diabetes research. *Transgenic Res* **23**, 27–38.

Wu, Z., Xu, Z., Zou, X., Zeng, F., Shi, J., Liu, D., Urschitz, J., Moisyadi, S., and Li, Z. (2013). Pig transgenesis by piggyBac transposition in combination with somatic cell nuclear transfer. *Transgenic Res* **22**, 1107–18.

Wuensch, A., Baehr, A., Bongoni, A. K., Kemter, E., Blutke, A., Baars, W., Haertle, S., Zakhartchenko, V., Kurome, M., Kessler, B., Faber, C., Abicht, J. M., Reichart, B., Wanke, R., Schwinzer, R., Nagashima, H., Rieben, R., Ayares, D., Wolf, E., and Klymiuk, N. (2014). Regulatory sequences of the porcine THBD gene facilitate endothelial-specific expression of bioactive human thrombomodulin in single- and multitransgenic pigs. *Transplantation* **97**, 138–47.

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Tissue Sampling Guides for Porcine Biomedical Models

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Supplemental material

CONTENTS

1. Introduction

1.1. Ethics statement

For the presented sampling images, eight pigs (four boars and four sows, aged 3-18 months) were euthanized in accordance with the German Animal Welfare Act. The pigs were bred, born and raised in the institute's own breeding facility, and housed in planar pens with straw litter. All pigs were fed a standard pig diet and had *ad libitum* access to water. Prior to necropsy, the pigs were anesthetized with ketamine (20 mg/kg BW i.m., Serumwerke Bernburg, Germany) and azaperone (2 mg/kg BW i.m., Elanco, Germany) and then euthanized by intravenous injection of T61 (5 ml/50 kg BW, Intervet, Germany).

1.2. Standard disease diagnostics in routine porcine necropsies

In every necropsy, a set of standard samples for testing for livestock epidemic, zoonotic diseases, and common infectious diseases of pigs (Jackson and Cockcroft, 2007) should routinely be generated. The diseases tested for should include common regionally occurring infections. Regular testing for infectious diseases is advantageous because it provides an overview of the health status of the pigs. Additionally, it should be normal practice to exclude diseases and infections that might interfere with the results of the experimental analyses in a specific study. Table S1 lists common pig diseases in middle Europe that are routinely tested for at the authors' institutes. However, this list is not comprehensive and should be adapted to the regional disease situation and updated regularly. For security reasons, livestock epidemic diseases such as Aujeszky's disease (pseudorabies), classical swine fever and African swine fever should be tested for and excluded at regular intervals.

Table S1. Standard disease diagnostics in routine pig necropsies at author's institution

Tissue sample and sample processing	Diagnostic investigation	Disease/pathogen
Lung, apical lobe	Bacteriology and PCR	General status -APP
Lung, main lobe	Virology and PCR	-Influenza-virus, PRSSV
	Bacteriology Culture and PCR	-M. hyopneumoniae -H. parasuis
Palatine tonsils (-20°C)	Real-time RT-PCR	-PRRSV
Superficial inguinal lymph nodes (FF-PE)	ISH	-PCV-2
Jejunum	Bacteriology and PCR	General status
	Virology and PCR	Rotavirus Coronavirus
Ileum	Bacteriology and PCR	General status -B. hyodysenteriae
Colon	Bacteriology and PCR	General status -Salmonella spp.
Nasal septum	Virology and PCR	SHV 2
Pleura, pericardium and peritoneum (swab test)	Bacteriology and PCR	General status -H. parasuis -M. hyosynoviae
Blood serum (-20°C; retained sample)	Serology* (as needed)	Antibody status
Liver and kidney tissue (-20°C; retained sample)	Toxicology (as needed)	Toxic agents

*APP: Actinobacillus pleuropneumoniae; M.: Mycoplasma; H.: Haemophilus; ISH: In situ hybridization; PRRSV: Porcine reproductive and respiratory syndrome virus; PCV-2: Porcine circovirus-2; B: Brachyspira; SHV: Suid herpesvirus.*See Jackson and Cockcroft (2007) for additional details.*

1.3. Adjustment of necropsy and sampling protocols

Given the considerable time, work, and personnel required to complete large-scale sampling from multiple organs/tissues, especially in biobank projects (Type-III studies), the development of a detailed step-by-step (check-list) work and sampling protocol in advance of the planned necropsies is strongly recommended (Abbott, 2015). This protocol should be hierarchically structured with regard to (1.) a meaningful chronological order of tissue and organ harvesting, (2.) the sampling procedures (sample locations, sizes, numbers, orientations, processing, etc.) for each organ, (3) the additional parameters to be recorded (organ weights, dimensions), (4) the sequence of operations, and (5) the personnel requirements for organs/tissues sampling. If appropriate, a “test run” with a “test pig” should be considered to ensure a rapid and smooth necropsy of the animals to be examined.

1.4. Systematic random sampling of porcine organs and tissues

For advanced studies (Type-II and -III studies), these guidelines include the generation of systematically randomly sampled tissue specimens (Gundersen and Jensen, 1987, Howard and Reed, 2005). The principal reason for the application of systematic random sampling strategies is to generate representative samples adequately reflecting the properties of interest of the tissue/organ or of defined organ compartments the samples were taken from (Gundersen et al., 2013, Howard and Reed, 2005). These properties may comprise the morphological and molecular features of the tissue samples, such as the cellular composition and volume proportions of distinguishable tissue compartments and the implicitly dependent abundance of proteins, RNA, and DNA. To ensure that the overall properties of an organ/tissue are adequately represented in the generated samples, a specific number of locations of the organ/tissue have to be sampled (Gundersen et al., 2013, Howard and Reed, 2005). In an optimal sampling procedure, each possible sampling location within the total volume (reference compartment) of the organ/tissue has exactly the same random chance to be sampled. For several porcine organs and tissues, this ideal volume-weighted sampling procedure can easily be performed by cutting the entire organ/tissue into parallel sections of equal thickness and systematic random determination of the sampling positions on the cut surfaces of the organ/tissue slices using cross grids (Howard and Reed, 2005). The location of the first sample is determined at random, whereas all subsequent samples are then systematically taken at a previously determined interval that depends on the size (volume, area or length) of the respective reference compartment (organ/tissue).

An example of the systematic random sampling of a fictitious parenchymal organ is shown in Fig. S2. The details are described in the figure legend. This sampling approach is used in study type-II and -III sampling protocols for most organs/tissues in these guidelines. Cross grids printed on plastic transparencies are used to facilitate sampling. A copy of templates of cross grids of 5 mm to 6 cm size is provided in the appendix.

The rules for cross counting (Howard and Reed, 2005) and the symbols used for illustration of the systematic random sampling in the sampling protocols are illustrated in Fig. S1. To provide a practical example, the systematic random sampling of the adrenal glands is extensively described in section 2.10.8. The sampling of long, tube-like structures/organs, such as the intestine (section 2.10.2.) or the Fallopian tube (section 2.11.2.2.2.), is slightly different and described in the relevant sections.

The described “organ-slabbing and subsampling” strategy for the systematic random sampling of porcine organs is easily comprehensible. With some practice, sampling can be carried out quickly, although the method is admittedly somewhat laborious. Notably, there are also alternative designs for systematic random sampling, such as the "Fast Fractionator", which represents a quick and highly efficient method to obtain unbiased samples from large organs at necropsy using cylindrical knives and perforated plates as cutting guides (Gundersen et al., 2013).

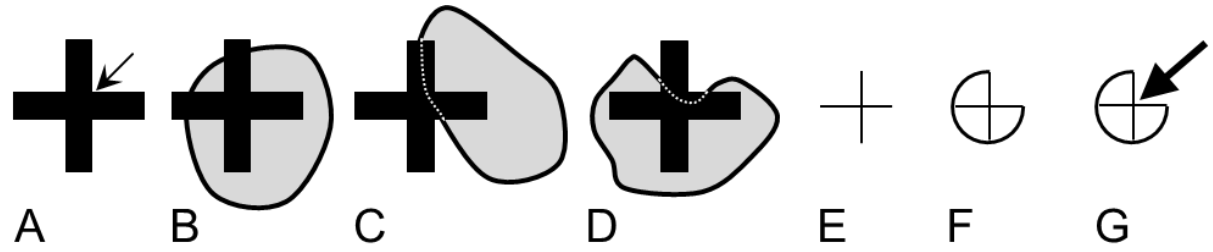
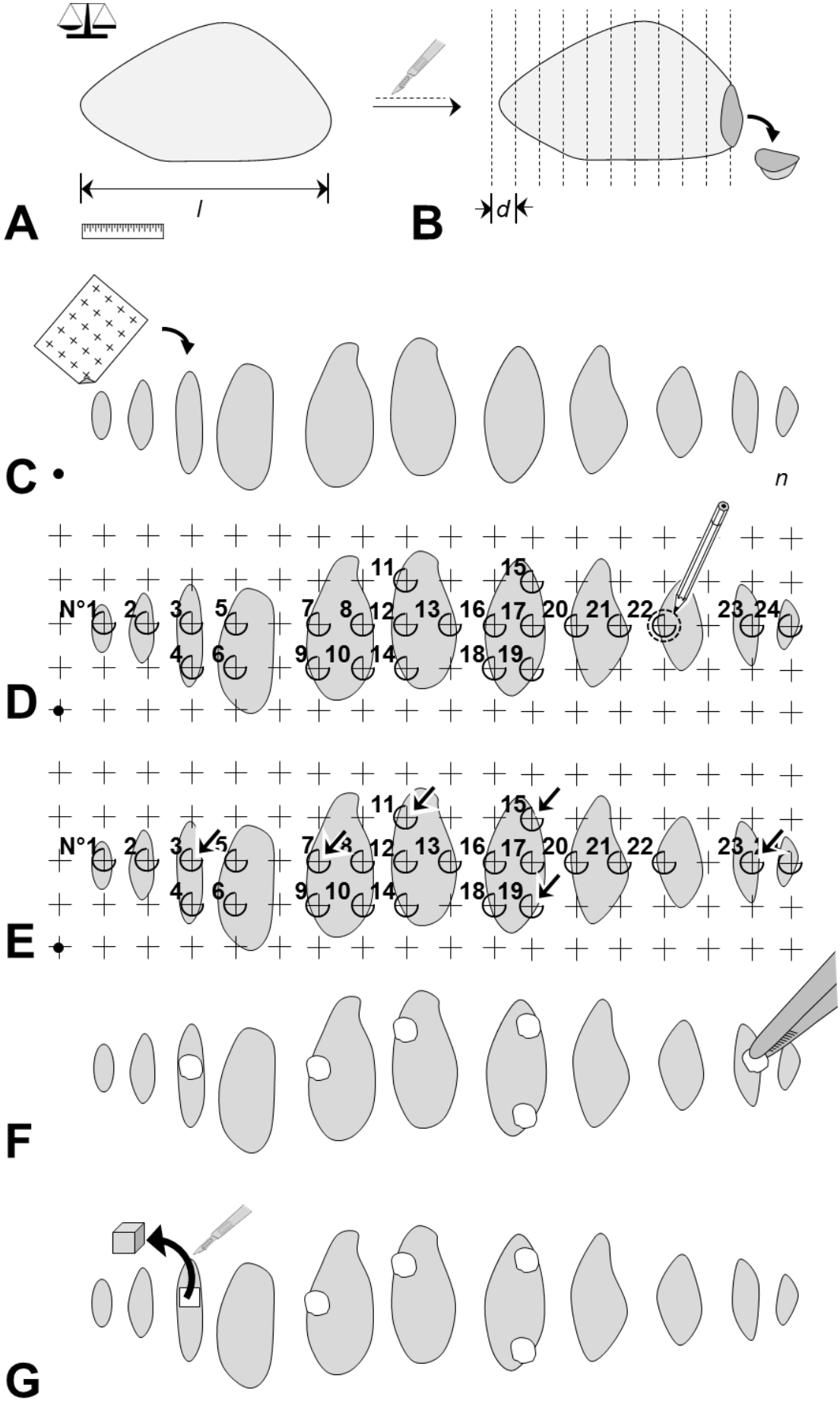


Fig. S1. The rules for the counting of crosses/points (A-D), and the symbols used for illustration of the systematic random sampling method (E-G). ***A****: A cross/point is counted as hitting the tissue if the right upper inner corner of the vertical and horizontal lines (arrow) covers the tissue.* ***B****,* ***C****: A cross hitting tissue.* ***D****: The cross in D is not counted as a hitting point.* ***E****: An example of the cross in a grid used for systematic random sampling.* ***F****: A cross hitting the reference compartment of a given structure is indicated by a circled cross.* ***G****: A sampling position selected by systematic random sampling is indicated by a circled cross with an arrow.*

Fig. S2 (↓). The systematic random sampling of a fictitious parenchymal organ.
A*: First, the excised organ is weighed. If appropriate, its volume and density are determined. Next, the organ is placed on a plain surface and the length (l) of the organ is measured along its longitudinal axis.* ***B****: The organ is then cut into equidistant parallel slices orthogonal to the longitudinal axis. The distance between two sections (sectioning interval) is abbreviated as "d". The first section is randomly positioned within a distance between 0 and the sectioning interval distance (d) from the margin of the organ. The tissue slabs are then laid on the same surface, and all samples are placed with either the left or right side facing down.* ***C****: The tissue slabs are counted (n) and overlaid with an appropriately sized cross grid printed on a plastic transparency. The outermost left lower cross of the grid is placed over a random point (•) out of the tissue.* ***D****: All crosses hitting the tissue or the tissue sub-compartment to be sampled are counted. In the present example, 24 crosses hit the tissue. For demonstration, the crosses hitting the tissue are numbered and marked/circled. Points (crosses) hitting the tissue are marked on the cross grid plastic transparency using a waterproof pen, as shown for position N°22. In the present example, six sampling positions are to be sampled. Therefore, every fourth position where a cross hits the tissue is sampled (24:6=4).The first sampling position is determined randomly in the 1-to-4 interval. This is done using a random-number table that can be conveniently and quickly generated using an online random number generator.* ***E****: In the present example, the position of the third cross (N°3) hitting the tissue is randomly sampled as the first sampling site. Next, the position of every fourth cross hitting the tissue is systematically sampled. In the present example, these include positions N°7, 11, 15, 19, and 23. In the present example and in the Figures illustrating the sampling procedures for diverse organs and tissues, the systematically selected sites are marked by arrows for illustration. During the sampling procedure, the respective positions are marked on the cross grid plastic transparency using a waterproof pen.* ***F****: The corresponding tissue locations can be tagged by slightly raising the cross grid plastic transparency and placing a small piece of clean, blank confetti paper on the surface of the tissue slice using a pair of tweezers.* ***G****: The tissue samples are excised from the selected sample sites. With some practice, the entire process can be completed quickly and efficiently. The plastic transparencies may be cleaned, dried, and reused.*


A
l
B
d
C
n
D
N°1 2 3 5 7 8 12 13 16 17 20 21 22 23 24
4 6 9 10 14 18 19
11
15
E
N°1 2 3 5 7 8 12 13 16 17 20 21 22 23
4 6 9 10 14 18 19
11
15
F
G

1.5. The volumetry and density of selected porcine organs and tissues

Except for certain sampling procedures, such as the fractionator method (Gundersen, 1986, Howard and Reed, 2005, Nyengaard, 1999, Nyengaard and Gundersen, 2006b), the total volume of the organ/tissue under examination (the volume of the reference compartment) is required to calculate the absolute quantitative stereological parameters, such as the absolute cell number or the total volume of a distinct sub-compartment in a respective organ. If the volume of an organ/tissue cannot be directly determined by volumetric measurement, it can be conveniently calculated from its weight and density (Scherle, 1970, Tschanz et al., 2014). The density of an organ/tissue can be determined using the submersion method (Scherle, 1970) which is extensively described in Fig. S3 and S4. Table S2 lists the measured densities of selected porcine organs and tissues. For solid parenchymal organs, organ weight can generally be used for volume estimations without introducing significant bias. In other organs, such as the lungs, density cannot be calculated because of the inconsistent air content. Instead, the organ volume (actual volume of the necropsied organ) can be adequately calculated from the cumulative sectional area and the mean height of the completely sectioned organ (Cavalieri principle, section 2.9.1.2., Lung) (Gundersen and Jensen, 1987, Tschanz et al., 2014).

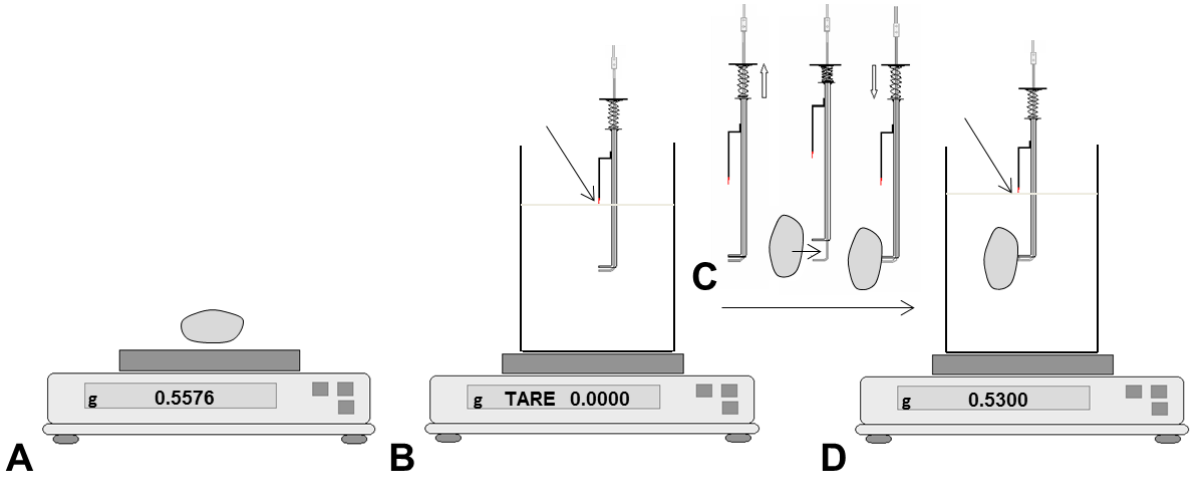


Fig. S3. A schematic illustration of the submersion method used for the determination of tissue density. ***A****: Depending on the size of the organ, a piece of tissue or the entire organ is weighed.* ***B****: A beaker filled with 20°C warm physiological saline solution is placed on the balance. A thin wire or clamping device is submerged in the liquid to a marked position (arrow). The scale is then tared to zero.* ***C****: The piece of tissue is attached to the wire or clamping device and submerged in the liquid to the marked position (arrow,* ***D****). The tissue must be submerged completely and must not touch the bottom or the walls of the beaker. The weight displayed on the balance is recorded. The density ρ is calculated from the weight (m) of the tissue piece and its volume (Vol.) as ρ = m/Vol. The volume is calculated from the weight of the NaCl solution displaced by the submerged tissue (D) and the density of the 0.9% NaCl solution at 20°C (1.0048 g/cm³).*

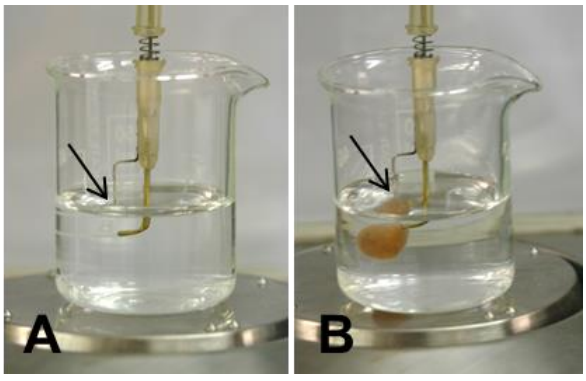


Fig. S4. Illustration of the submersion method for determination of tissue density. ***A****: An image of the step described in Fig. S3B.* ***B****: An image of the step described in Fig. S3D.*

Table S2. **The density of selected porcine organs and tissues**

Organ/tissue	ρ (g/cm³)	Organ/tissue	ρ (g/cm³)
Adrenal gland	1.063 +/- *0.025*	Stomach (full wall thickness)	
Liver	1.071 +/- *0.007*	Cardiac portion	1.041 +/- *0.062*
Pancreas	1.062 +/- *0.016*	Fundus	1.037 +/- *0.022*
Ventricular myocardium	1.036 +/- *0.014*	Pyloric portion	1.045 +/- *0.008*
Testis	1.047 +/- *0.001*	Intestine (full wall thickness)	
Epididymis	1.052 +/- *0.002*	Duodenum	1.052 +/- *0.026*
Thymus	1.055 +/- *0.019*	Jejunum	1.092 +/- *0.083*
Uterus*	1.040 +/- *0.011*	Ileum	1.037 +/- *0.013*
Urinary bladder	1.043 +/- *0.017*	Cecum	1.030 +/- *0.023*
Vesicular gland	1.104 +/- *0.047*	Colon	1.005 +/- *0.026*
Bulbourethral gland	1.068 +/- *0.006*	Parotid gland	1.040 +/- *0.005*
Prostate gland	1.068 +/- *0.004*	Mandibular gland	1.054 +/- *0.004*
Skeletal muscle **	1.074 +/- *0.003*	Lymph node (superficial inguinal lymph node)	1.042 +/- *0.031*
Diaphragm (left crus)	1.063 +/- *0.001*	Thyroid gland	1.061 +/- *0.007*
Spleen	1.069 +/- *0.010*	Brain	1.051 +/- *0.007*
Kidney (fresh) total	1.044 +/- *0.006*	Spinal cord	1.046 +/- *0.010*
Kidney cortex	1.048 +/- *0.033*	Adipose tissue	
Kidney medulla	1.030 +/- *0.034*	Visceral adipose tissue***	0.921 +/- *0.032*
Kidney (perfusion fixed)	1.077 +/- *0.071*	Subcutaneous adipose tissue****	0.961 +/- *0.011*

*The data are shown as the mean +/- SD. Specific organ/tissue weights were determined in 18 pigs (14 sows and four boars; 60 days-2 years of age; body weight 30-250 kg) using the "submersion method" (Scherle, 1970, Tschanz et al., 2014). At least three measurements of different specimens from different animals were performed with each tissue. *Wall of the uterine horn, not gravid. **Mean values for the longissimus lumborum muscle, the tibialis cranialis muscle, the triceps brachii muscle, and the gluteobiceps muscle. ***Adipose tissue from the jejunal mesentery. **** Adipose tissue from the middle of the back.*

1.6. Generation of Isotropic Uniform Random (IUR) sections and Vertical Uniform Random (VUR) sections for quantitative stereological analyses

In addition to the sample location, the orientation of a specimen is important for the estimation/determination of distinct quantitative morphological tissue properties, such as the mean, relative and absolute numbers, volumes, lengths, thicknesses, and surfaces of different cell types or tissue compartments (Howard and Reed, 2005). These parameters can be reliably and unbiasedly determined using a quantitative stereological analysis. The application of several quantitative stereological methods depends on distinct technical prerequisites concerning the processing and orientation of the samples. For several quantitative stereological analyses, tissue embedding in plastic resin, such as Epon (Glycidether) or GMA/MMA (glycolmethacrylate/methylmethacrylate) (Hermanns et al., 1981), is advantageous as compared to paraffin embedding, due to the lower, and more uniform embedding related shrinkage of the tissue (Nielsen et al., 1995, Schneider and Ochs, 2014). Accordingly, these guidelines consistently employ plastic embedding for samples designated for quantitative stereological analyses. Alternatively, there are stereological sampling designs, such as different "Fractionator" approaches, that avoid the issue of embedding-related tissue shrinkage and allow for the generation of paraffin-embedded tissue samples that are suitable for various quantitative stereological analyses. A detailed description of these methods is provided by Gundersen et al. in "Stereological principles and sampling procedures for toxicologic pathologists" in the third edition of "Haschek and Rousseaux´s Handbook of Toxicologic Pathology" (Gundersen et al., 2013). Virtually all quantitative stereological parameters can be assessed in so-called isotropic or independent uniform random (IUR) sections (Howard and Reed, 2005, Nyengaard and Gundersen, 2006b). In IUR sections,

the orientation of the sectional tissue plane is randomized in all three directions of space. In organs/tissues with a naturally present or clearly identifiable vertical axis, such as skin or mucosal samples strictly sectioned orthogonal to their natural surface, the use of so-called vertical uniform random (VUR) sections is advantageous (Baddeley et al., 1986). In contrast to IUR sections, a vertical axis of the sample can be freely defined by the investigator in VUR sections, and the sample is randomized in the two remaining dimensions of space. VUR sections are simple to generate and optimal for surface density/area estimations. VUR sectioning may be preferred because a particular orientation can provide information that is absent in other sections. Except for length-parameters (Howard and Reed, 2005), all quantitative stereological parameters that can be estimated in IUR sections can also be determined in VUR sections.

Thus, a routinely performed, low-effort generation of IUR/VUR-sectionable tissue specimens in advance enables the assessment of quantitative stereological parameters that might be of interest in the later course of a study, and could not be adequately determined without the respective specimen. A complete discourse of the theoretical principles of IUR/VUR sampling or a comprehensive description of the downstream quantitative stereological analyses of IUR/VUR sections lies outside the scope of these guidelines. The interested reader is referred to the literature of quantitative stereology, including the standard textbooks (Gundersen et al., 2013, Howard and Reed, 2005), and several recently published reviews and papers cited in the present work. Therefore, sections 1.6.1.-1.6.4. and Figures (Fig. S5-S13) provide short and practical instructions for the preparation of IUR- and VUR-sectionable samples using methods and materials adapted to the processing of typical sample sizes of porcine organs/tissues.

1.6.1. Generation of Isotropic Uniform Random (IUR) sections, using the "ISECTOR" method

In the ISECTOR method (Nyengaard and Gundersen, 1992), the tissue sample is cast into a spherical mold. The isotropic sphere is then cut at a random position, which results in a randomly isotropic sectional plane. In these guidelines, the ISECTOR method may be used to generate IUR sections from:

1.) Small samples for plastic resin (Epon or GMA/MMA) embedding or from tissue specimens that were previously embedded in Epon or GMA/MMA. The samples are embedded in Epon or GMA/MMA using self-constructed silicone casting molds (Fig. S6). The resulting spheres are then embedded in Epon or GMA/MMA in a random position and sectioned.

2.) Larger fixed tissue samples are cast in agar and cut at a random position (Fig. S5, S6 and S7).

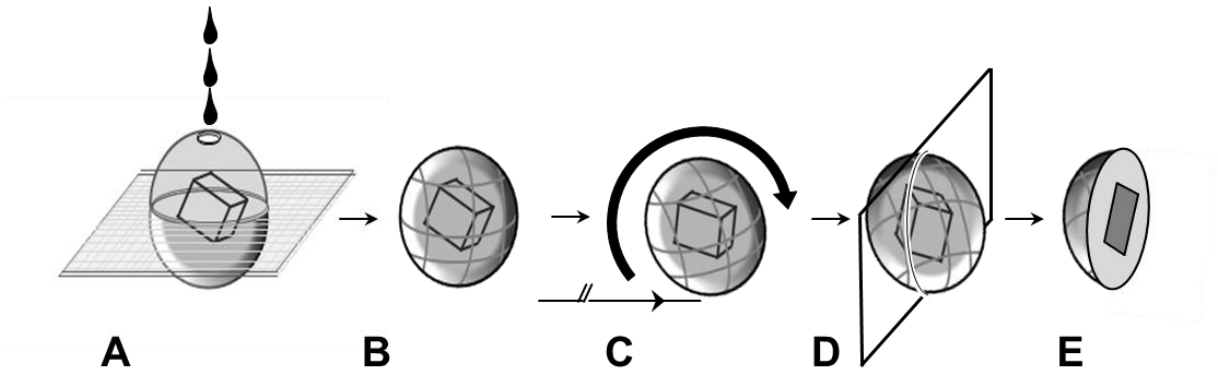


Fig. S5. The schematic illustration of ISECTOR section preparation. A*: A sample of fixed tissue (gray cube) is embedded in liquid agar using a spherical casting mold.* ***B****: After hardening of the agar, the resulting agar sphere is removed from the mold.* ***C****: The agar sphere is rolled across the table and stopped at a random position.* ***D****: The agar sphere is then cut at this random position.* ***E****: This results in a randomly isotropic sectional plane. The tissue sample is then embedded in Epon or in GMA/MMA and sectioned parallel to the ISECTOR-generated IUR section plane.*

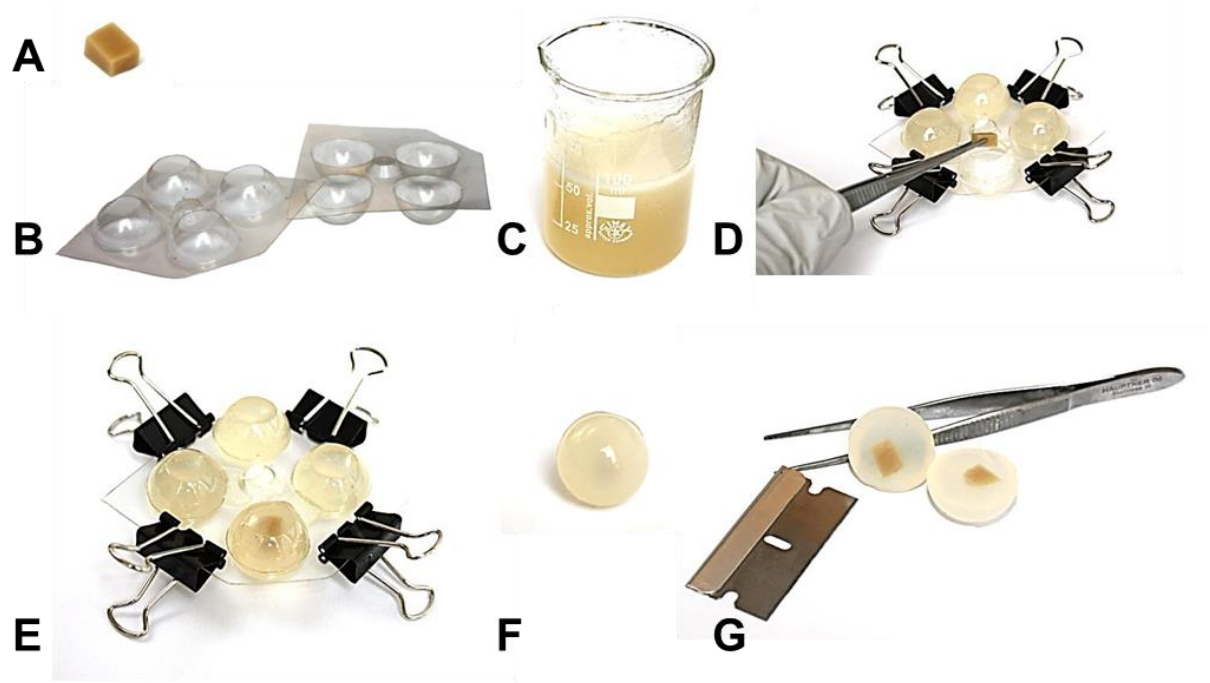


Fig. S6. An illustration of ISECTOR section preparation from formalin-fixed porcine kidney tissue. A*: An excised, systematically randomly sampled specimen of fixed kidney tissue.* ***B****: Spherically shaped casting molds. The casting molds shown above are casting molds for pralines and can be obtained from confectioner suppliers.* ***C****: Liquid agar in a beaker.* ***D-E****: The samples are cast in agar.* ***F****: The hardened agar sphere is removed from the casting mold.* ***G****: The agar sphere is rolled across the table and then stopped and sectioned at a random position, which results in a randomly isotropic sectional plane.*

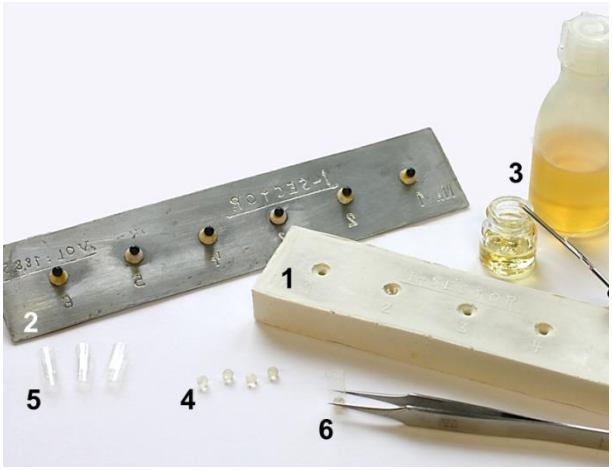


Fig. S7. The preparation of ISECTOR spheres using Epon as an embedding medium. 1*: A self-constructed silicone block with spherical casting molds.* ***2****: The self-constructed casting mold for preparation of the silicone casting molds (1).* ***3****: Epon embedding resin.* ***4****: Epon spheres removed from the silicone casting molds after polymerization.* ***5****: Gelatin capsules.* ***6****: Epon spheres are randomly rotated and then re-embedded in Epon using the gelatin capsules. After polymerization, the Epon blocks are sectioned with a microtome (not shown).*

1.6.2. Generation of Isotropic Uniform Random (IUR) sections using the "ORIENTATOR"

In the ORIENTATOR method (Mattfeldt et al., 1990, Nyengaard and Gundersen, 2006b), the tissue sample is sectioned twice in randomly orientated directions to generate IUR sections. Use of the ORIENTATOR method for formalin-fixed, agar-embedded tissue specimens is shown in detail in Fig. S8 and Fig. S9. The agar embedding of tissue samples prior to sectioning is advantageous for small tissue samples but can be omitted for larger samples. A copy of templates of the used equiangular and cosine-weighted circles are provided in Nyengaard and Gundersen (2006) and Gundersen et al. (2013).

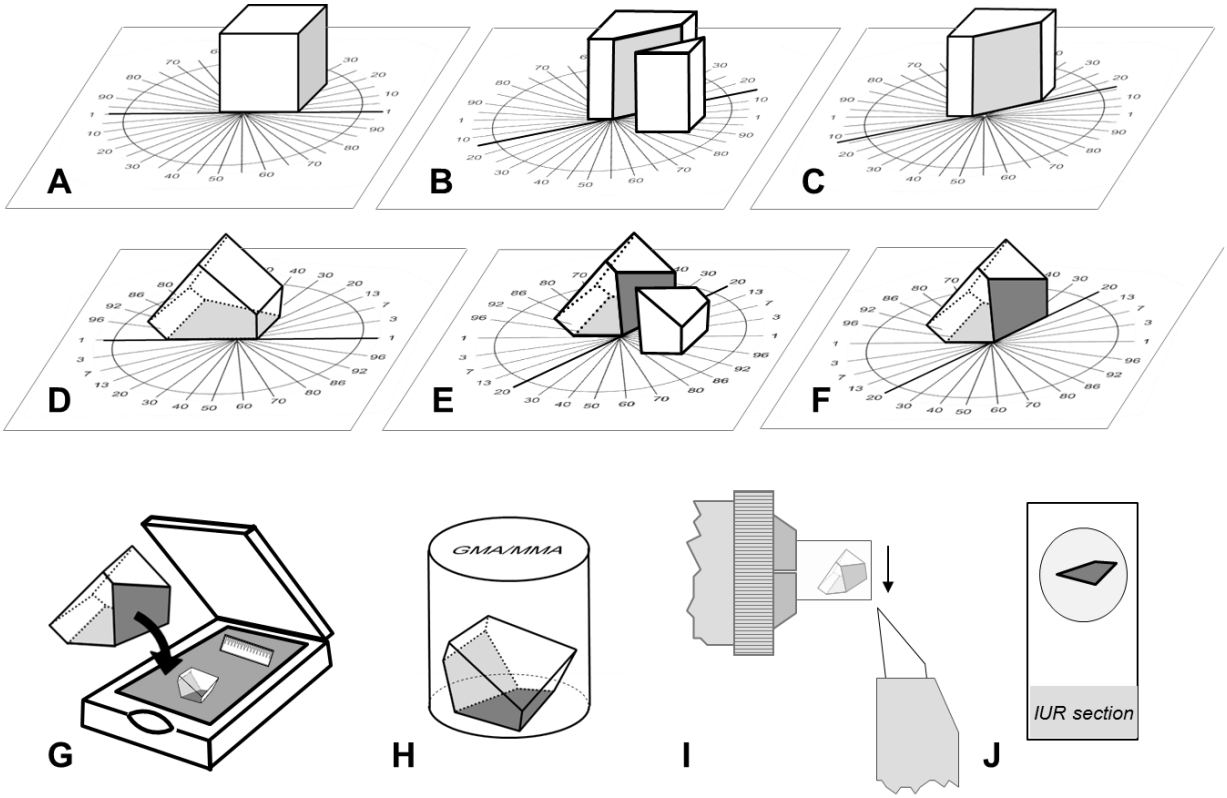


***Fig. S8. The schematic illustration of IUR section preparation using the ORIENTATOR technique. A**: A sample of fixed tissue (gray cube) is placed on an equiangular circle with an edge parallel to the 0°-180° direction marked with a thick horizontal line. **B**: The tissue block is then sectioned at a random angle marked by a thick oblique line. The sectional plane is orthogonal to the table. The random angle is determined using a random number table. **C**: Fresh section surface from step B. **D**: The tissue block is laid on the sectional surface cut in B on a cosine-weighed circle with the edge of the resting surface in C placed parallel to the 1-1 direction. **E**: A new cut is made at a random angle that was determined using a random number table. **F**: This results in a randomly isotropic sectional plane (dark gray). **G**: The area of the IUR section can be determined by scanning the tissue sample with the IUR section surface face down and a length standard. This process is completed for subsequent determination of embedding-related tissue shrinkage. **H**: The tissue sample is embedded in plastic resin (Epon or GMA/MMA), and sections parallel to the IUR plane are cut on a microtome (I). **J**: The IUR sections are mounted on glass slides and stained. The area of the IUR section of the GMA/MMA-embedded tissue is determined as shown in **G**, or directly by planimetric measurement using a commercially available morphometry system. The embedding-related tissue shrinkage is calculated from the sectional area of the fixed tissue prior to embedding in the plastic resin and the corresponding sectional area of the GMA/MMA-embedded tissue sample.*

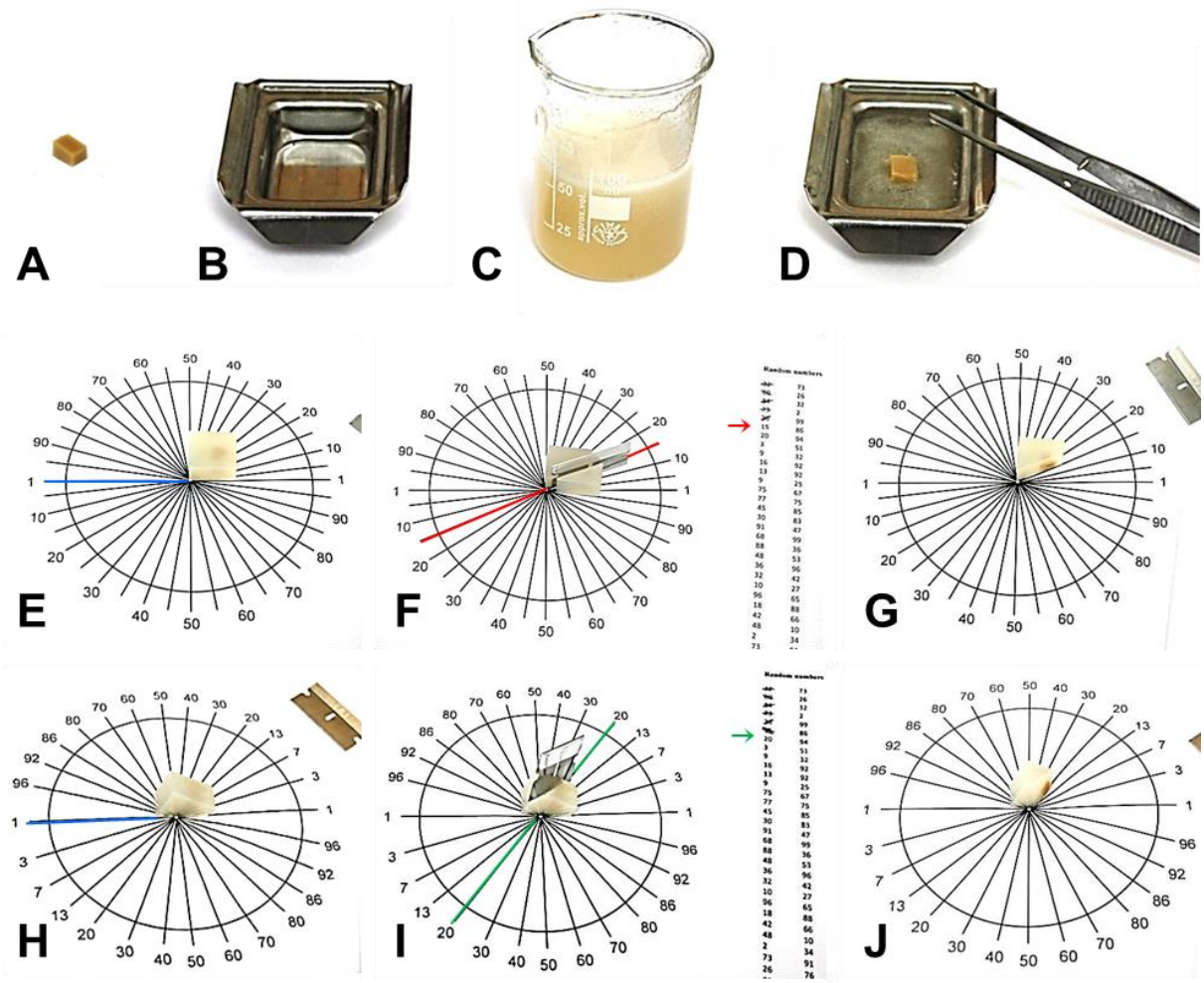


Fig. S9. An illustration of IUR section preparation using the ORIENTATOR technique with an agar-embedded tissue specimen. *The illustrated technique can be applied to larger tissue specimens that are not embedded in agar. Embedding in agar prior to sectioning is advantageous for small-sized tissue samples. If the ORIENTATOR method is applied to very small tissue samples, the agar block should be large enough that both random sections can be placed within the agar without cutting the tissue.* ***A****: An excised, systematically randomly sampled specimen of fixed porcine cortical kidney tissue.* ***B****: Casting mold. The casting mold used here is a casting mold typically used for paraffin embedding.* ***C****: Liquid agar in a beaker.* ***D****: The sample is cast in agar.* ***E****-****J****: ORIENTATOR sectioning of the agar-embedded tissue.* ***E****: The agar block is placed on an equiangular circle with an edge parallel to the 1-1 direction marked by the blue line.* ***F****: The block is sectioned at a random angle with the sectional plane orthogonal to the table. The random angle is determined using a random number table (red arrow). In this example, the random angle is 15-15 and marked by the red line.* ***G****: The resulting section surface.* ***H****: The sectioned block is laid on the section surface cut in F on a cosine-weighted circle with the edge of the resting surface in G placed parallel to the 1-1 direction marked by the blue line.* ***I****: A new cut is made at a random angle indicated by the green line. In this example, the random angle is 20-20 and is indicated by a green arrow in the random number table.* ***J****: This results in a randomly isotropic sectional plane.*

1.6.3. Generation of Orthogonal Triplet Probes (ORTRIPS) for quantitative stereological analyses

Orthogonal Triplet Probes (ORTRIPS) are tissue samples sectioned in three dimensions, which are orthogonal to each other (one x-y plane, one x-z plane and one z-y plane), with the first section plane being an IUR section (Mattfeldt et al., 1985). Basically, ORTRIPs are generated by cutting an IUR section through a sample which is the first of the three ORTRIP section planes. Next, two additional sections are cut orthogonal to the first IUR section and to each other. For this, the sample is first embedded in agar in a cylindrical casting mold. For the size of most porcine samples, a 20 ml syringe with a cut off tip can be used (Fig. S11). The agar cylinder is rotated around its longitudinal axis at a random angle (θ: Longitude in radian, with $0 \leq \Phi \leq 2\pi$). Thus, in the present approach, the tissue sample is rotated, whereas in the previously described ORIENTATOR approach, the section plane is randomized. Next, the agar cylinder with the tissue specimen is rotated again at a random angle (Φ: Colatitude in radian, with $0 \leq \theta \leq \pi$), but this time around its vertical axis. The tissue in the agar cylinder is then sectioned in this orientation. The resulting section is an IUR section and the first of the three ORTRIP sections. Next, two additional sections are cut, as illustrated in the Fig. S10 and Fig. S11 below.

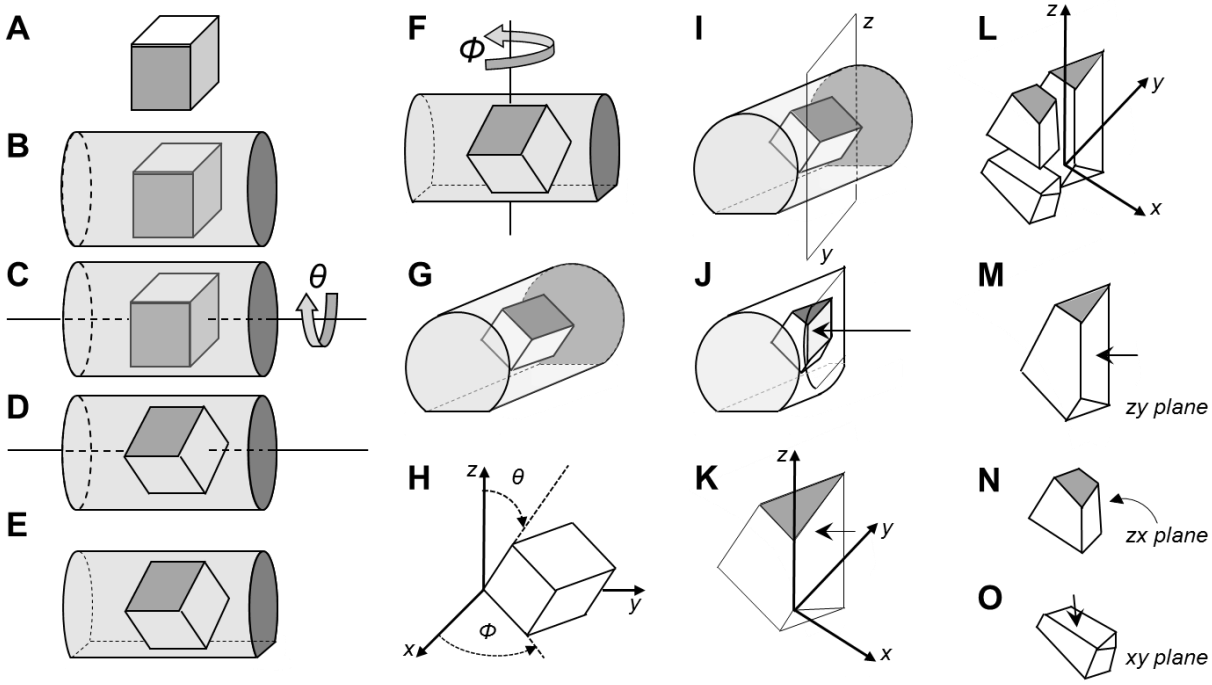


Fig. S10. A schematic illustration of preparation of Orthogonal Triplet Probes (ORTRIPS). A*: Sample of fixed tissue (gray cube).* ***B****: The tissue block is embedded in an agar cylinder.* ***C****: The agar cylinder is then rotated around its longitudinal axis at a random angle (θ).* ***D****: A small piece of the bottom of the agar cylinder is cut off to generate a resting surface.* ***E****: Randomly rotated agar cylinder resting on the surface cut in* ***D****.* ***F****: The agar cylinder is rotated around its new vertical axis at a random angle (Φ).* ***G****: Agar cylinder randomly rotated around its longitudinal axis (angle θ) and its new vertical axis (angle Φ).* ***H****: ORTRIPS geometry I. The 3D-position of the original tissue sample block from A is shown, with the angles θ and Φ indicated.* ***I****: The agar cylinder with the embedded tissue specimen is now cut, with the section plane being parallel to the initial longitudinal axis of the agar cylinder in* ***B-E****, and orthogonal to the table.* ***J****: The resulting section plane (z-y plane, arrow) is an IUR section.* ***K****: ORTRIPS geometry II. The 3D-position of the IUR sectioned (in the z-y plane) tissue sample block from J is shown. The surrounding agar is not depicted. The directions of the x, y, and the z axes are indicated.* ***L-O****: ORTRIP geometry III.* ***L****: Two more section planes (z-x plane and x-y plane) are cut orthogonal to each other and orthogonal to the first IUR-ORTRIP section plane from J.* ***M-O****: Final ORTRIP section planes indicated by arrows. The tissue pieces are then embedded in plastic resin (Epon or GMA/MMA) and histological sections are cut parallel to the ORTRIP section planes.*

To determine the two angles θ and Φ, two random numbers (x_1, x_2) within the interval [0, 1] are drawn from a random number table. The angles Φ and θ are then calculated as: $\theta = \arccos(1-2x_1)$ and $\Phi = 2\pi x_2$ (Mattfeldt et al., 1985). A copy template of a radian-scaled circle used for sectioning of tissue samples is provided in the appendix.

As demonstrated by Mattfeld et al. (1985), the use of ORTRIPS for estimation of different quantitative stereological parameters of myocardial capillarization is far more efficient (by a factor of 10-35) than directionally independent IUR sections. In these sampling guides, generation of ORTRIPS is recommended in the study Type-II and -III sampling protocols of the heart (section 2.9.2.2.), adipose tissue (section 2.3.2.), thymus (section 2.5.2.2.), skeletal muscle (section 2.6.1.2.), tendon (section 2.6.4.2.), and peripheral nerve (section 2.8.2.2.).

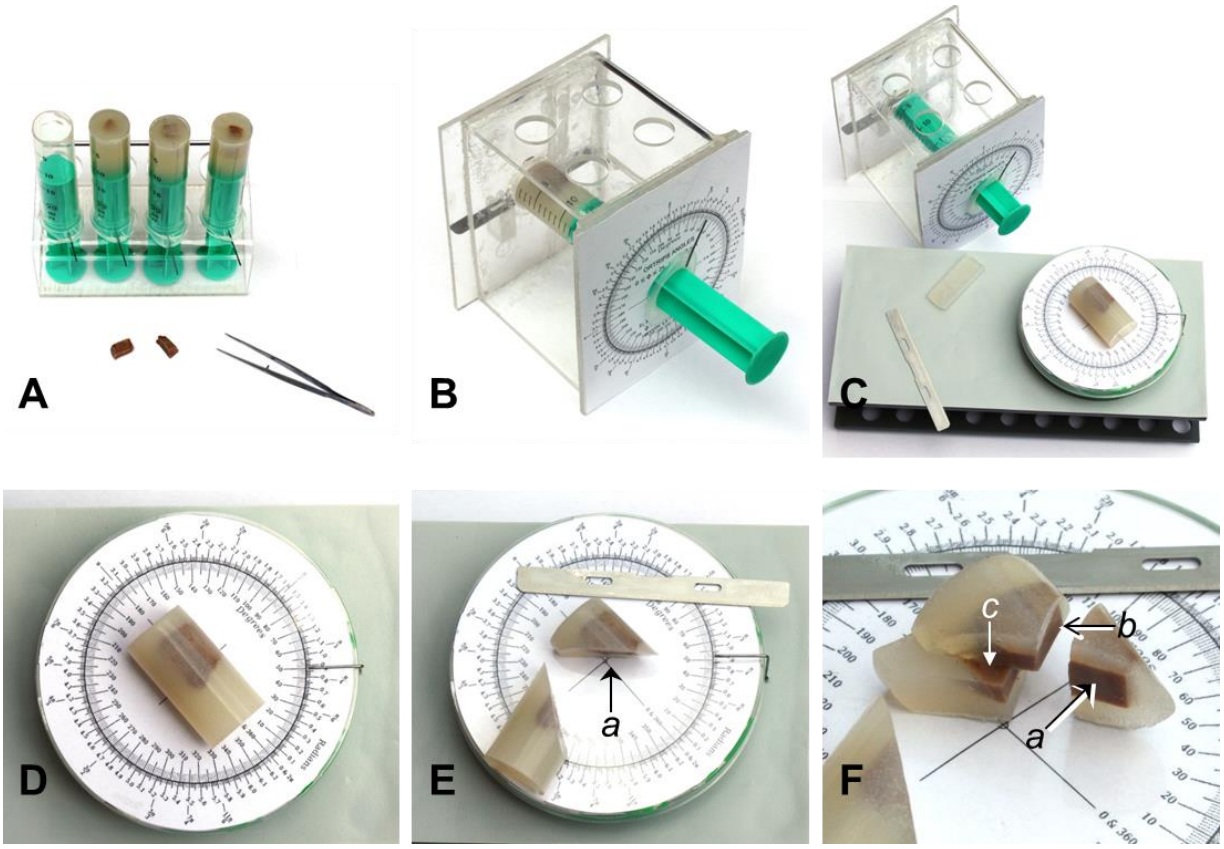


Fig. S11. Illustration of preparation of Orthogonal Triplet Probes (ORTRIPS) using self-constructed devices. A*: Samples of fixed porcine heart are cast in liquid agar in cylindrical casting molds. 20 ml syringes with cut off tips are used as casting molds.* ***B****: After hardening of the agar, the syringes are placed into a mounting support (holder). A radian-scaled circle is printed on the front of the holder, orthogonal to the longitudinal axis of the syringe. A small indicator adapted to the syringe barrell is showing the position of the syringe at the radian-scaled circle. The syringe is rotated, until the indicator points to the 0 (=2π) position. The angle θ is determined, as described above, and the syringe is rotated accordingly. The back of the holder has a round opening which is slightly larger than the diameter of the agar cylinder inside the syringe. At the bottom of this opening, an exchangeable microtome blade is mounted. While maintaining its orientation, the agar cylinder is pushed out of the syringe through the opening in the back of the holder so that the microtome blade cuts off a thin slice of agar from the bottom of the agar cylinder, providing a new resting surface.*

C*: With its new resting surface facing downwards, the agar cylinder is then placed on a radian-scaled circle on a rotatable sample plate, so that the longitudinal axis of the agar cylinder is parallel to the 0 (=2π) position of the radian-scaled circle. The angle Φ is determined as described above and the sample plate is rotated accordingly (****D****). Note the position of the angle indicator of the sample plate in* ***C****-* ***E****.* ***E****: The agar cylinder and the embedded tissue specimen is cut with the section plane being parallel to the initial longitudinal axis of the agar cylinder in B-E, and orthogonal to the table. The section plane a (arrow) is an IUR section and represents the first of the three ORTRIP sections.* ***F****: ORTRIPS. Two more sections (b, c) are cut orthogonal to the section plane a and orthogonal to each other. The tissue pieces are then embedded in plastic resin (Epon or GMA/MMA) and histological sections are cut parallel to the ORTRIP section planes (a-c).*

1.6.4. Generation of Vertical Uniform Random (VUR) sections

VUR sections are commonly used for the estimation of surface densities, e.g., of different skin interfaces or intestinal mucosa, in combination with cycloid test systems (Baddeley et al., 1986, Howard and Reed, 2005). In these guidelines, the generation of VUR sections is recommended in study Type-II and -III sampling protocols for the skin (section 2.2.1.2.) and the mucosa of the stomach (section 2.10.1.2.), intestine (section 2.10.2.2.) and urinary bladder (section 2.10.7.2.). The sampled tissue is placed flat onto a planar surface. A vertical axis that must be always recognizable in the sample/sections during the subsequent steps is freely defined by the investigator. The axis vertical to the natural surface of the tissue structure under investigation is typically chosen as the vertical axis, thus coinciding with a direction of particular biological interest. The tissue is sectioned at a random angle with the sectional planes positioned parallel to the vertical axis.

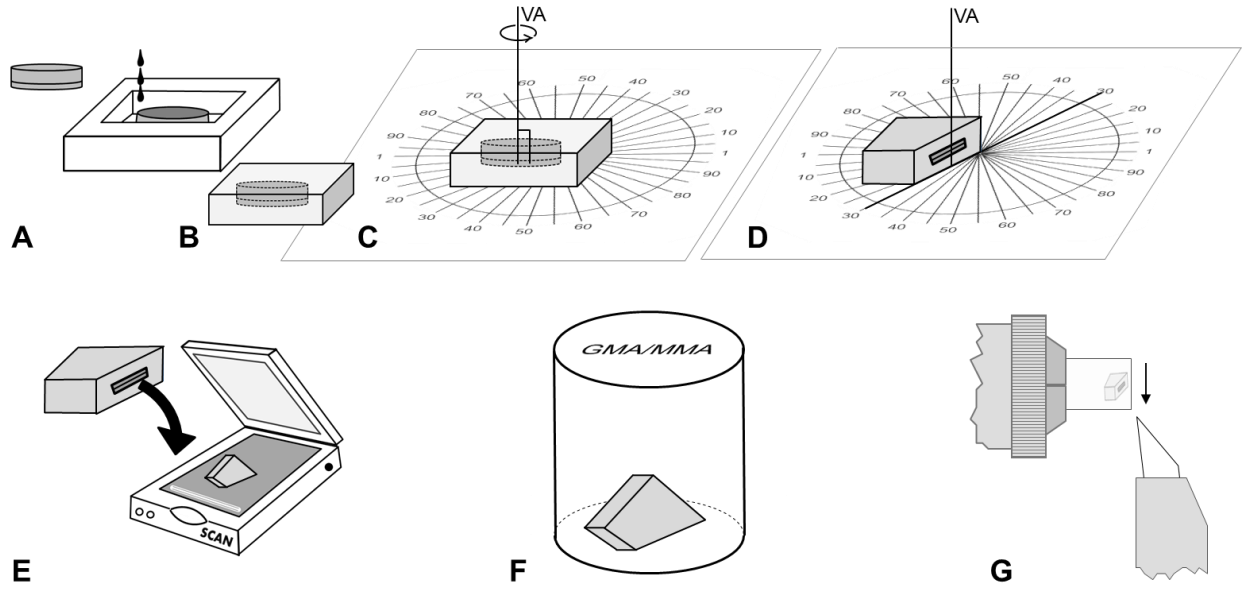


***Fig. S12. A schematic illustration of VUR section preparation. A**: A punched out fixed tissue sample, e. g., the intestinal wall, is embedded in agar in a flat orientation. **B**: A hardened agar block with the embedded tissue sample. **C**: The agar-embedded tissue sample is placed on an equiangular circle. The vertical axis (VA) is shown. **D**: The sample is cut at a random angle with the sectional plane orthogonal to the table and parallel to the vertical axis. The random section angle is determined using a random number table. In this example, the random angle is 30-30 as indicated by a thick oblique line. The resulting sectional plane is VUR. **E**: The area of the VUR section can be determined by scanning of the tissue sample with the VUR section surface face down and a length standard. This procedure is completed to determine the embedding-related tissue shrinkage. **F**: The tissue sample is embedded in plastic resin (Epon or GMA/MMA), and the sections parallel to the VUR plane are cut on a microtome (**G**). The VUR sections are mounted on glass slides and stained. The area of the VUR section of the GMA/MMA-embedded tissue is determined as shown in E or directly by planimetric measurement using a commercially available morphometry system. The embedding-related tissue shrinkage is calculated from the sectional area of the fixed tissue prior to embedding in plastic resin and the corresponding sectional area of the GMA/MMA-embedded tissue sample.*

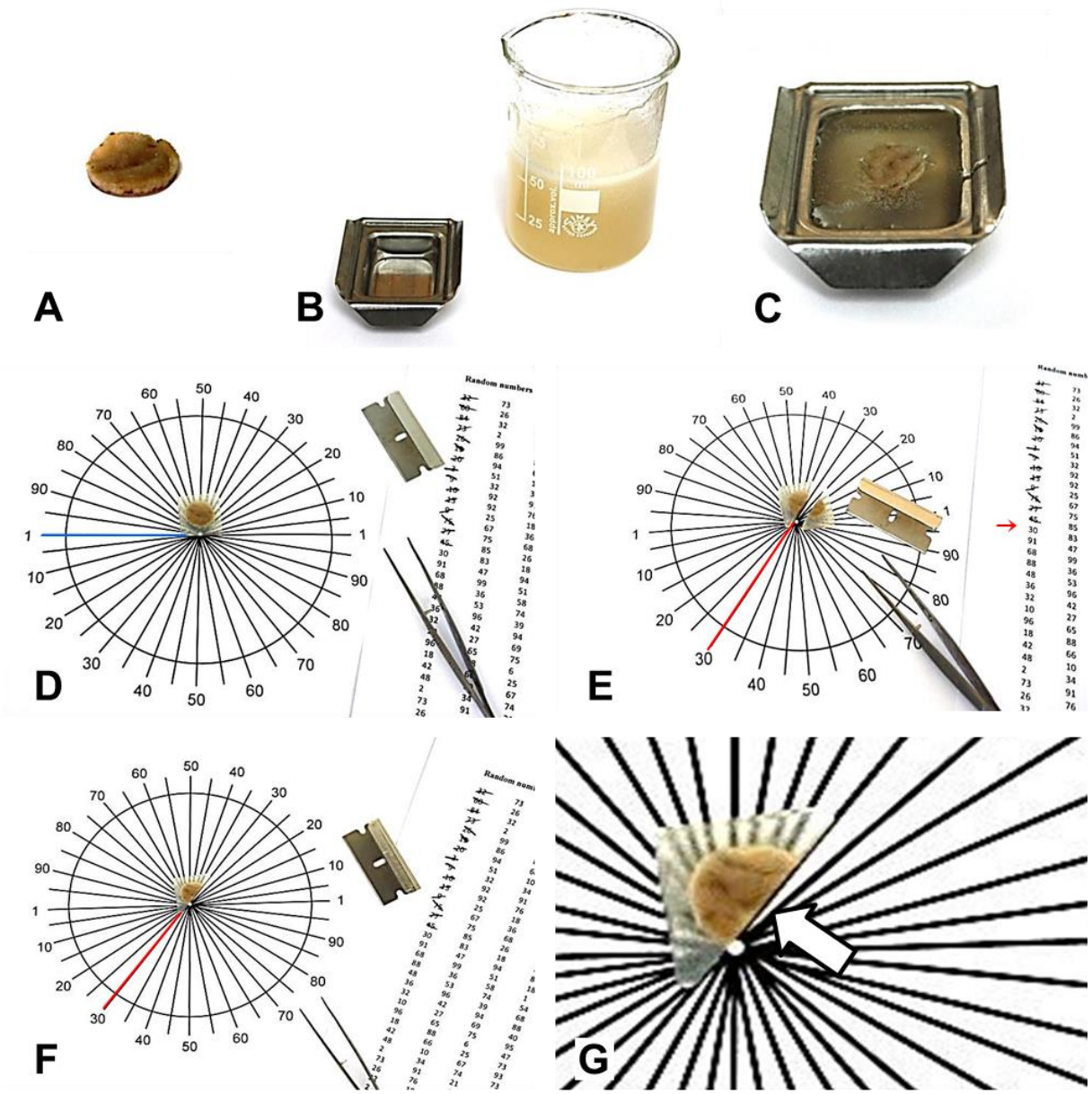


Fig. S13. An illustration of VUR section preparation *(compare to Fig. S12).* ***A****: A punched out sample of fixed tissue from the colon wall.* ***B****-****C****: The tissue specimen is embedded in agar in a flat orientation.* ***D****: The agar block is placed on an equiangular circle with an edge parallel to the 1-1 direction marked by the blue line.* ***E, F****: The block is then sectioned at a random angle with the section plane orthogonal to the table and parallel to the vertical axis. The random angle is determined using a random number table (red arrow). In this example, the random angle is 30-30 as indicated by a red line.* ***G****: The resulting VUR section surface is marked with an arrow.*

2. Tissue sampling guides for porcine biomedical models

The order of sections is not related to the sequence of organ/tissue dissection during the necropsy.

2.1. Section planes, cutting directions, pictograms, and symbols

Figures S14 and S15 illustrate the designations of sample orientations and cutting directions; in addition, the pictograms and symbols used in the sampling protocols to indicate different analysis types, tools, examinations, and sample processings are shown.

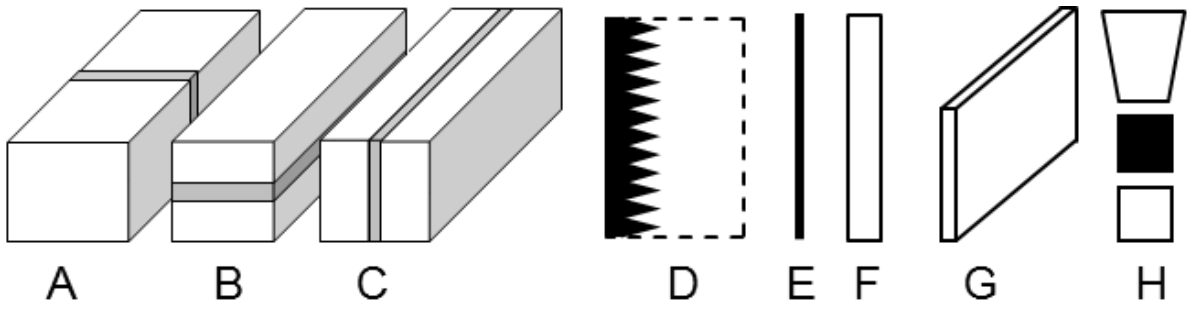


Fig. S14. *Position/orientation designations, cutting planes/section directions and other symbols used in the sampling guidelines.* ***A****: A transverse, cross, or frontal sectional plane.* ***B****: A horizontal sectional plane.* ***C****: A vertical (sagittal) sectional plane.* ***D****: The cutting level parallel to the plane of the picture.* ***E****: The cutting level of histological sections orthogonal to the picture plane.* ***F****: The cutting level of tissue slabs orthogonal to the plane of the picture for molecular analyses.* ***G****: A 3-dimensional cutting level.* ***H****: The outlines of the sampled specimen.*

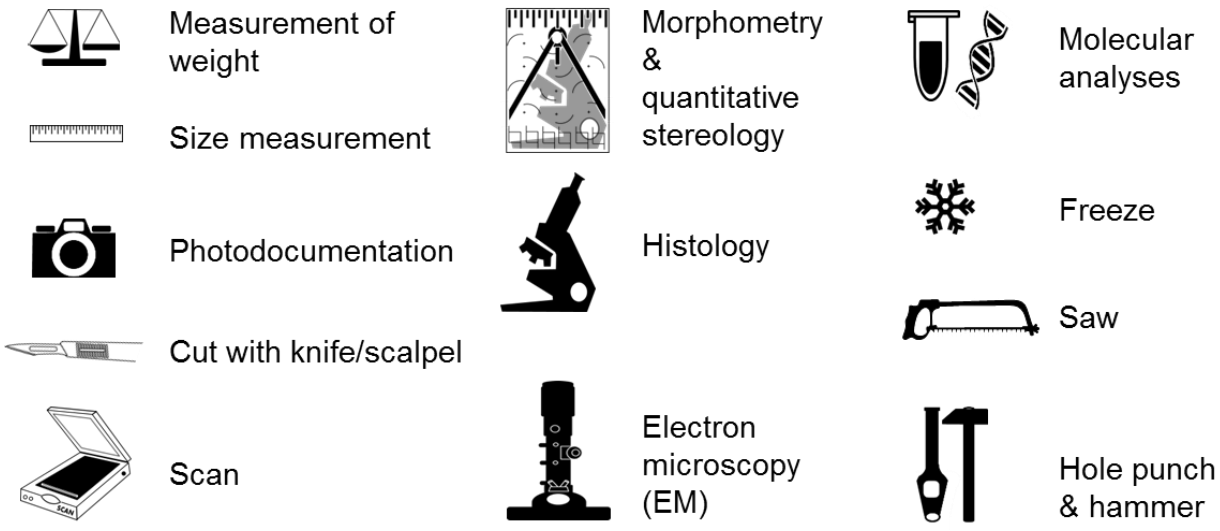


Fig. S15. *Analyses and sample-processing pictograms.*

2.2. Integument

2.2.1. Skin and hoofs

Relevant anatomical features/preparation

Apart from a few anatomical differences, the pig is generally considered a very good model of human skin (Summerfield et al., 2015). Prior to sampling, the skin may be washed to remove dirt. In Type-I, -II, and -III studies, skin samples are taken from defined locations of the body using easily identifiable anatomic landmarks. The locations are specified in Tables S3 and S4 and Fig. S16. For standardization and for the reduction of work effort, it is recommended that skin samples be consistently collected from one side (left) of the body. This refers to flank and abdominal skin samples as well as skin samples taken from the extremities.

Table S3. Skin sampling locations.

N°	Location
1	Snout
2	Interorbital nasal bridge
3	Back
4	Flank
5	Abdomen
6	Inner thigh
7	Tarsus
8	Perineum
9	Hoof

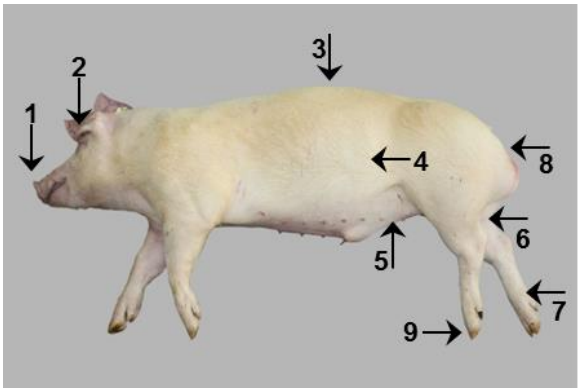


Fig. S16. *Skin sampling locations.*

Table S4. Skin sampling locations in Type-I-III studies.

Study type	Location	Anatomic landmarks /size of sample
III	Snout	Dorsal tip of the snout (Fig. S17, S20, and S22).
III	Interorbital nasal bridge	Triangular area between the eyes: Dorsal to the eyes, middle of the nasal bridge, ventral to the lower eyelid (Fig. S17 and Fig. S20).
I, II, III	Back	Middle of the back (left side) and caudal to the last rib.
I, II, III	Flank	Middle of the left flank between the last rib and the thigh.
II, III	Abdomen	Caudal to the right or left penultimate teat.
I, II, III	Inner thigh	Inner thigh of the left hind leg (Type-I studies) or both hind legs (Type-II/III studies) (Fig. S17).
II, III	Tarsus	Plantar side of the left tarsus between the hoofs and the dewclaws (Fig. S20).
II, III	Perineum	(Fig. S17 and Fig. S21).
II, III	Hoof	Midsagittal section through the medial hoof of the left hind leg (study Type-II) or midsagittal section through the medial and lateral hoof of the left hind leg (study Type-III) (Fig. S17 and Fig. S23).

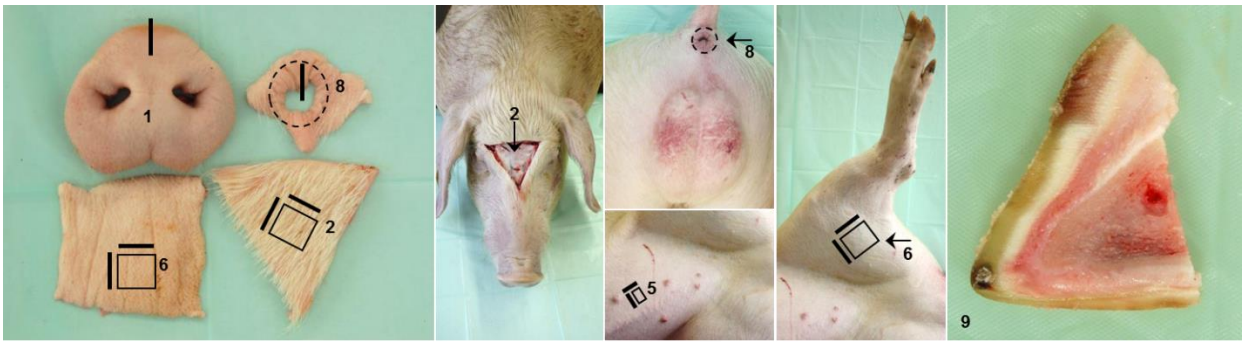


Fig. S17. Skin sampling locations. *The indicated numbers refer to the positions in Table S3. Black lines indicate the orientation of sections parallel and orthogonal to the direction of hair growth. The locations of samples for molecular analyses are indicated by black rectangles.*

General examination parameters

The skin should be examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) when required.

2.2.1.1. Skin samples in Type-I studies

In Type-I studies, skin samples of the hairy thick skin of the back and of the left flank, and of the scantily haired thin skin of the left inner thigh are examined (Table S4 and Fig. S18). After sectioning of the fixed tissue for histology section preparation, thick hairs should be removed from the sample prior to paraffin embedding.

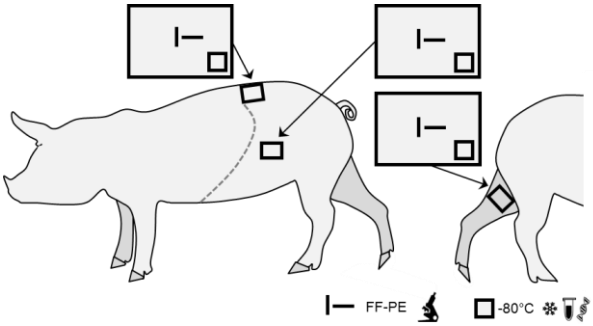


Fig. S18. *Skin sampling locations at the left back, the left flank, and the left inner thigh in Type-I studies. The costal arch is indicated by a dashed line.*

Samples for molecular analyses

Location:	Left back, left flank, left inner thigh as indicated in Fig. S16, S17, S18, and Table S4.
Number of samples:	One sample is collected from each location.
Remarks:	Full thickness specimens of the epidermis and dermis are cut. If required, separate samples of subcutaneous tissue are also collected from the same locations for molecular analyses (see also sections 2.2.1.2. and 2.3.2.).
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Two sections are prepared for each location: One section in the direction of hair growth (longitudinal section) and one section orthogonal to the direction of hair growth (cross section). The sections should contain the epidermis, dermis and subcutaneous tissue. Sampling locations are indicated in Fig. S16, S17, S18 and Table S4.
Fixation/Embedding:	FF-PE (Fig. S19).

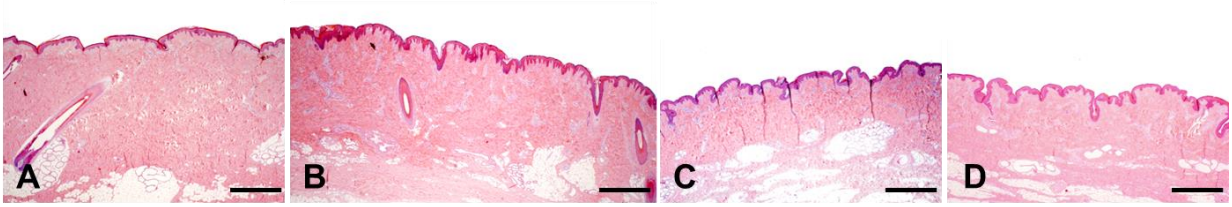


***Fig. S19. Skin histology**. **A**, **B**: Back (A, longitudinal section; B, cross section). **C**, **D**: Inner thigh (C, longitudinal section; D, cross section). FF-PE. HE. Scale bars = 1 mm.*

The comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Ruehl-Fehlert et al., 2003) recommend the collection of two skin samples from the inguinal region. One section is taken in the transverse plane, and one section is taken from a longitudinal orientation of hair growth.

In comparison, the porcine model Type-I-study sampling protocols recommend the sampling of additional skin locations to represent the large skin surface of pigs and the different morphology of the skin at different body locations.

2.2.1.2. Skin samples in Type-II and Type-III studies

General sampling strategy

In Type-II and -III studies, samples are generated from additional skin locations, including the snout, tarsus, perineum, interorbital nasal bridge, and ventral abdomen, to sample areas with different skin thickness, hair density, and innervation-density patterns. Unlike many of the other organs and tissues listed in this guide, the sampling locations of skin specimens for Type-II and -III studies are not determined by systematic random sampling and do not include the entire surface area of the skin; in addition, the surface of a defined skin area is not determined. If needed, this can be done in quantitative stereological skin studies by calculating the skin surface area from the metabolic body weight, analysis of whole-body 3D-CT scans, whole-body castings of a molding compound (Cavalieri principle), or by directly skinning the pig. These approaches have been previously applied to mice (Blutke et al., 2014, Wanke et al., 1999) and, in principle, could be applied to pigs. However, these methods are regarded as too complex and elaborate for the sampling approaches outlined in these guidelines.

Skin samples for histopathological examinations and molecular analyses in Type-II and Type-III studies

Standard FF-PE histology samples are prepared from skin of the ventral abdomen, the left tarsus, and the inter-orbital nasal bridge, with sectional directions parallel and orthogonal to the direction of hair growth. The sectional directions for histology sections of the snout and the perineum are indicated in Fig. S17. FF-decalcified-PE-histology samples are prepared from both the medial and lateral hoof of the left hind leg. Decalcification protocols are provided in section 2.6.2. In Type-II/III studies, two skin samples from the right and left side of the back, flanks, and inner thighs are examined. Additionally to the FF-PE histology samples, specimens for molecular analyses are taken from the back, the flank, and the inner thigh as in in type I studies, and additionally from the tarsus. If required, separate samples of subcutaneous tissue are additionally collected for molecular analyses from the same locations (see also section 2.3.1.).

Samples for cryo-histology, with section directions parallel and orthogonal to the direction of hair growth, and specimens for quantitative stereological analyses are collected (Fig. S20). The latter comprise three round skin specimens from each location that are used to generate VUR-GMA/MMA-embedded skin sections punched out mechanically from the skin using a hole punch and a hammer and three VUR sections of smaller (max. 3x3x3 mm) glutaraldehyde-fixed Epon-embedded specimens (GA-VUR-EE). Skin sampling locations and orientations are schematically shown in Fig. S20.

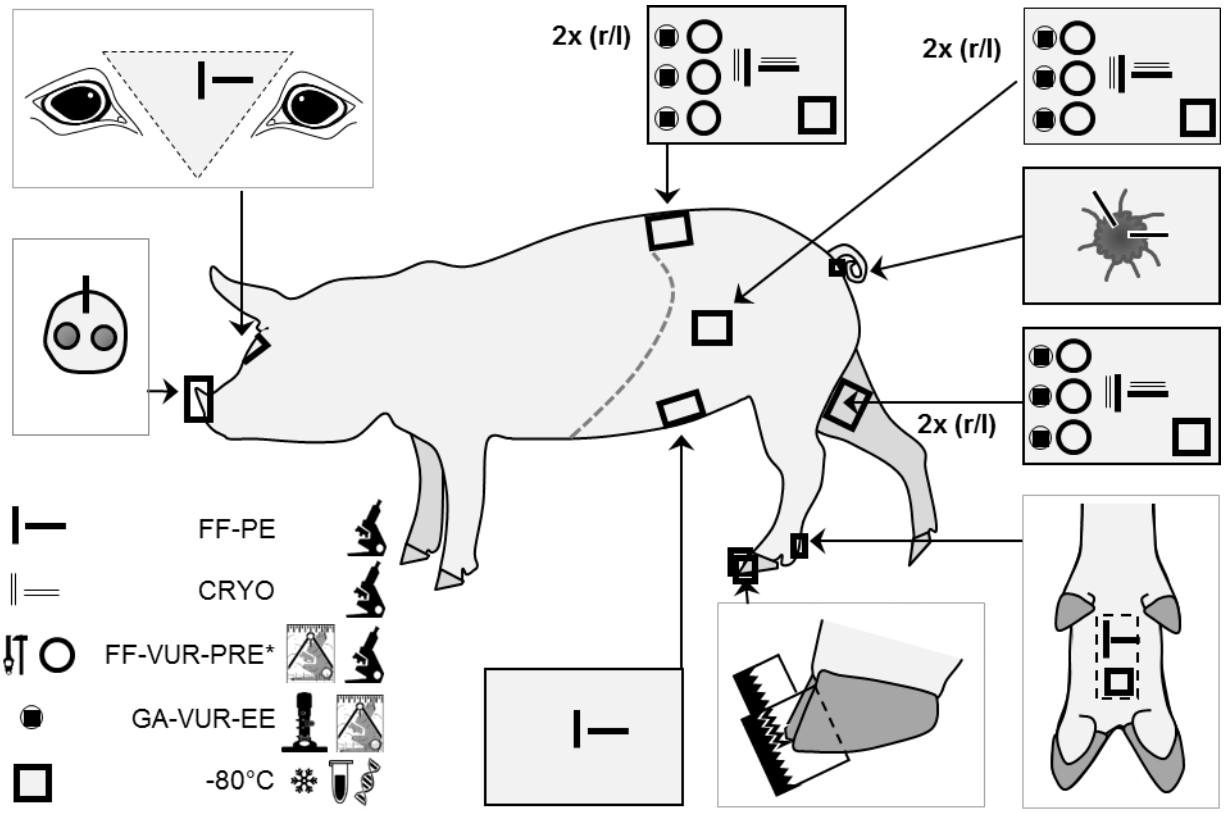


Fig. S20. *A schematic illustration of the skin samples to be collected in Type-II/III studies.*PR = GMA/MMA.*

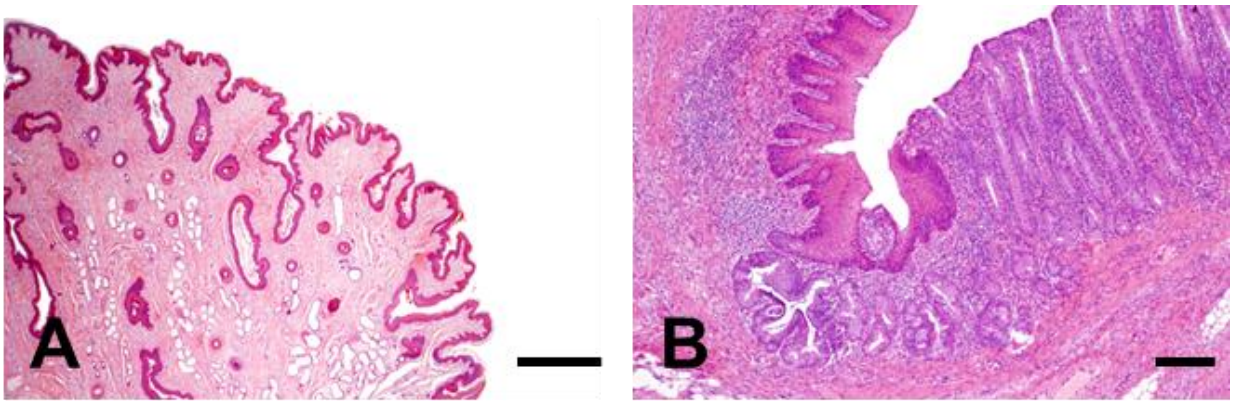


Fig. S21. Perianal skin histology. ***A****: Overview. Scale bar = 1 mm.* ***B****: Higher magnification of the muco-cutaneous junction. FF-PE. HE. Scale bar = 1 mm.*

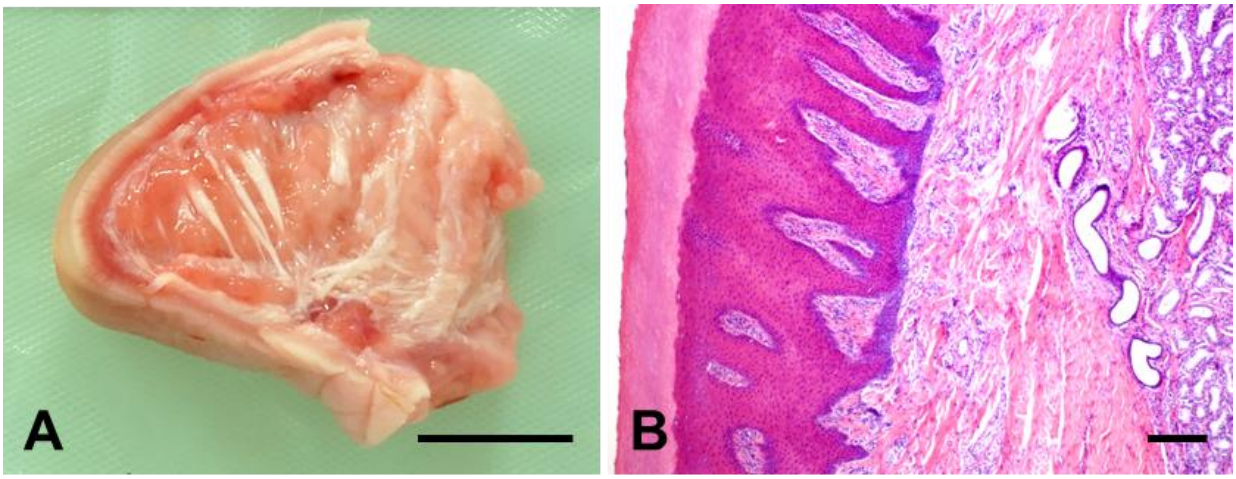


Fig. S22. Midsagittal section through the snout (A) and snout histology (B). ***B****: FF-PE. HE. Scale bars = 1 mm.*

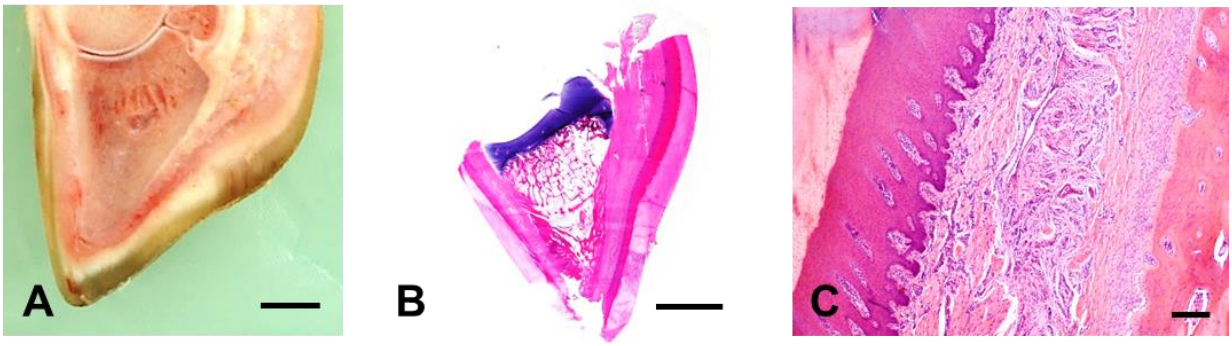


Fig. S23. Midsagittal section through the hoof (A), an image of a histology slide with a FF-DECAL-PE hoof section (B), and hoof histology (C). *Scale bars = 1 cm (A, B), 1 mm (C).* ***C****: FF-PE. HE. Scale bar = 1 mm.*

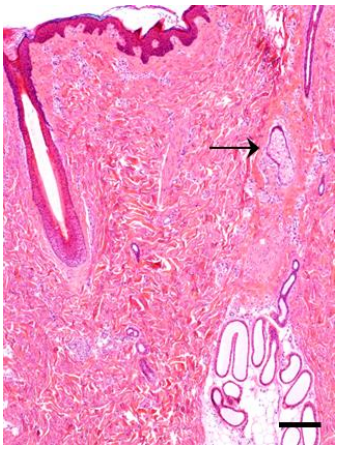


Fig. S24. Histology of the interorbital nasal bridge skin*. The arrow indicates a sebaceous gland sectional profile. FF-PE. HE. Scale bar = 100 µm.*

Time and personnel requirements

At least 20 minutes must be scheduled for the execution of a complete Type-II/III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimens; however, this estimate does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Mecklenburg et al., 2013, Ruehl-Fehlert et al., 2003, Summerfield et al., 2015, Turner et al., 2015)

2.2.2. Mammary gland

Relevant anatomical features/preparation
There are 12-16 mammary gland complexes in the pig. In the protocols for Type-I, -II, and -III studies, mammary gland specimens from female pigs are routinely taken from the left penultimate complex (Fig. S26).

General examination parameters
All mammary gland complexes are examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required. The size and weight of the excised penultimate mammary complex are determined.

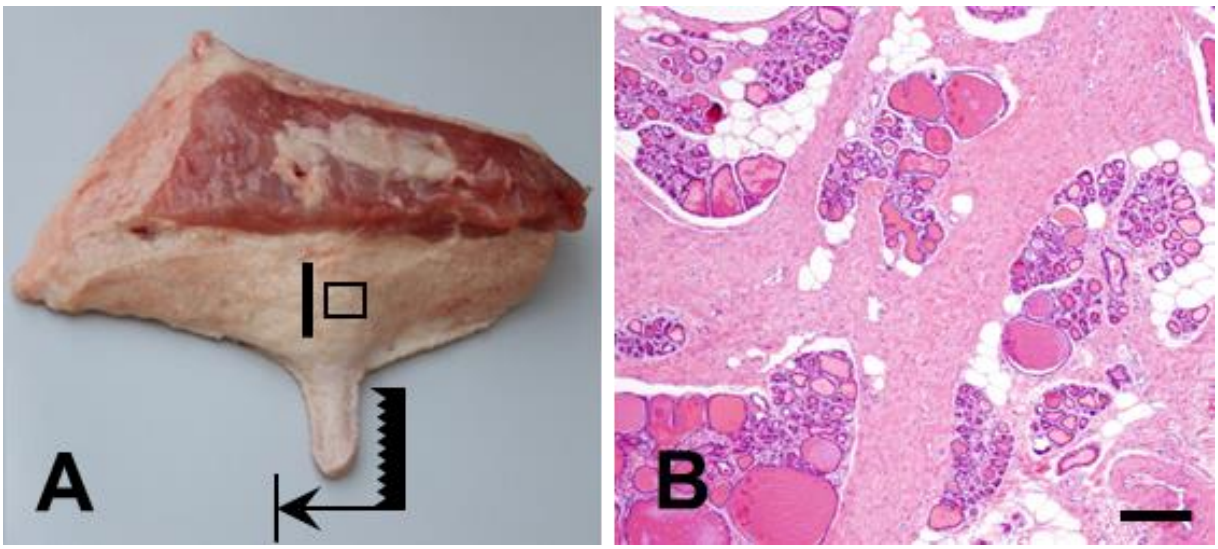


Fig. S25. Mammary gland. A*: A sagittal section of the left penultimate mammary gland complex of a nulliparous six-month-old sow. The locations and orientations of the histology (black lines) and molecular analyses samples (black rectangle) for Type-I studies are indicated.* ***B****: Histology of the mammary gland parenchyma. FF-PE. HE. Scale bar = 100 µm.*

2.2.2.1. Mammary gland samples in Type-I studies

In Type-I studies, samples of mammary gland tissue for molecular analyses and histopathological examination are collected from the left penultimate complex (Fig. S26).

Samples for molecular analyses

Location:	Left penultimate complex (Fig. S25 and Fig. S26).
Number of samples:	One.
Remarks:	A homogeneous sample is cut from the mammary gland parenchyma.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One sagittal section through the teat and one transverse section through the parenchyma of the left penultimate mammary gland complex as indicated in Fig. S25 and Fig. S26.
Fixation/Embedding:	FF-PE (Fig. S25).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Ruehl-Fehlert et al., 2003) recommend the collection of two samples of mammary gland tissue in both sexes. The samples are taken from the 5th inguinal mammary gland complex and usually include samples for skin examination. The same sampling recommendations are described in the INHAND (International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice) publication on proliferative and non-proliferative lesions of the rat and mouse mammary, Zymbal's, preputial, and clitoral glands (Rudmann et al., 2012). Comparable sampling efforts to examine the porcine mammary gland are recommended in the Type-I-study sampling protocols.

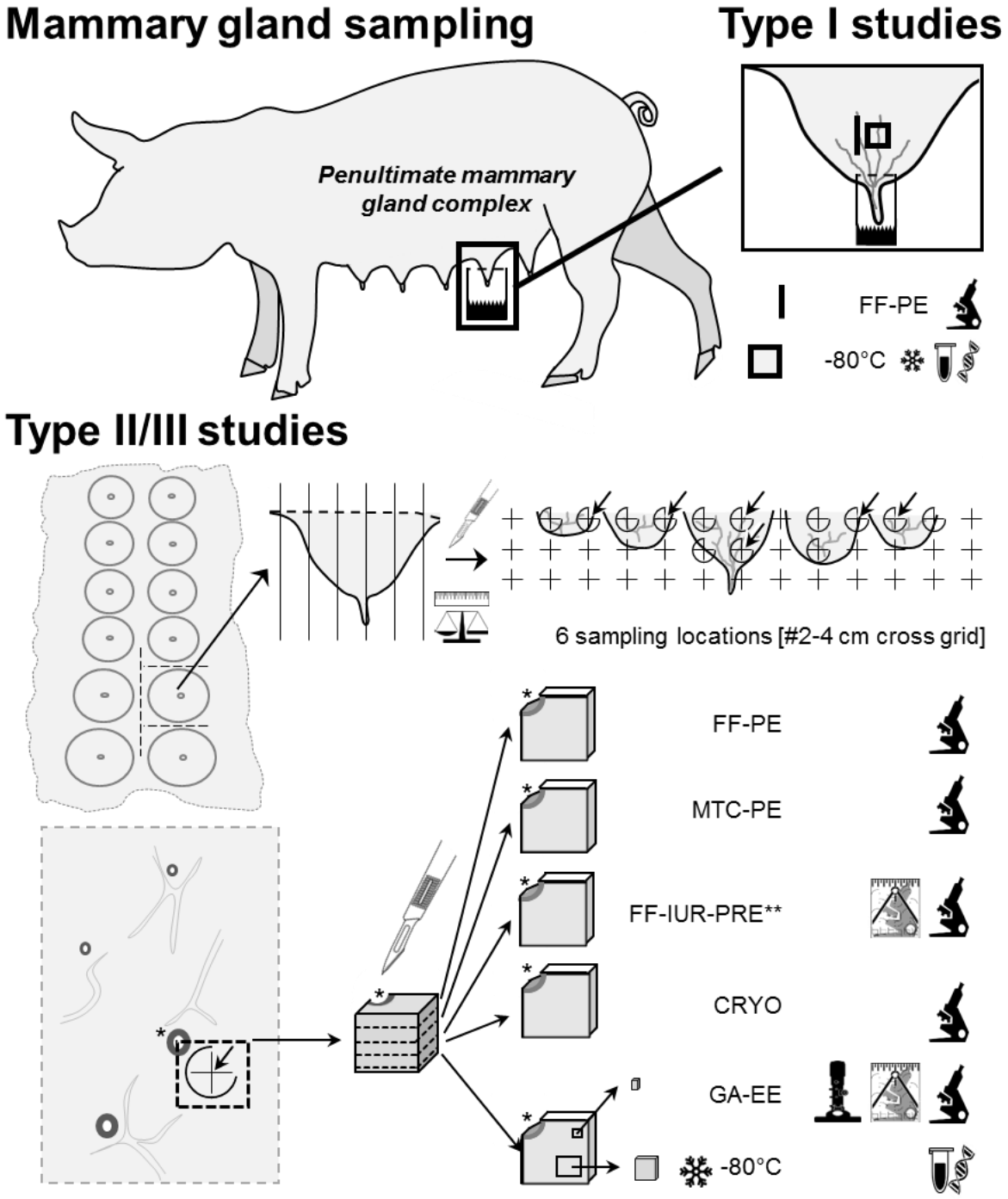


Fig. S26 (↑). *A schematic illustration of subsample preparation for different downstream analyses (Type-I-III studies) from one systematically randomly sampled location of mammary gland tissue. *Duct section profile. **PR = GMA/MMA.*

2.2.2.2. Mammary gland samples in Type-II and Type-III studies

General systematic random sampling strategy

The left penultimate mammary gland complex is sampled. The sampling positions and tissue size-dependent sample numbers are determined by systematic random sampling. The weight and dimensions of the skin-covered mammary gland complex is determined, and the tissue is cut into parallel equidistant slabs approximately 10-20 mm thick. The cuts are orthogonal to the width axis of the complex with the first section randomly positioned between 0 and 20 mm from the margin of the tissue (Fig. S26). The tissue slabs are then placed on the same side and counted.

Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-4 cm depending on the size and number of tissue slabs). The systematic random sampling of the mammary parenchyma is performed as described in section 1.4. and schematically shown in Fig. S26. The sampled positions can be marked by placing confetti paper pieces on the respective positions. An approximately 2x2x1-2 cm sized tissue piece is excised from each of the sampled locations. The sampled positions can be marked by placing pieces of confetti paper on the respective positions. An approximately 2x2x1-2 cm piece of tissue is excised from each of the sample locations. The excised tissue specimens are further subdivided into slabs to generate subsamples for multiple downstream analyses. These samples include FF-PE, MTC-PE, FF-IUR-PRE (GMA/MMA), CRYO, GA-EE and molecular analyses (Fig. S26).

Table S5. Types and numbers of mammary gland samples in Type-II and Type-III studies.

	Number of sampled locations/samples per location	
Sample type	**Type-II studies**	**Type-III studies**
	6	*6*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 per sampled location
CRYO	1 from every 3rd location	1 per sampled location
GA-EE	1 per sampled location	1 per sampled location
FF-PRE (IUR)	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

Remarks

For studies that explicitly examine the complete mammary gland in detail, samples can be generated from the parenchyma of all complexes; however, this would exceed the workload outlined in these guidelines.

Time and personnel requirements

At least 15-20 minutes must be allotted for the execution of the complete Type-II/III-study sampling procedure by an experienced investigator. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of sampled specimens; however, it does not include the time required for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Rudmann et al., 2012, Ruehl-Fehlert et al., 2003)

2.3. Adipose tissue

Relevant anatomical features/preparation

As a unique feature, pigs lack brown adipose tissue (Berg et al., 2006, Trayhurn et al., 1989). White adipose tissue specimens are taken from defined locations. Subcutaneous adipose tissue samples are harvested from the back and ventral abdomen. Visceral adipose tissue samples are taken from the perirenal and mesenteric adipose tissues. These guidelines do not recommend the sampling of adipose tissue depots, such as epididymal adipose tissue, which is frequently used in rodent studies. The locations of the storage adipose tissue depots to be sampled are specified in Table S6 and Fig. S27, S28 and S31. These depots are present in both sexes, easily accessible, and sufficient amounts of adipose tissue can typically be harvested from these locations. In pigs, the subcutaneous adipose tissue of the back has a rather firm consistency, whereas adipose tissue from other locations is very soft. The preparation of adipose tissue samples for quantitative stereological analyses is illustrated in Fig. S30 in section 2.3.2.

General examination parameters

Adipose tissue depots are examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, and molecular analyses are taken from the altered site(s) if required.

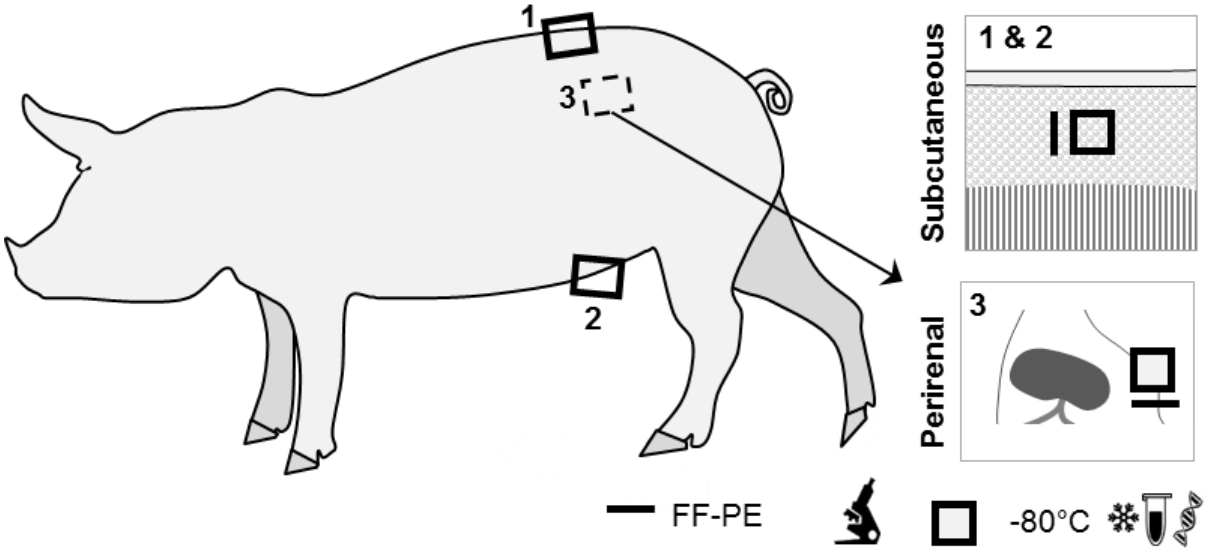


Fig. S27. The adipose tissue sampling locations for routine screenings. *The costal arch is indicated with a dashed line.*

Table S6. Adipose tissue sampling locations in Type-I-III studies.

Study type	Location	Anatomic landmarks
I, II, III	Back (subcutaneous)	Middle of the back (left side) and caudal to the last rib.
I, II, III	Abdomen (subcutaneous)	Caudal to the left penultimate teat.
I, II, III	Perirenal (visceral)	Caudal to the left kidney.
II, III	Mesenteric (visceral)	Mid-jejunal mesentery.

In Type-I studies, the samples are taken from one location (left side). In Type-II and -III studies, both sides are sampled.

2.3.1. Adipose tissue samples in Type-I studies

Samples for molecular analyses

Location:	One sample of subcutaneous adipose tissue is taken from the back (left side), and one sample is taken from the left abdomen. One sample of perirenal adipose tissue is taken from the left kidney. The sampling locations are indicated in Fig. S27, Fig. S28, and Table S6.
Number of samples:	One per location.
Remarks:	Homogeneous samples of adipose tissue are cut from the middle of the respective adipose tissue layer.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One sample of subcutaneous adipose tissue is taken from the back (left side) and left abdomen. One sample of perirenal adipose tissue is taken from the left kidney. Section orientations are indicated in Fig. S27 and Fig. S28.
Fixation/Embedding:	FF-PE (Fig. S29). Alternatively, fixed adipose tissue samples can be defatted in a 1:1 (vol) mixture of ethyl alcohol (100%) and xylene (100%) for 2-4 d at RT prior to processing for paraffin embedding to ensure the complete defatting of the sample.

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies

Adipose tissue is not commonly evaluated by histopathology in regulatory toxicity studies in rodents. Chemical/toxicological analyses of adipose tissue might be important in chronic toxicity studies; however, a selective generation of specimens from macroscopically unaltered adipose tissue for histopathological examination in regulatory toxicity studies in pigs is not necessary because adipose tissue can be evaluated in other samples, such as the skin. The collection and examination of adipose tissue specimens from different storage adipose tissue depots, such as subcutaneous and intraabdominal adipose tissue, should be used when animals of “newly developed” pig models, particularly models displaying metabolic disorders, are necropsied for an initial pathological examination. Even if the focus of interest includes the molecular analyses of adipose tissue samples, the corresponding histology samples should be collected to allow for the evaluation of possible morphological alterations. Therefore, the collection of adipose tissue samples for molecular analyses and histopathological evaluation was included in the porcine Type-I-study sampling protocols.

2.3.2. Adipose tissue samples in Type-II and Type-III studies

General sampling strategy

In Type-II and -III studies, adipose tissue samples are generated from the same locations as in Type-I studies. Additional samples are collected from mesenteric adipose tissue to sample adipose tissue from depots with presumably different metabolic properties (Ouchi et al., 2011, Wang et al., 2013). Unlike Type-I studies in which samples are taken from one location (left side), both sides (right and left) are sampled in Type-II and -III studies. Unlike other organs and tissues in this guide, the sampling locations in Type-II and -III studies adipose tissue specimens are not determined by systematic random sampling, and the entire volume or weight of the respective adipose tissue depot is not calculated. If the reference compartment volume of the respective adipose tissue depot is needed for the calculation of the total number of fat cells within a given depot using quantitative stereological analyses, the complete removal and weighing of the adipose tissue from an adipose tissue depot can be attempted. Due to the rather unclear delimitation of adipose tissue from its adjacent tissue, this is usually not possible with sufficient precision. Alternatively, the volume of a respective adipose tissue depot can be calculated from 3D-CT scans (Cavalieri principle).

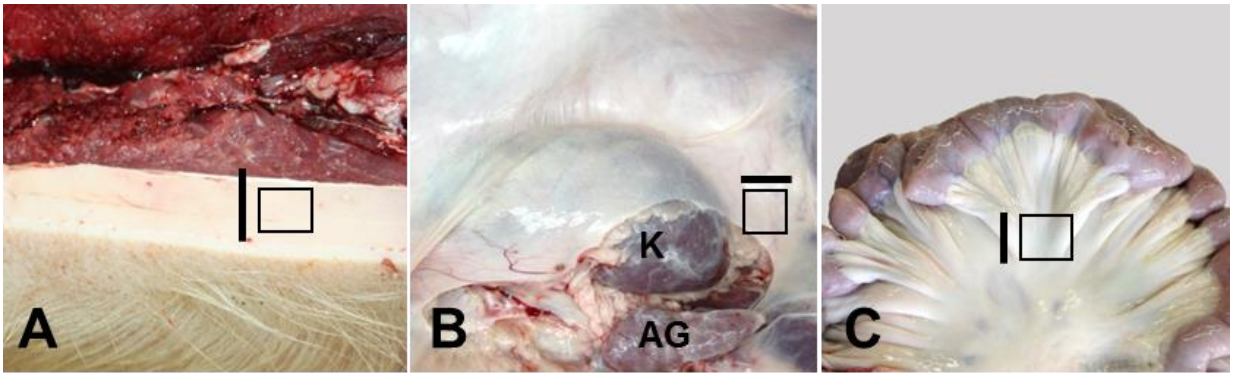


***Fig. S28. Adipose tissue sampling locations**. The locations and orientations of the histology (black lines) and molecular analyses samples (black rectangles) for Type-I studies are indicated. **A**: Subcutaneous adipose tissue (back). **B**: Perirenal adipose tissue. Ventral aspect of the left kidney (K) and adrenal gland (AG). **C**: Mesenteric adipose tissue in the jejunal mesentery.*

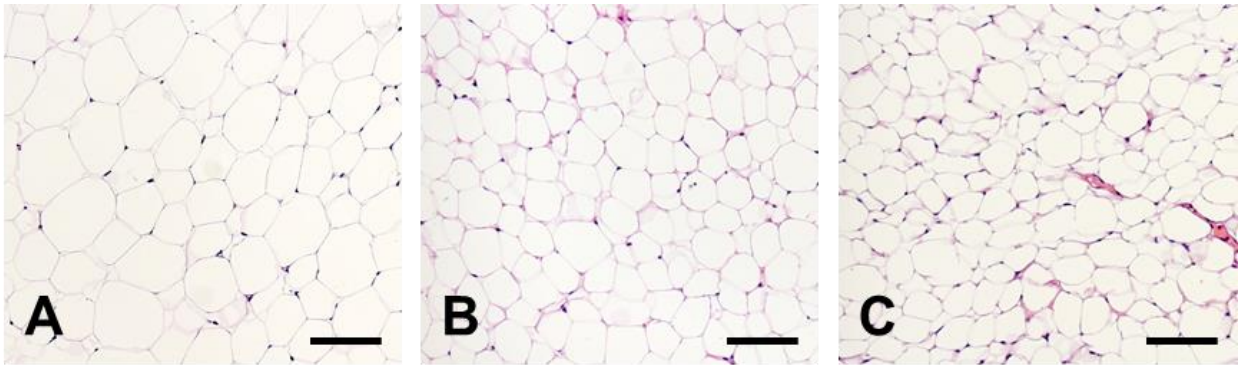


***Fig. S29. Adipose tissue histology**. **A**: Subcutaneous adipose tissue from the abdomen. **B**: Perirenal adipose tissue. **C**: Mesenteric adipose tissue. Note the different mean fat cell profile areas in A vs. B and C. FF-defatted-PE tissue. HE. Scale bars = 100 µm.*

Adipose tissue samples for histopathological examination and molecular analyses in Type-II and Type-III studies

All adipose tissue samples to be generated in Type-I-III studies are listed in Table S7. In both Type-II and Type-III studies, FF-PE, CRYO and molecular analysis samples are generated. For quantitative stereological analyses, three adipose tissue specimens from the right and the left side of the body are processed for generation of FF-ORTRIP-PRE (GMA/MMA) sections as indicated in Fig. S31 and shown in section 1.6.3.

To facilitate the embedding of adipose tissue samples in plastic resins, such as GMA/MMA, the formalin-fixed adipose tissue specimens are defatted in a ethyl alcohol-xylene mixture (section 2.3.1.) for 2-4 d at RT, depending on the sample size. Defatting of adipose tissue samples will prevent the floating of samples during the embedding procedure. For non-defatted adipose tissue samples, it may be necessary to weigh them down in the casting mold while the liquid constituents of the plastic embedding medium harden. Regardless of fresh or formalin-fixed conditions, these specimens are easily deformable due to the soft consistency of adipose tissue. For the analysis of embedding-related tissue shrinkage in quantitative stereological analyses, the fixed and defatted adipose tissue samples are embedded in ink-blackened agar prior to IUR-sectioning and embedding in plastic resin (Fig. S30). The final section surface of the sample is then scanned with a size scale. It is advantageous to use agar blackened with dark ink. This method will increase the contrast between the adipose tissue and the surrounding agar, thereby facilitating the identification of the adipose tissue excision borders in the scanned images of the adipose tissue section profiles prior to the plastic resin embedding and the corresponding histological sections (Fig. S30). The sample size for Epon-embedded specimens is usually restricted to few mm^3, and adipocytes are comparably large cells with small nuclei; thus, only a few section profiles will contain adipocytes, and even fewer section profiles of adipocyte cell nuclei will be present in sections of Epon-embedded adipose tissue specimens. These characteristics substantially limit the suitability of Epon-embedded adipose tissue specimens for quantitative stereological methods, such as physical disector analyses (Gundersen, 1986) that estimate the numerical volume densities of fat cell nuclei (fat cells) in the adipose tissue. Therefore, the proposed Type-II and -III sampling protocols do not recommend the generation of GA-EE samples of adipose tissue for quantitative stereological analyses. Generation of GA-EE specimen of adipose tissue is only scheduled in Type-III studies for performance of ultrastructural examination.

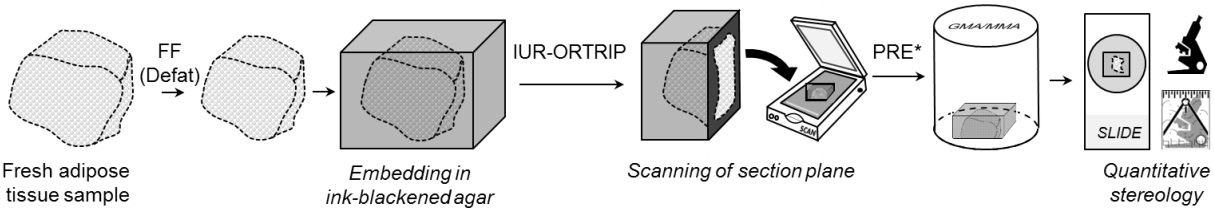


Fig. S30. *The processing of adipose tissue samples for preparation of FF-(defatted)-ORTRIP-PRE sections for plastic histology and quantitative stereological analyses of adipose tissue specimens in Type-II/III studies. The fixed and defatted adipose tissue sample is cast in ink-blackened agar, IUR-sectioned (ORTRIP), and the final sectioned surface scanned. The sample is then embedded in plastic resin (GMA/MMA) and sectioned. The sectional area of the GMA/MMA-embedded adipose tissue sample is determined (Fig. S8). The embedding-related tissue shrinkage is calculated from the sectional area of the fixed adipose tissue prior to embedding in plastic resin and the corresponding sectional area of the GMA/MMA-embedded tissue sample.*

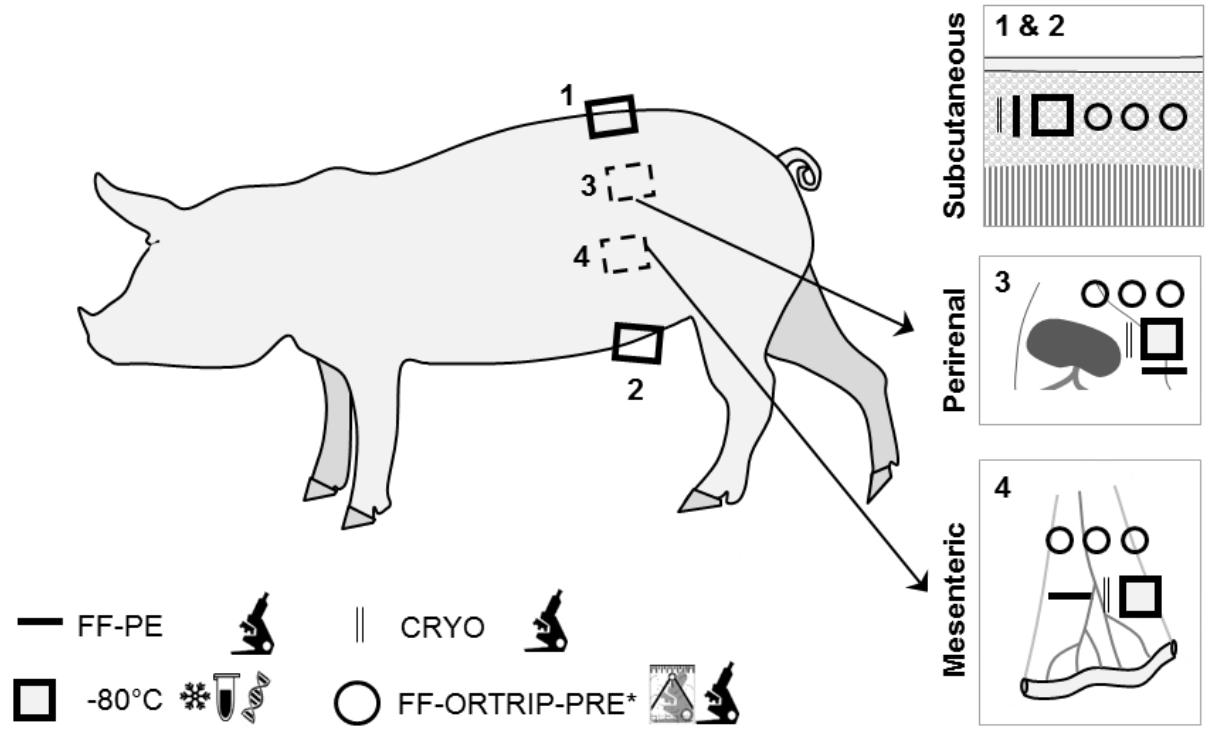


Fig. S31. The schematic illustration of adipose tissue sampling in Type-II studies. **PR = GMA/MMA. Generation of GA-EE specimen (●) is recommended only for Type-III studies.*

Table S7. The type and number of adipose tissue samples in Type-I-III studies.

	Number of samples per location*		
Sample type	**Type-I studies**	**Type-II studies**	**Type-III studies**
FF-PE	1	1	1
CRYO	-	1	1
GA-EE	-	-	1
FF-ORTRIP-PRE (GMA/MMA)	-	3	3
Molecular analyses (-80°C)	1	1	1

**Locations: Subcutaneous adipose tissue of the back and ventral abdomen and perirenal adipose tissue (Type-I-III studies) and adipose tissue from the jejunal mesentery (Type-II and -III studies). In Type-I studies, samples are taken from one location (left side). In Type-II and -III studies, both sides (right and left) are sampled, except for the adipose tissue from the jejunal mesentery.*

Time and personnel requirements

At least 15 minutes must be scheduled for the complete Type-II/III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of sampled specimens; however, it does not include the time needed for the preparation and labeling of necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Greaves et al., 2013, Ouchi et al., 2011, Wang et al., 2013)

2.4. Blood vessels (large arteries and veins)

Generally, pigs are regarded as valuable models for human vascular disorders, such as atherothrombotic disease (Vilahur et al., 2011).

Relevant anatomical features/preparation

Depending on the location of the blood vessels (Table S8), samples are collected at different phases of the necropsy. The samples of the aorta and of the caudal caval vein are collected after evisceration of the thoracic and the abdominal cavities. The coronary vessels are sampled with the heart, whereas the carotid artery and the jugular vein are dissected with other cervical organs and tissues, such as the thyroid gland and thymus. After the vessels have been localized and identified (Table S8), the samples are taken according to the sampling protocols for Type-I, -II and -III studies. Before sampling, larger blood vessels are cut open with a longitudinal incision using scissors with the blunt scissor end inside the vessel. All sampled vessels and sampling positions are shown in Table S8 and in Fig. S32 and Fig. S33.

General examination parameters

The blood vessels are examined for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required. Note that cervical vessels might be affected by injection-related thrombosis/thrombo-phlebitis/arteritis.

Table S8. Sampled blood vessels (large arteries and veins) in Type-I-III studies.

Study type	Vessel	Location
I	Thoracic aorta	Directly distal to the aortic arch (Fig. S33).
II, III	Abdominal aorta	Proximal to the terminal branching site (Fig. S33).
I-III	Caudal caval vein	Next to the sampling location of the abdominal aorta (Fig. S33).
II, III	Carotid artery	Left side, middle third of the neck (Fig. S33).
II, III	Jugular vein	Left side, middle third of the neck (Fig. S33).
I*-III	Coronary vessels	As indicated in Fig. S34.

**In Type-I studies, coronary vessel morphology is examined in standard histology sections of the heart (no samples for molecular analyses are generated).*

2.4.1. Blood vessel samples in Type-I studies

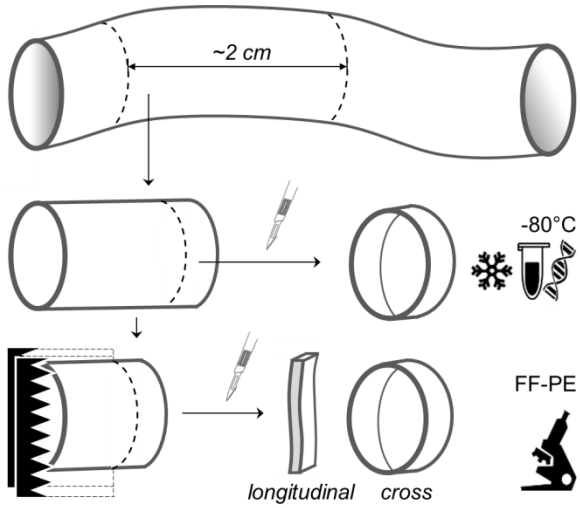


Fig. S32. *A schematic illustration of blood vessel sampling in Type-I studies.*

Samples for molecular analyses

Location:	Locations are specified in Table S8, Fig. S33 and Fig. S34.
Number of samples:	One per location.
Remarks:	If the vessel diameter is too large to use an entire cross section as a sample, four small (1x1 mm x full thickness) subsamples from the cross section at the 3, 6, 9, and 12 o'clock positions are excised.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Locations are specified in Table S8, Fig. S33 and Fig. S34. From each location, one cross section and one longitudinal section is cut as indicated in Fig. S33 and Fig. S34.
Fixation/Embedding:	FF-PE (Fig. S35).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Morawietz et al., 2004) recommend the collection of one cross-section sample of the thoracic aorta. In Type-I-study sampling protocols for porcine models, the caudal caval vein is also sampled for the evaluation of large veins.

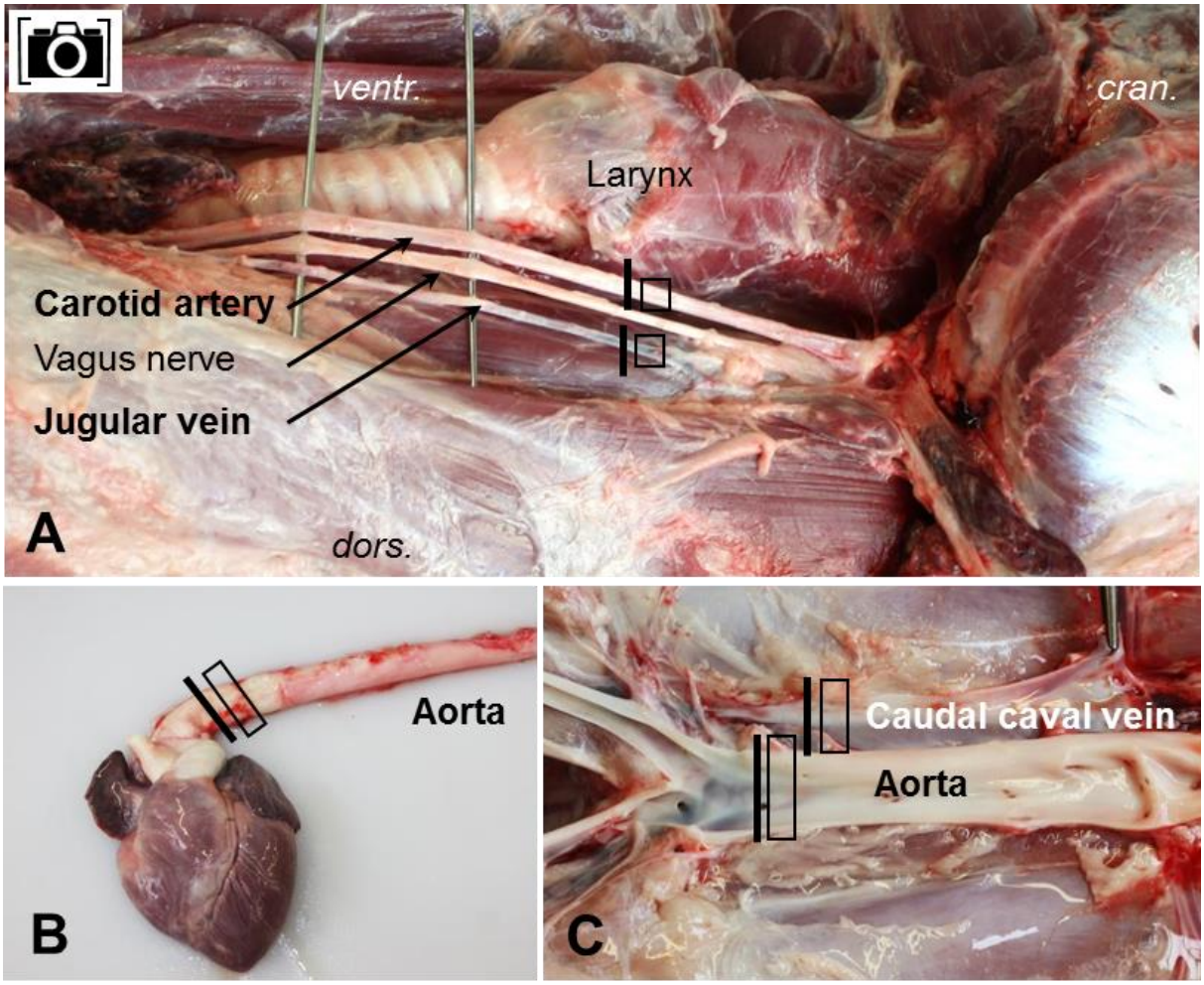


Fig. S33. *Macroscopic images of large blood vessels and sampling positions.* ***A****: Left carotid artery and jugular vein at the level of the larynx.* ***B****: Thoracic aorta.* ***C****: Abdominal aorta and the caudal caval vein proximal to the terminal branch of the aorta. Directions in A are indicated (ventral, dorsal, cranial).*

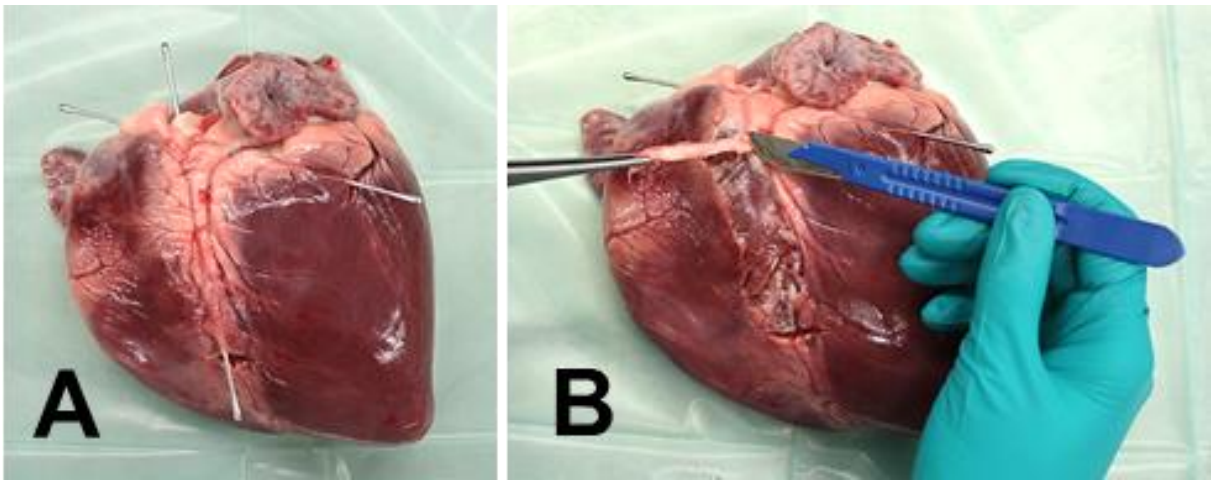


Fig. S34. *Dissection of coronary vessels.* ***A****: For illustration purposes, probes were inserted into the coronary arteries.* ***B****: Dissection of coronary vessels using a scalpel. Note that the preparation of isolated coronary vessel samples should be coordinated with the generation of heart samples (section 2.9.2.). The specimens of the coronary vessels are often harvested after sampling of the myocardium.*

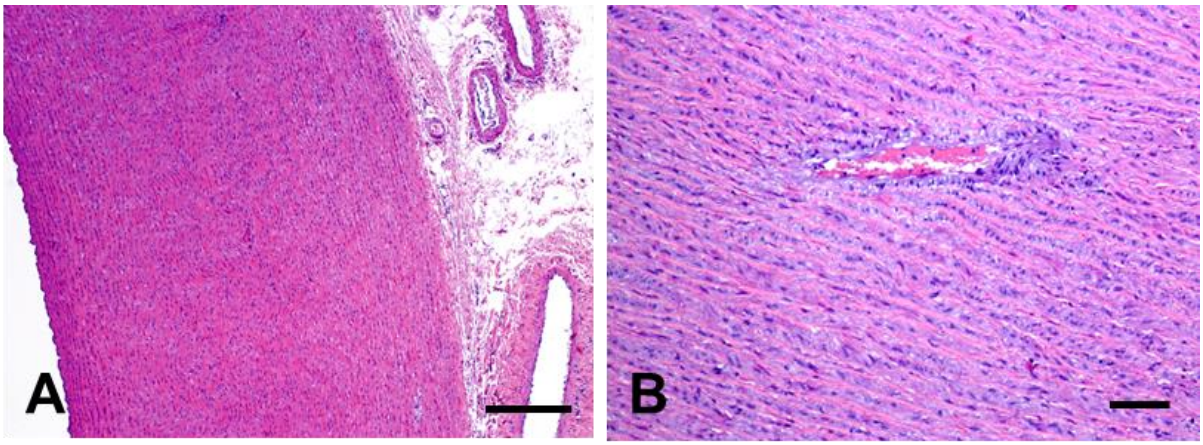


Fig. S35. Histology of the aorta. *FF-PE. HE.* ***A****: Overview. Cross section. Scale bar = 1 mm.* ***B****: Detailed enlargement of the tunica media with a section of the vasa vasorum. Cross section. Scale bar = 100 µm.*

2.4.2. Blood vessel samples in Type-II and Type-III studies

The type and number of samples for histopathological examination and molecular analyses of blood vessels in Type-II and Type-III studies

The same locations used in Type-I studies (Table S8) are sampled. From each vessel, a piece approximately 2-4 cm long is excised and further divided into subsamples for different downstream analyses. For details, refer to Fig. S36 and Table S9.

Table S9. The type and number of blood vessel samples in Type-II and Type-III studies.

	Number of samples taken per blood vessel	
Sample type	**Type-II studies**	**Type-III studies**
FF-PE	1 l*, 1c*	1 l*, 1c*
MTC-PE	-	1 l*, 1c*
CRYO	1 l*, 1c*	1 l*, 1c*
GA-VUR-EE	-	6
FF-PRE	1 l*, 1c*	1 l*, 1c*
Molecular analyses (-80°C)	1 (4 pieces)	1 (4 pieces)

**l: Longitudinal section; c: Cross section.*

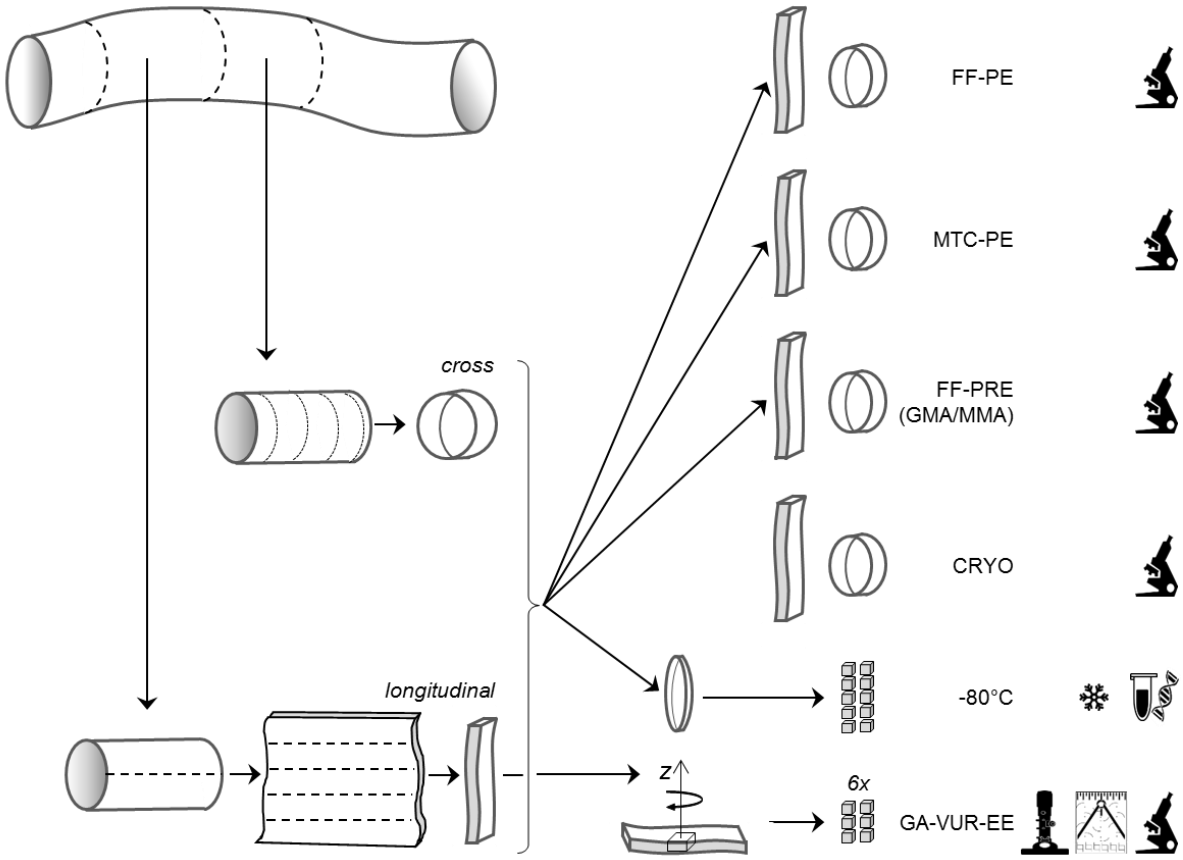


Fig. S36. *A schematic illustration of preparation of subsamples for different downstream analysis types (Type-II/III studies) from an excised piece of blood vessel. The sample is divided into two pieces. One sample is cut open longitudinally and then cut into 5-6 parallel longitudinal strips. The other piece is cut into 5-6 cross sections. One FF-PE, MTC-PE, FF-PRE (*PR = GMA/MMA), and CRYO sample is prepared from the longitudinal section and cross section pieces of the blood vessel wall. Several small samples (~1x1x2 mm) without a preferred section orientation are prepared for molecular analyses and frozen at -80°C. VUR-EE samples are prepared from a piece of longitudinally sectioned GA-fixed sample of the vessel wall.*

Time and personnel requirements

At least 25 minutes must be scheduled for execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of sampled specimen; however, it does not include the time required for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Al-Mashhadi et al., 2015, Berridge et al., 2013, Vilahur et al., 2011)

2.5. Immune and hematopoietic system

In this section, the sampling of lymph nodes, thymus, palatine tonsils, and bone marrow are described. The sampling of the spleen is described in section 2.10.5.

2.5.1. Lymph nodes

Relevant anatomical features/preparation

In pigs, afferent lymphatics enter the center of the lymph node and efferent lymphatics leave the lymph node from its periphery (Meurens et al., 2012). These guidelines recommend the sampling of distinct lymph nodes that are relatively easy to access and identify, including lymph nodes of the head, hindlimbs, lungs and intestinal tract (Fig. S37 and Table S10). For the routine assessment of systemic and local immunotoxicity in general toxicity studies, the lymph nodes are sampled according to the route of administration of the drug or xenobiotic (section 2.5.1.1.) (Haley et al., 2005). For bilateral lymph nodes, such as mandibular, inguinal, and popliteal lymph nodes, it is generally recommended that the lymph nodes are consistently sampled from one side (left) of the body to standardize sample collection and reduce workload.

General examination parameters

If a marked enlargement or reduction in lymph node size is evident, the sizes (dimensions) and weights of the sampled lymph nodes are determined (Haley et al., 2005). Lymph nodes are examined for pathological alterations. If present, these are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered lymph nodes if required.

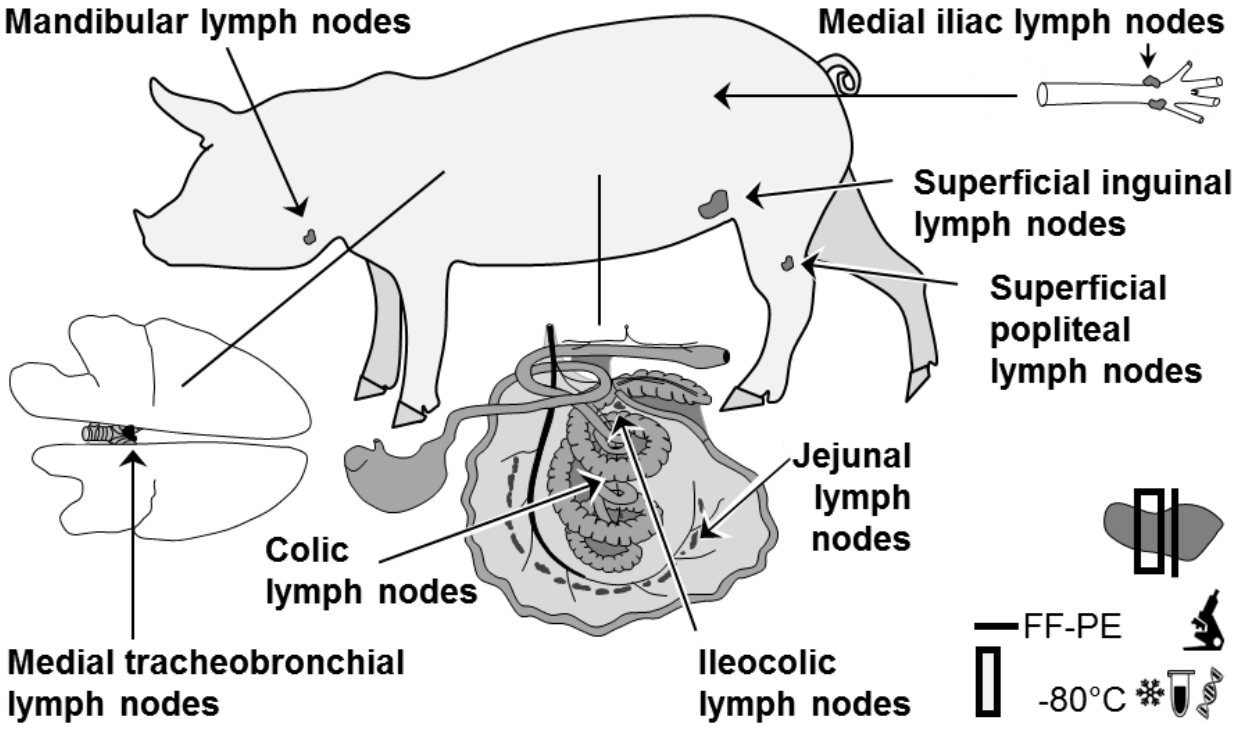


Fig. S37. A schematic illustration of the anatomical location of the different lymph nodes examined in Type-I-III studies. *The location of lymph nodes, orientation of the histology samples (black lines) and molecular analyses samples (black rectangles) for Type-I studies are indicated.*

Table S10. The anatomical locations of lymph nodes.

Study type*	Lymph nodes	Anatomic landmarks/size of sample
I-III	Superficial inguinal lymph nodes	Group of 3-4 nodules located in the subcutaneous tissue of the inguinal region (Fig. S37 and Fig. S39).
I-III	Jejunal lymph nodes	In the jejunal mesentery (Fig. S37 and Fig. S194).
II, III	Mandibular lymph nodes	At the angle of the jaw (Fig. S37).
II-III	Superficial popliteal lymph nodes	Deep in the adipose tissue of the popliteal fossa (may be small or occasionally absent) (Fig. S37 and Fig. S40).
I-III	Medial tracheobronchial lymph nodes	Caudal to the bifurcation of the trachea (Fig. S37 and Fig. S171).
II, III	Colic lymph nodes	In the center of the colonic spiral (Fig. S37 and Fig. S38).
I- III	Ileocolic lymph nodes	Adjacent to the ileocecal junction (Fig. S37 and Fig. S38).
II, III	Medial iliac lymph nodes	At the terminal branching of the abdominal aorta (Fig. S37 and Fig. S38).

**Not including routine toxicity studies. The sampling of lymph nodes in toxicity studies is described separately in section 2.5.1.1.*

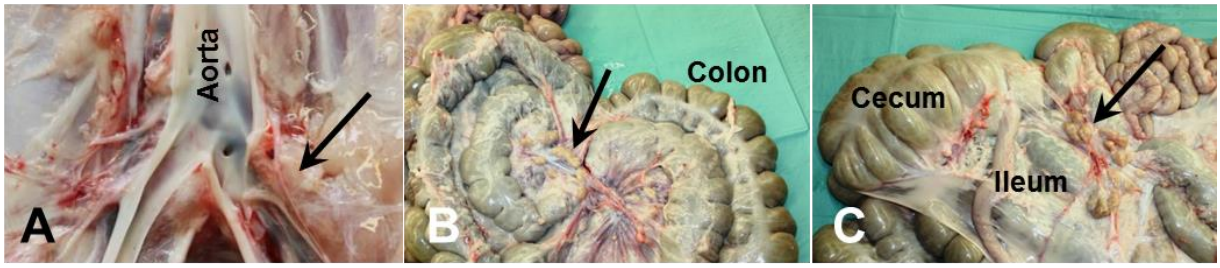


Fig. S38. *Anatomical location of the medial iliac lymph nodes (**A**), colic lymph nodes (**B**), and ileocolic lymph nodes (**C**).*

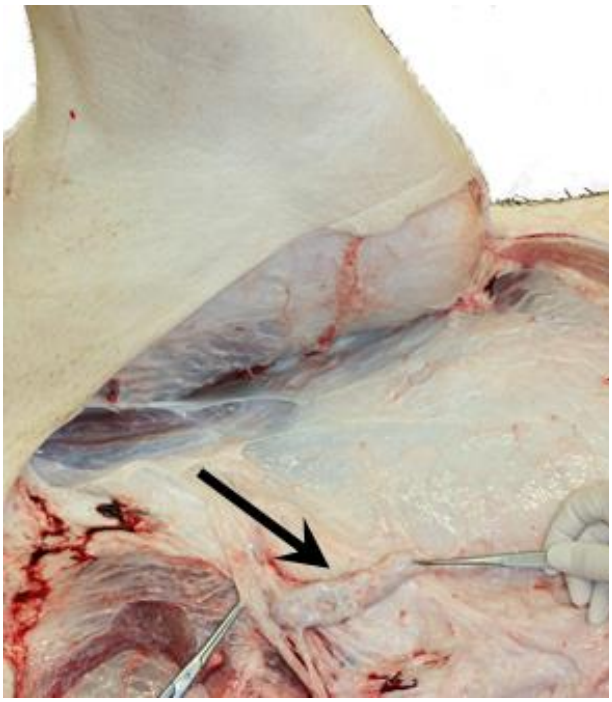


Fig. S39. Location of the superficial inguinal lymph nodes *(arrow).*

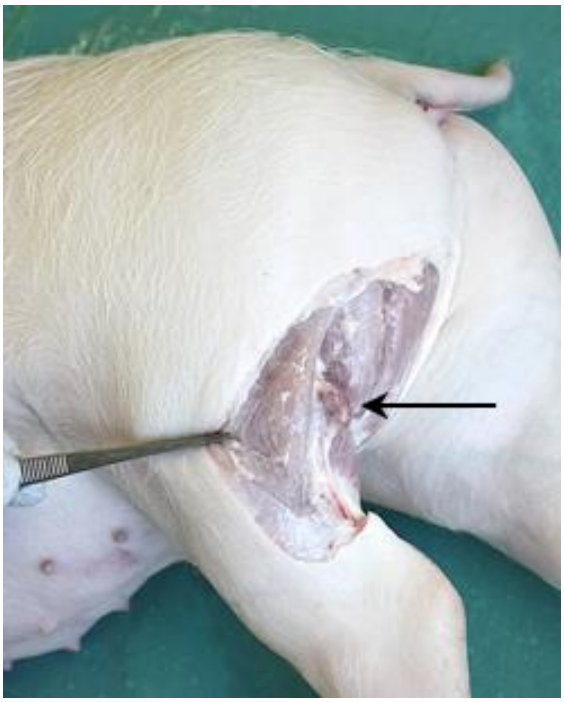


Fig. S40. Location of the superficial popliteal lymph nodes *(arrow).*

2.5.1.1. Lymph node samples in Type-I studies

In non-toxicological studies, superficial inguinal lymph nodes, medial tracheobronchial lymph nodes (section 2.9.1.1.), jejunal lymph nodes, and ileocolic lymph nodes (section 2.10.2.1.) are sampled on a routine basis for histopathological examination. Samples for molecular analyses are collected from only the left superficial inguinal lymph nodes.

Samples for molecular analyses in non-toxicological studies

Location:	One sample of a left superficial inguinal lymph node is taken as indicated in Table S10 and Fig. S39.
Number of samples:	One.
Remarks:	A thin strip of tissue containing all lymph node compartments is collected.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples in non-toxicological studies

Location, number and orientation of sections:	One cross section each for the left superficial inguinal, medial tracheobronchial, jejunal, and ileocolic lymph nodes are taken as indicated in Table S10, Fig. S38, Fig. S39 and Fig. S40, and sections 2.9.1.1. and 2.10.2.1. The sections should contain all lymph node compartments.
Fixation/Embedding:	FF-PE (Fig. S41).

Samples for molecular analyses and histopathology in routine toxicity studies.

For the routine assessment of systemic and local immunotoxicity in general toxicity studies, the samples for the histopathological examination of lymph nodes are collected according to the route of administration of the drug or xenobiotic. The samples for molecular analyses are collected from all lymph nodes sampled for histopathological examination.

Xenobiotic/drug administration route/location and number of samples

Oral administration:	One (left) mandibular lymph node, 2-3 jejunal lymph nodes, and one ileocolic lymph node.
Inhalation study:	2-3 medial tracheobronchial lymph nodes.
Local and parenteral application:	1-2 draining lymph nodes (evaluation of local effects) and 2-3 lymph nodes distant from the site of application (evaluation of systemic effects).
Sample processing, and downstream analyses:	For molecular analyses, a thin strip of tissue containing all lymph node compartments is cut from the lymph node, frozen on dry ice and stored at -80°C for subsequent DNA, RNA, and protein analyses (OMICS profiling). The histology samples consist of one cross section per lymph node and contain all lymph node compartments. FF-PE (Fig. S41).

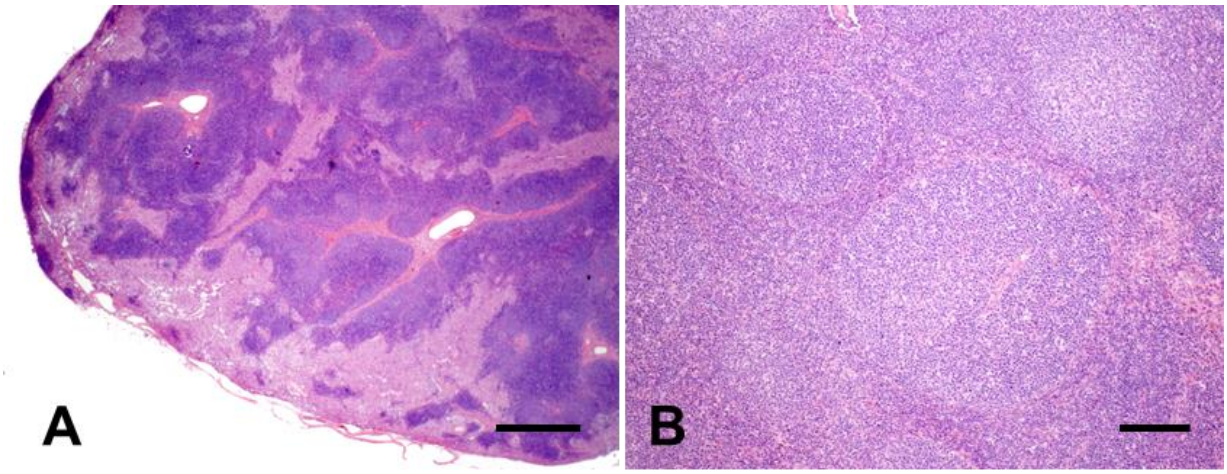


***Fig. S41. Histology of a medial tracheobronchial lymph node. A**: Overview. **B**: Detailed enlargement of the lymph node cortex. FF-PE. HE. Scale bars = 100 µm.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

The Immunotoxicity Screening Working Group (STP IWG) of the Society of Toxicologic Pathology (STP) recommends the sampling of lymph nodes based on the drug application site in toxicity studies (Haley et al., 2005). For orally administered drugs, the mesenteric lymph nodes should be sampled, and the most proximal draining peripheral lymph nodes should be sampled in studies with cutaneous, subcutaneous, or intradermal applications of xenobiotics. For histopathological evaluation, the sections should contain all separate lymph node compartments.

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Morawietz et al., 2004) recommend the collection of mesenteric lymph node samples for oral studies, lung-associated lymph nodes for inhalation studies, and additional lymph nodes based on the route of drug application. One longitudinal section is prepared per lymph node. The proposed Type-I-study sampling protocols for non-toxicological studies in porcine biomedical models and general toxicity studies in pigs recommend comparable sampling procedures.

2.5.1.2. Lymph node samples in Type-II and Type-III studies

General systematic random sampling strategy

Lymph node samples for advanced analyses (Type-II and -III studies) are taken from the left and right superficial inguinal lymph nodes or other appropriate lymph nodes based on the specific study. The lymph nodes of both sides are excised, weighed and measured. One lymph node is chosen randomly, fixed in formaldehyde solution, and then completely sectioned in parallel, equidistant sections with an approximate thickness of 5 mm. For quantitative and qualitative histopathological analyses, the sections are embedded in paraffin following the procedures outlined in the Type-II/III study ovary-sampling protocol (section 2.11.2.2.1.). Notably, all quantitative stereological parameters cannot be estimated using this sample material. The other lymph node is sectioned using the same procedure. The tissue slabs are then placed on the same side and counted. One slab is randomly chosen, divided into two halves and processed for MTC-PE and CRYO (Fig. S42). Next, the remaining slabs are overlaid with a cross grid printed on a transparency (grid size: 3-10 mm, depending on the organ size and number and area of tissue slabs). The systematic random sampling of six tissue locations is performed as described in section 1.4. and indicated in Fig. S42. An approximately 0.5x0.5x0.5-cm tissue piece is excised from each of the sampled locations, regardless of the lymph node compartment. The excised tissue specimen is further subdivided into one sample for GA-EE and one sample for molecular analyses (Fig. S42).

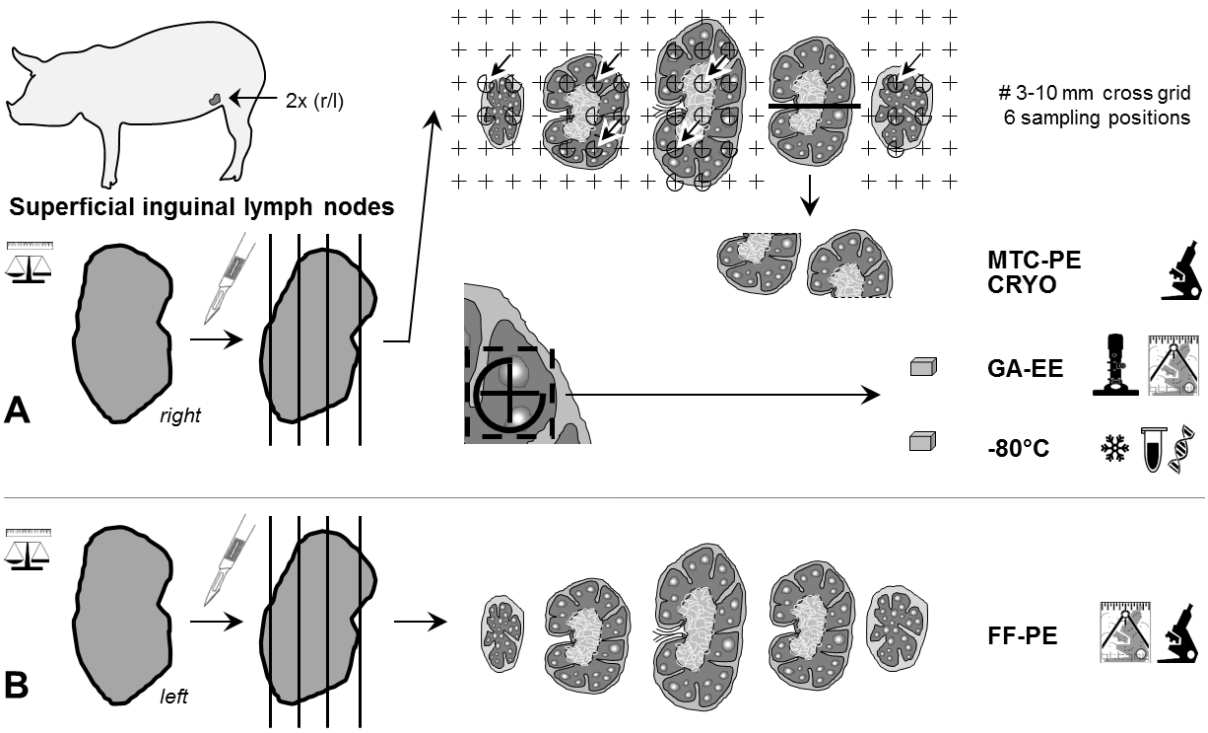


Fig. S42. *A schematic illustration of superficial inguinal lymph node sampling in Type-II/III studies.*

Time and personnel requirements

At least 20 minutes must be scheduled for the execution of the complete Type-II/III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of sampled specimen; however, it does not include the time needed for the preparation and labeling of necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time required for further sample processing after fixation.

Related literature

(Blutke et al., 2015, Cheng et al., 2015, Docampo et al., 2014, Haley et al., 2005, Meurens et al., 2012, Morawietz et al., 2004)

2.5.2. Thymus

Relevant anatomic features/preparation

In pigs, the well-developed cervical part of the thymus has a cranial and a caudal portion (Liebich and König, 2008). These guidelines recommend the sampling of the caudal part of the cervical portion of the thymus because it is reasonably well-delimited (Fig. S43, Fig. S44). The thymus is a tan, white or beige, soft lobulated tissue mass that can usually be easily identified and differentiated from adjacent adipose tissue. The thymus is removed from the body with the tongue, larynx, esophagus, trachea, lungs and heart (section 2.9.).

General examination parameters

The size (dimensions) and weight of both (right and left) caudal parts of the cervical portion of the thymus are determined. The thymus is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

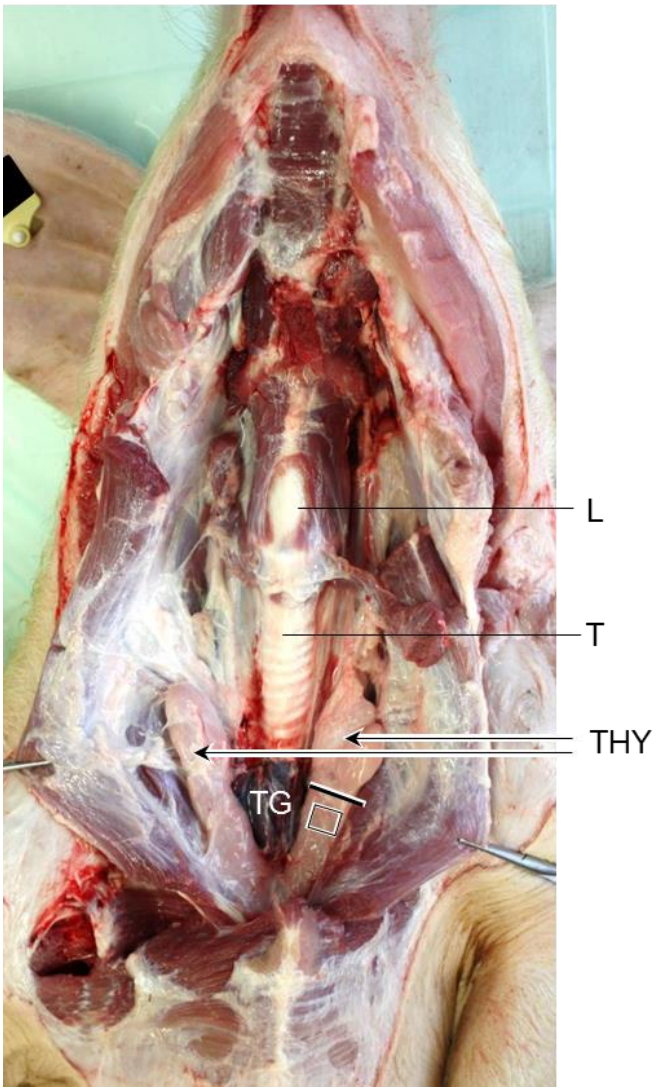


Fig. S43. *The anatomical location of the caudal part of the cervical portion of the thymus (THY) in a six-month-old pig (ventral aspect of the neck). L: Larynx; T: Trachea; THY: Thymus; TG: Thyroid gland. The locations and orientations of the histology (black line) and molecular analyses samples (black rectangle) for Type-I studies are indicated.*

2.5.2.1. Thymus samples in Type-I studies

Samples for molecular analyses

Location:	Left caudal part of the cervical portion of the thymus as indicated in Fig. S43 and Fig. S44.
Number of samples:	One.
Remarks:	A homogeneous sample is cut from the thymus parenchyma.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One cross section of the left caudal part of the cervical portion of the thymus is taken for histopathological examination. The sampling location is indicated in Fig. S43 and Fig. S44.
Fixation/Embedding:	FF-PE (Fig. S45).

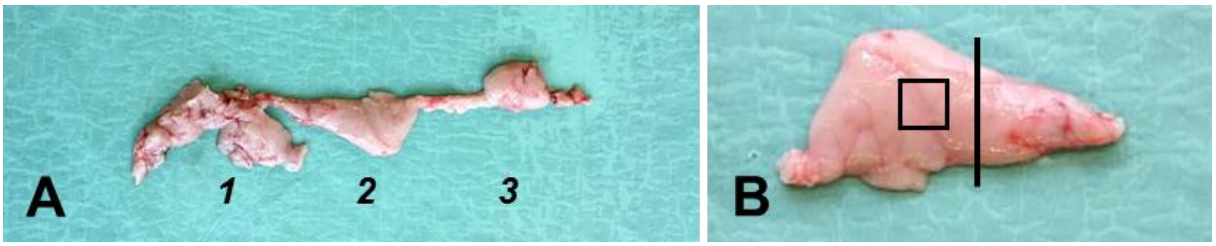


Fig. S44. *Left thymus (3-month-old-pig).* ***A****: Thoracic part (1), and caudal part of the cervical portion of the thymus (2), cranial part of the cervical portion of the thymus (3).* ***B****: Caudal part of the cervical portion of the thymus. The location and orientation of the histology (black line) and molecular analyses samples (black rectangle) for Type-I studies are indicated.*

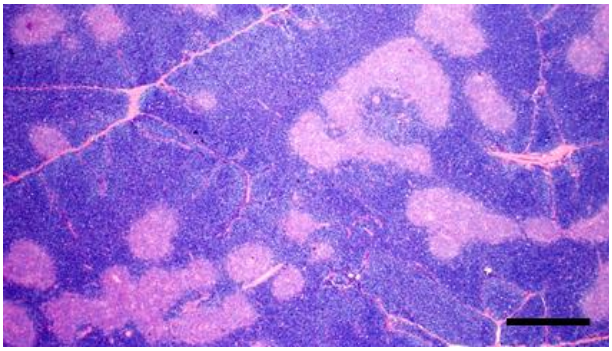


Fig. S45. Thymus histology*.*
FF-PE. HE. Scale bar = 1 mm.

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

The Immunotoxicity Screening Working Group (STP IWG) of the Society of Toxicologic Pathology (STP) recommends the routine histopathological evaluation of the thymus using sections containing all separate thymus compartments (Haley et al., 2005).

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Morawietz et al., 2004) recommend the preparation of one longitudinal section through the entire length of one lobe of the thymus to obtain a standardized section showing all anatomical structures.

In comparison, the samples recommended in Type-I-study sampling protocols for porcine biomedical models should also allow for an adequate evaluation of the thymus in routine studies.

2.5.2.2. Thymus samples in Type-II and Type-III studies

General systematic random sampling strategy

The caudal parts of the cervical portions of the thymus of both sides are excised and weighed. Sampling positions and tissue-size dependent sample numbers are determined by systematic random sampling. Depending on the size of the animal, the tissue is cut into parallel equidistant slabs approximately 5-20 mm thick and orthogonal to the longitudinal axis of the organ. The first section is randomly positioned between 0 and 20 mm from the margin of the organ (Fig. S46). The tissue slabs are placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-4 cm, depending on the organ size and number and area of slabs). The systematic random sampling of six tissue locations is performed as described in section 1.4. The sampled positions can be marked by placing pieces of confetti paper on the respective positions. One tissue piece approximately 2x2x1-2 cm in size is excised from each location, regardless of the structures contained in the sample. Thus, if the sampled location contains vascular structures, these structures must be included in the rest of the sample to maintain representativity. The excised tissue specimens are further subdivided into slabs for downstream analyses (Fig. S47).

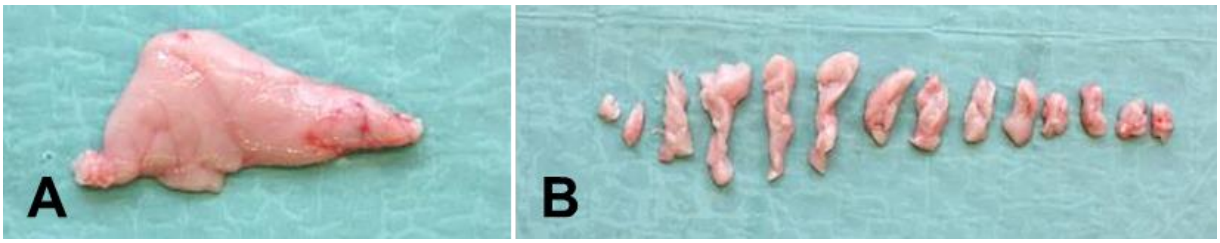


Fig. S46. ***A***. *Left caudal part of the cervical portion of the thymus (3-month-old-pig).* ***B****: The serially sectioned caudal cervical part of the thymus for systematic random sampling in Type-II and -III studies.*

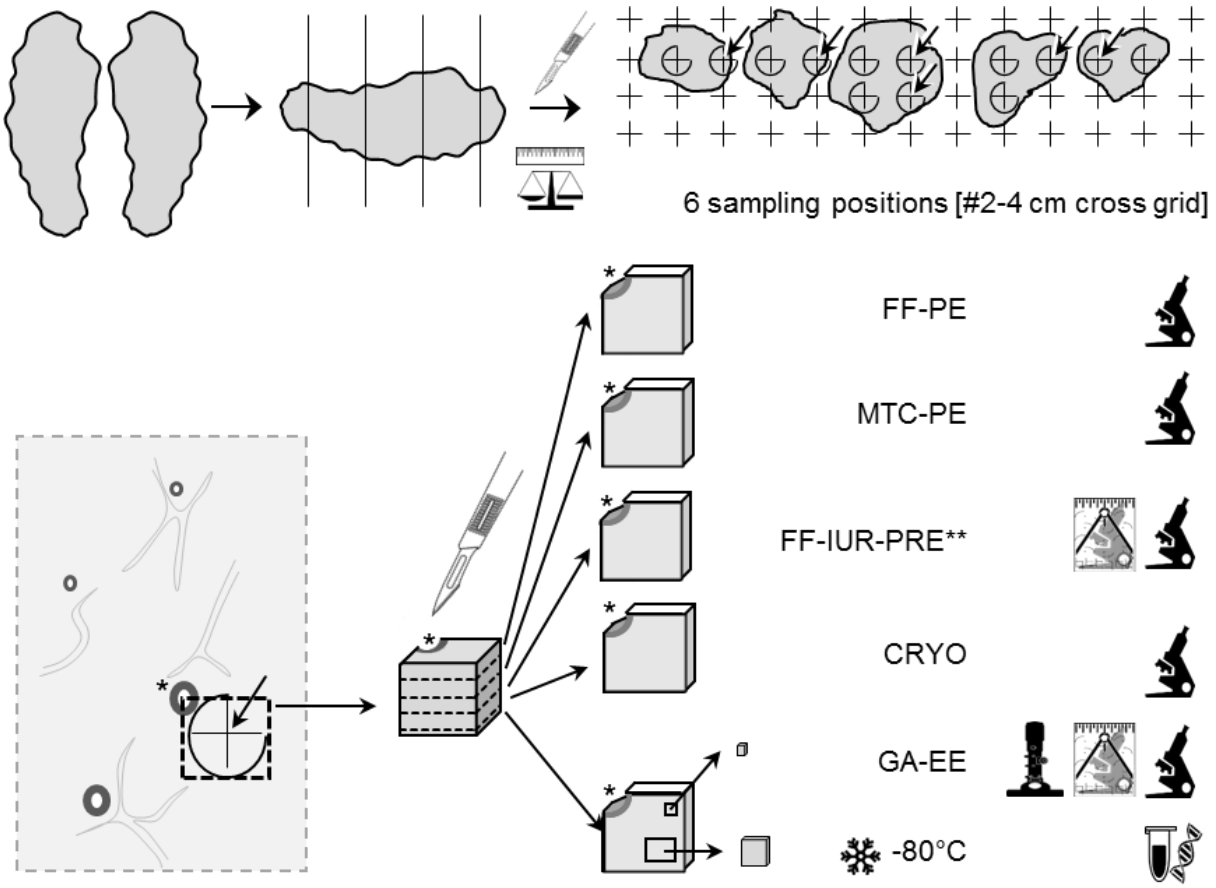


Fig. S47. *A schematic illustration of thymus sampling in Type-II/III studies. *Blood vessel section. **PR = GMA/MMA.*

Table S11. The type and number of thymus tissue samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

	Number of samples per sampled location*	
Sample type	**Type-II studies**	**Type-III studies**
FF-PE	1	1
MTC-PE	-	1 out of every 2nd location
CRYO	1 out of every 2nd location	1 out of every 2nd location
GA-EE	1	1
FF-IUR-PRE (GMA/MMA)**	1	1
Molecular analyses (-80°C)	1	1

**Six locations are sampled: Three from the left and three from the right caudal cervical part of the thymus.*
***ORIENTATOR or ORTRIPS (section 1.6.3.).*

Time and personnel requirements
At least 15 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature
(Docampo et al., 2014, Haley et al., 2005, Kuper et al., 2013, Morawietz et al., 2004)

2.5.3. Pharynx and palatine tonsils

This sampling guide outlines the preparation of standard FF-PE histology samples from the pharynx/palatine tonsils (Type-I study). Advanced analyses (Type-II/III-study sampling protocols) are not recommended.

Relevant anatomical features/preparation
The palatine tonsils are located on the dorsal and lateral side of the pharynx (Fig. S48).

General examination parameters
The pharynx is examined for pathological alterations. If present, the alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

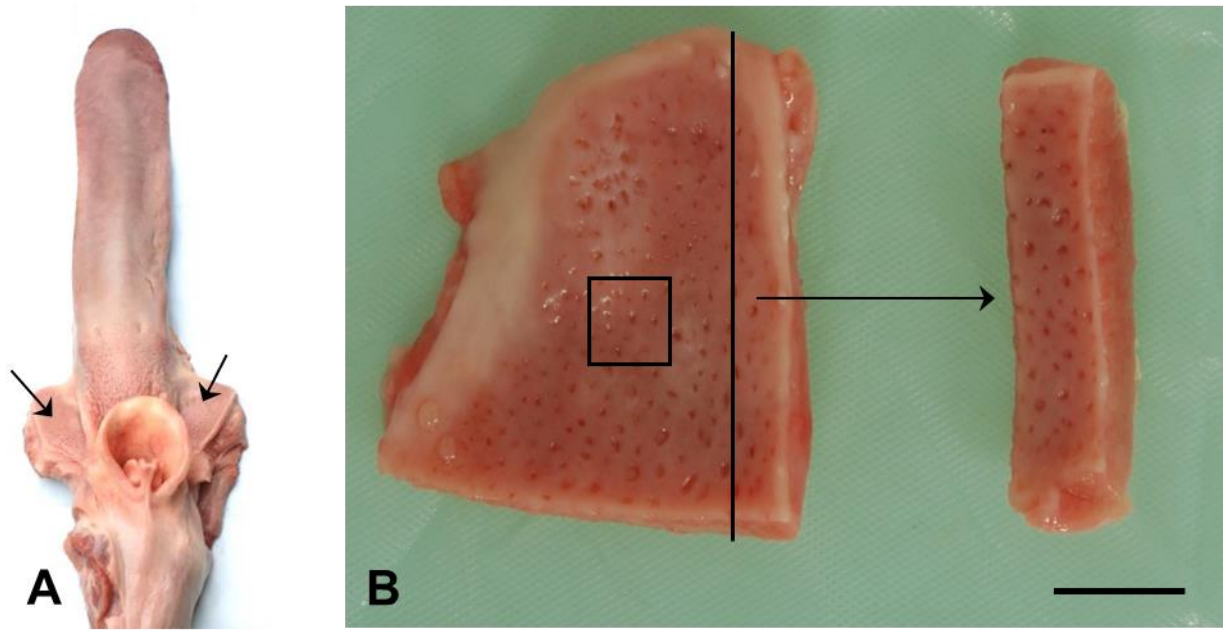


***Fig. S48. A**: Anatomical location of the palatine tonsils (arrows) at the pharynx. **B**: Detailed enlargement of excised tonsillar tissue. The Type-I-study sampling locations for molecular analyses (black rectangle) and the location/section orientation (black line) of the histology samples are indicated. Scale bar = 1 cm.*

Sample for molecular analyses

Number and location of samples:	One sample from the left palatine tonsil is collected, as shown in Fig. S48.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).
Remarks:	An identical sample of the tonsil may be taken for the routine virological examination for PRRSV infection (by PCR).

Histology sample

Location, number and orientation of sections:	One cross section is cut from the left palatine tonsil next to the sample location for molecular analyses (Fig. S48).
Fixation/Embedding:	FF-PE (Fig. S49).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Ruehl-Fehlert et al., 2003) recommend the collection of one transverse section through the middle of the larynx to allow for the histopathological evaluation of the pharynx. A histology sample generated according to the Type-I-study sampling protocols for porcine biomedical models allows for adequate evaluation of the pharynx and palatine tonsils in routine studies.

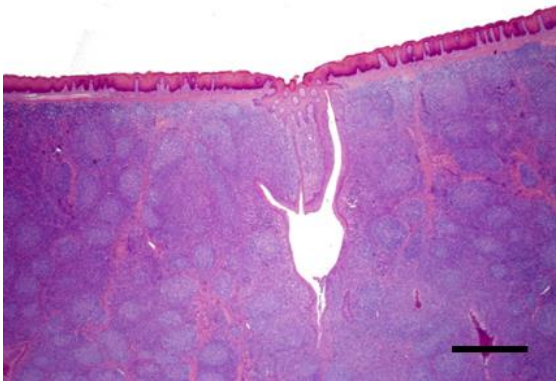


Fig. S49. Palatine tonsil histology*. FF-PE.HE. Scale bar = 1 mm.*

Related literature

(Cheng et al., 2015, Docampo et al., 2014, Kuper et al., 2013, Ruehl-Fehlert et al., 2003)

2.5.4. Sternal bone and bone marrow

In adult healthy pigs, hematopoietic bone marrow is usually present in the sternum, ribs, bones of the pelvis, and the extremities of several long bones. For the molecular and histopathological analyses in Type-I-III studies, these guidelines recommend the generation of bone marrow samples from the third sternebra. Additionally, bone marrow smears for cytological examination are prepared from the sternal or costal bone marrow in all study types.

2.5.4.1. Sternal bone and bone marrow samples for molecular and histopathological analyses

Relevant anatomical features/preparation

Bone marrow samples should be collected as quickly as possible after the animal's death. For histopathological analyses, the marrow should be collected within 20 minutes of death. For removal of the sternum from the thorax, the sterno-costal boundaries are cut using a sharp knife or saw. Next, the sternum is cut longitudinally using a saw (Fig. S50) and examined for pathological alterations. Where appropriate, gross pathological findings are documented by photography. In Type-I and Type-II studies, the third sternebra is then excised and two approximately 5-mm-thick midsagittal slabs are cut. In larger animals, the samples are sawed from the third sternebra (Fig. S50 and Fig. S51). In piglets or young minipigs, it is possible to embed a complete longitudinal slab from the third sternebra in paraffin or plastic resin for histopathological analysis, which should be done whenever possible to avoid damaging the tissue morphology. However, in most cases, the size of the sternebra will exceed the size of a standard embedding cassette. Therefore, smaller samples of spongiosa are cut from the sternebra. For molecular analyses in Type-I-III studies and plastic histology (Type-II and -III studies) not involving decalcification of the samples, the samples are excised from the fresh unfixed spongiosa (section 2.5.4.4.). Here, it is important not to squeeze or contuse the tissue samples because this will destroy the histomorphology of the spongiosa. The spongiosa samples are excised using a hammer and sharp scalpel or microtome blade (Fig. S50 and Fig. S51). The sample excised for plastic histology should not be smaller than 10x10x5 mm to ensure that the center of the sample is not damaged by squeezing artifacts. The histological sections should also be prepared from the centers of the embedded samples. For paraffin histology, the samples can be conveniently trimmed after decalcification.

General examination parameters

The sternum is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

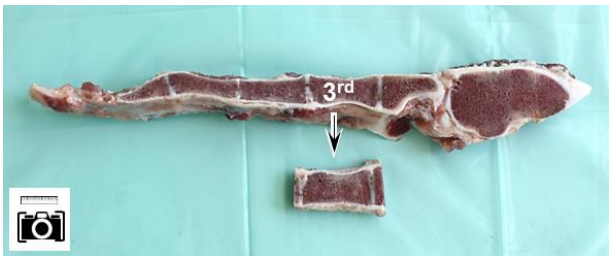


Fig. S50. *Longitudinal section of the sternum and excised third sternebra.*

2.5.4.2. Sternal bone and bone marrow samples in Type-I and Type-II studies

Samples for molecular analyses

Location:	One spongiosa sample is excised from a fresh, unfixed, longitudinally sectioned, ~5-mm-thick slab of the third sternebra, as described above and indicated in Fig. S51.
Number of samples:	One.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

*Location, number, size and orientation, fixation, and embedding of samples in **Type-I** studies:*	A ~5 mm thick, longitudinally sectioned slab of the third sternebra is fixed in 4% formaldehyde and then decalcified. Decalcification protocols are provided in section 2.6.2., Bones. After decalcification, the tissue sample is trimmed to fit into an embedding cassette using a scalpel and then embedded in paraffin (Fig. S52).
*-in **Type-II** studies:*	After excision of the sample for molecular analysis, one sample of spongiosa (>10x10x5 mm) is excised from the rest of the fresh, unfixed, longitudinally sectioned, ~5-mm-thick slab of the third sternebra for plastic histology using a hammer and a scalpel or microtome blade. The sample is then fixed in 4% formaldehyde, not decalcified and embedded in plastic resin (GMA/MMA). For paraffin histology, another approximately 5-mm-thick, longitudinally sectioned slab of the third sternebra is fixed in 4% formaldehyde, decalcified, and trimmed exactly as described above (Fig. S52).

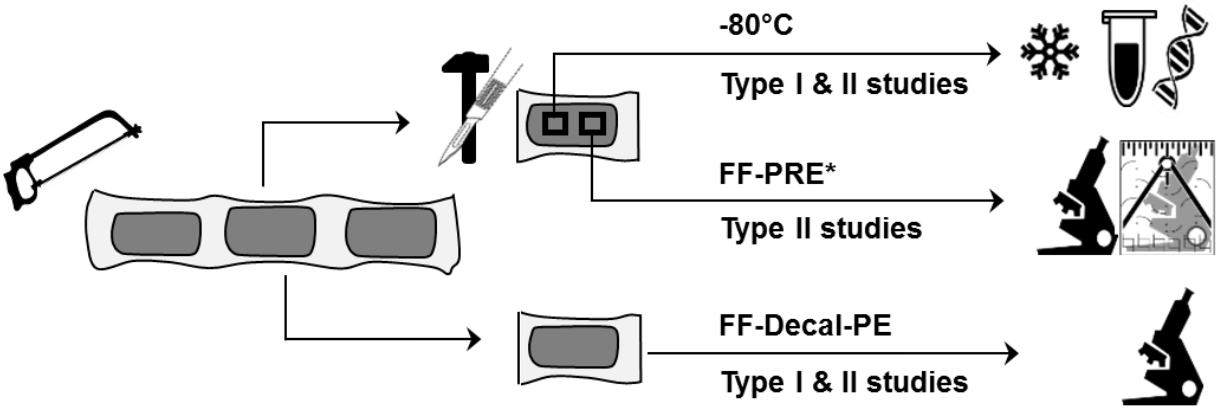


Fig. S51. A schematic illustration of sampling of the third sternebra *in Type-I and II studies. *PR = GMA/MMA. A FF-PRE sample is collected in only Type-II studies. For details, refer to the text above.*

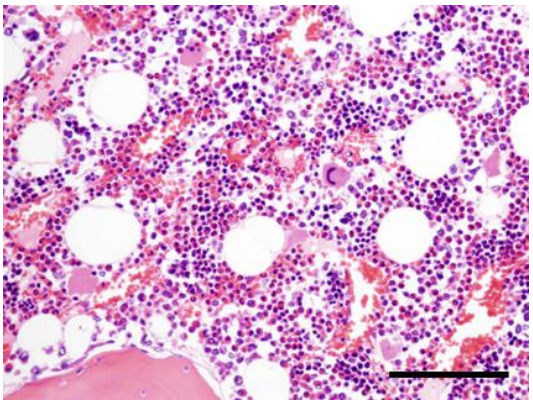


Fig. S52. Bone marrow histology. *Spongiosa of the third sternebra (six-month-old pig). FF-DECAL-PE. HE. Scale bar = 100 µm.*

2.5.4.3. Bone marrow smears

In toxicity studies and advanced studies of the bone marrow, cytological examination of bone marrow smears is performed as a valuable addition to histopathological analyses. These guidelines recommend the preparation of bone marrow smears from costal or sternal bone marrow in Type-I, -II and -III studies.

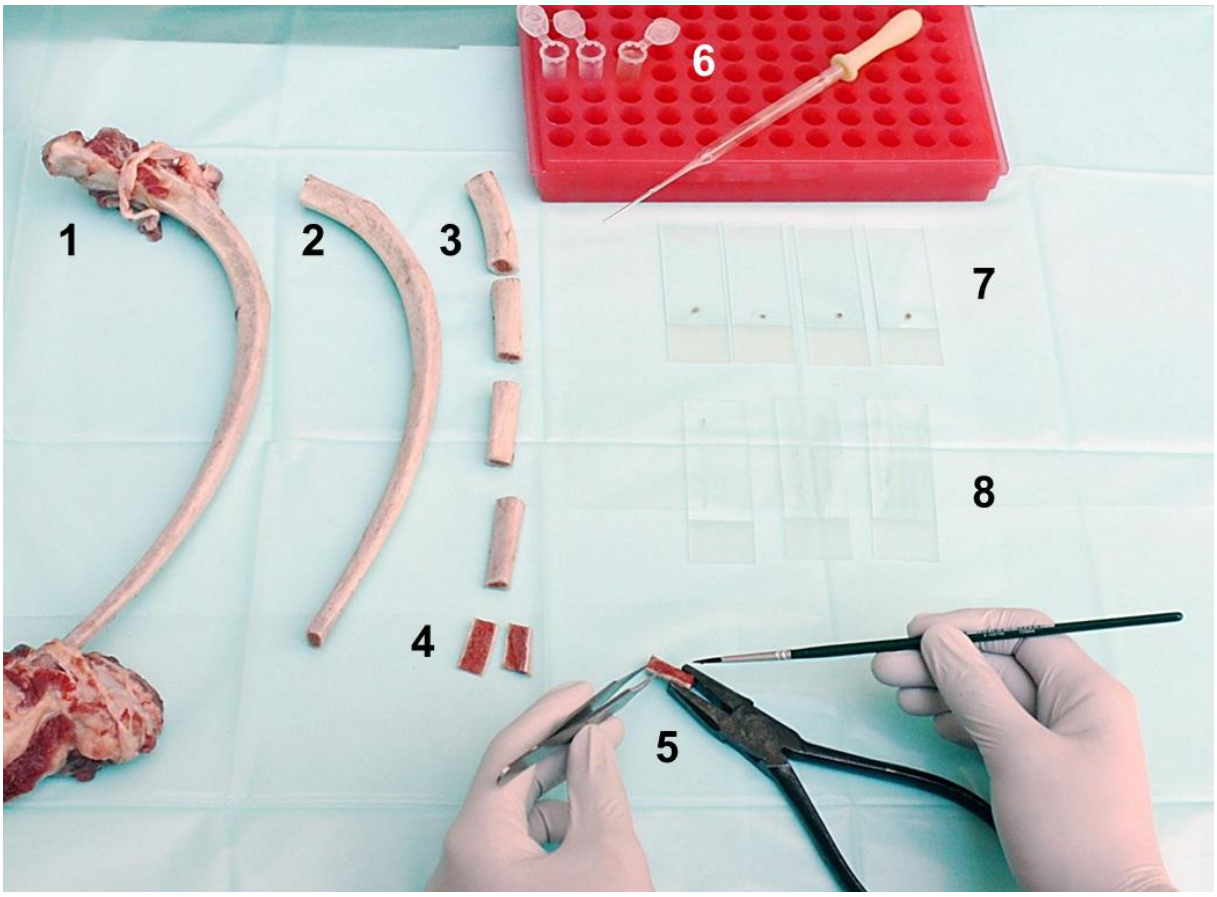


Fig. S53. Preparation of bone marrow smears from a rib. 1*: Excised rib with removed adjacent soft tissue.* ***2-3****: "Cleaned" rib-bone, cut into segments of approximately 4 cm.* ***4****: Distal segment of rib bone cut open longitudinally.* ***5****: Bone marrow is "squeezed" from the cut surface of the spongiosa using pliers and collected with a paintbrush or tweezers.* ***6****: Pig serum is used as diluent.* ***7****: Slides with bone marrow samples for the preparation of smears using the push slide technique and the squash or pull technique.* ***8****: Unstained paint brush bone marrow smears.*

Preparation of costal or sternal bone marrow smears

In pigs, bone marrow smears can generally be prepared as described for other animals, including mice and rats (refer to the literature cited below and to Figures S53-S55). The collection of bone marrow should be performed within five minutes after the animal's death to provide optimal cytomorphology with minimal post-mortem artifacts. For preparation of bone marrow smears, bone marrow can be collected from a rib or sternebra. The tenth left rib or the fourth sternebra is excised from the body and adjacent soft tissues, such as musculature, adipose tissue and the periosteum, are removed by scraping the surface of the bone using the back of a knife. Next, a suitable piece of bone (~4 cm long) is excised using a saw. The piece of bone is then split longitudinally using a sharp knife and hammer. The bone is carefully squeezed to extrude the marrow from the exposed surface of the spongiosa using pliers. Depending on the smear preparation technique, the marrow is collected using a pair of fine tweezers or a paintbrush of appropriate size. The smears should be prepared immediately. The different techniques for bone marrow smear preparation (paint brush, push slide, and squash or pull technique) are illustrated in Figures S53 and S54 and extensively described in the literature cited below. Solutions of 5% bovine serum albumin, pig serum, or fetal calf serum mixed with 7.5% EDTA (2:1) can be used as diluents. At least two slides should be made per specimen regardless of the technique used. The smears are air-dried and then stained using modified Wright's-Giemsa, May-Gruenwald Giemsa, or a Romanowsky stain for routine evaluation.

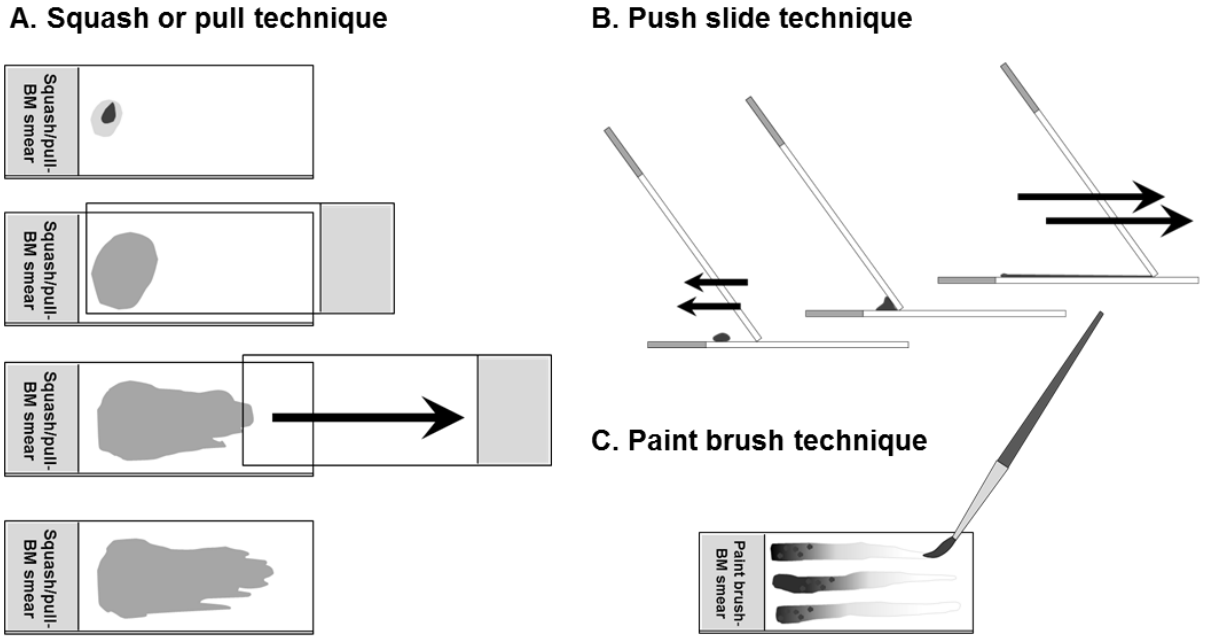


***Fig. S54. A schematic illustration of different techniques of bone marrow smear preparation. A**: **Squash or pull technique. B**: **Push slide technique.** For the "Squash or pull technique" and the "Push slide technique", a small drop of diluent is placed on the slide. The bone marrow sample is then gently mixed with the diluent on the slide before the smears are produced. **C**: **Paint brush technique.** A clean paintbrush is dipped into a diluent, such as pig serum, and then blotted on tissue paper. The bone marrow is collected from the bone medullary cavity using the tip of the paintbrush and applied to the slide in 3-4 lines. Separate brushes are used for different animals and are rigorously cleaned after a necropsy.*

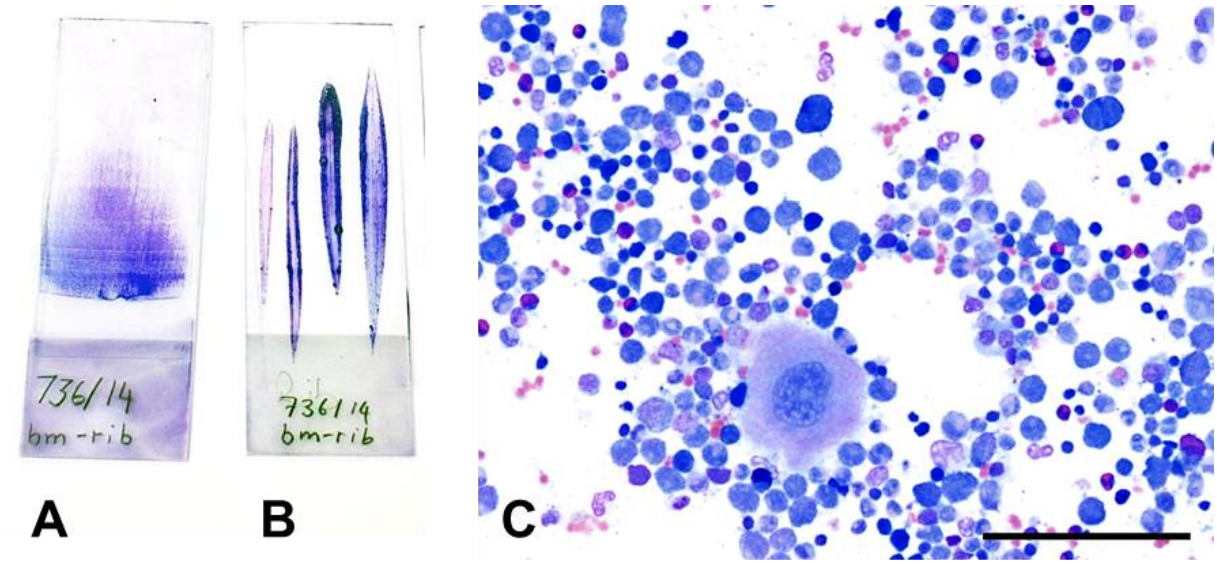


***Fig. S55. A**, **B**: Smears from the costal bone marrow of a healthy adult pig prepared with the "Squash or pull technique" (**A**) and "Paint brush technique" (**B**). Wright's-Giemsa stain. **C**: Cytology of the bone marrow smear from A. Wright's-Giemsa stain. Scale bar = 100 µm.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one bone marrow smear from the femoral diaphysis and the histopathological examination of decalcified, longitudinal horizontal sections of the 2-3 sternebrae (Morawietz et al., 2004). The Immunotoxicity Screening Working Group (STP IWG) of the Society of Toxicologic Pathology (STP) recommends the histopathological examination of bone marrow for routine pathological evaluations of the immune system in rodents and other species, such as dogs and monkeys using sections that contain all bone marrow compartments (Haley et al., 2005). The Best Practices for Evaluation of Bone Marrow in Nonclinical Toxicity Studies (Reagan et al., 2011) recommends the collection of bone marrow samples for the preparation of bone marrow smears from the femur and the sternum of rodents or the rib and sternum in larger animal species. For histopathological evaluation, the same bones can be sampled. Chelating agents, such as ethylene diamine tetraacetic acid (EDTA) or a weak organic acid, is recommended for tissue decalcification. Comparable sampling efforts and extents are recommended in the porcine Type-I-study sampling protocols for an adequate evaluation of bone marrow in routine studies.

2.5.4.4. Sternal bone and bone marrow samples in Type-III studies

In Type-III studies, sampling positions are determined by systematic random sampling. The length of the third sternebra is measured along the longitudinal axis, and the sternebra is then transversally sawed into ~1 cm thick slabs (Fig. S56). The organ slabs are then placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 1-2 cm, depending on the size and number of tissue slabs). The systematic random sampling of six tissue locations (spongiosa) is performed as indicated in Fig. S56. An approximately 2x1x1-cm tissue piece is excised from each of the sampled locations. Each of the excised tissue specimens is further subdivided into four pieces using a hammer and a sharp knife, scalpel, or microtome. One of the four specimens is frozen at -80°C for molecular analyses. Two pieces are fixed in a formaldehyde solution (FF) and decalcified. The decalcification protocols are provided in section 2.6.2., Bones. One of the FF-DECAL-specimens is embedded in paraffin (FF-DECAL-PE), and the other specimen is embedded in plastic resin (PR: GMA/MMA). The fourth piece of tissue is fixed in GA and IUR-EE sections are generated for semithin section histology, electron microscopy and quantitative stereological analyses (Fig. S56).

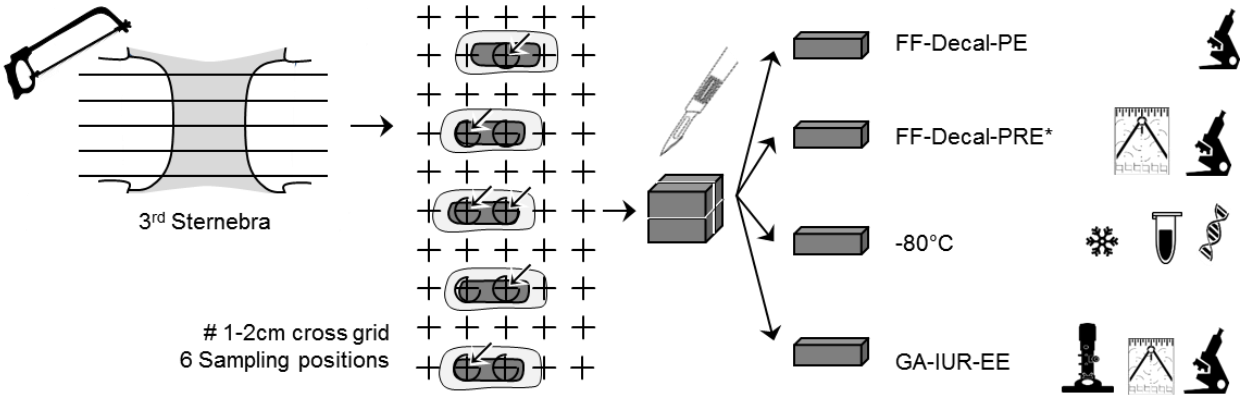


Fig. S56. *A schematic illustration of third sternebra sampling in Type-III studies. *PR = GMA/MMA.*

Time and personnel requirements

To prepare samples for molecular analyses, histopathological examination and bone marrow smears, at least 25 minutes must be scheduled for the execution of the complete Type-III-study sampling procedures by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Bolliger, 2004, Dalle Carbonare et al., 2005, Morawietz et al., 2004, Ramaiah et al., 2013, Reagan et al., 2011)

2.6. Musculoskeletal system

2.6.1. Skeletal muscles

Skeletal muscles are sampled after the spontaneous postmortal muscle twitchings have stopped but before the onset of rigor mortis. In routine studies (Type-I studies), the skeletal muscle samples should be consistently collected from one side (left) of the body for standardization and the reduction of work effort. In Type-I studies, samples are collected from the gluteobiceps muscle (Fig. S57) and the left longissimus lumborum muscle (Fig. S61). Skeletal muscles sampled for advanced analyses (Type-II and Type-III studies) include additional muscles of the extremities and trunk and respiratory muscles (i.e., diaphragm). This guide recommends the sampling of the following muscles: The gluteobiceps muscle (Fig. S57), the triceps brachii muscle (Fig. S58), the tibialis cranialis muscle (Fig. S59, S60), the longissimus lumborum muscle (Fig. S61), and the left crus of the diaphragm (Fig. S62). In studies particularly focused on the muscular system, the sampling should include additional muscles based on their fiber type (oxidative (type 1), glycolytic (type 2B) and mixed (type 2A)) composition (Lefaucheur and Vigneron, 1986).

Relevant anatomical features/preparation
The following images show the sampled muscles (highlighted by white dotted lines), main fiber orientation (white open arrows), and location and section orientation for sample generation (solid white lines). For histopathological analyses, cross sections and longitudinal sections are prepared based on the muscle fiber orientation. For standardized fixation conditions, the muscle samples are slightly stretched out on a wooden spatula or similar surface prior to transfer into the fixation solution to prevent contraction (Fig. S63).

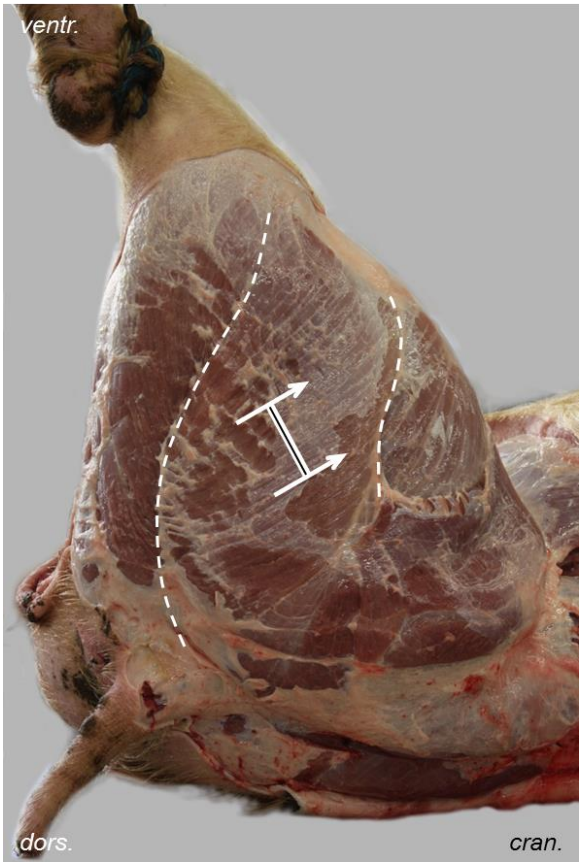


Fig. S57. Gluteobiceps muscle *(left side). Samples are collected from the middle of the muscle belly.*

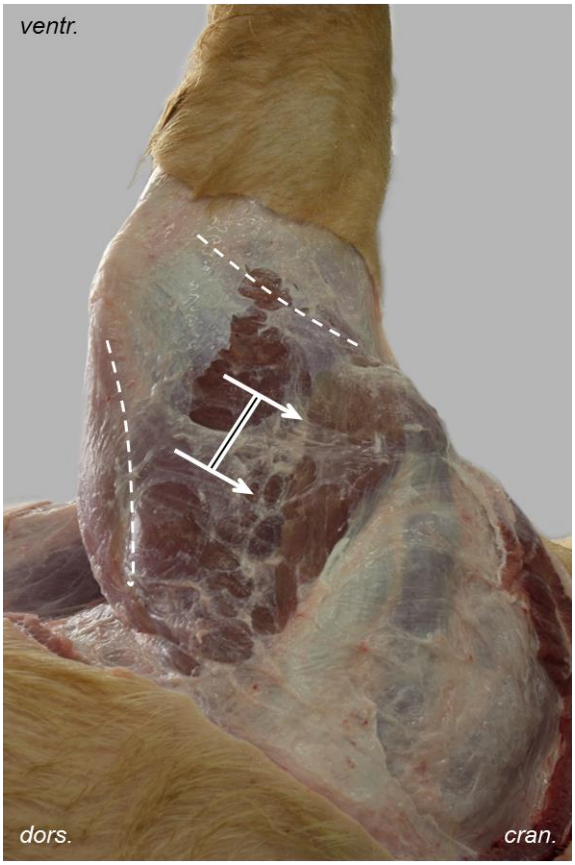
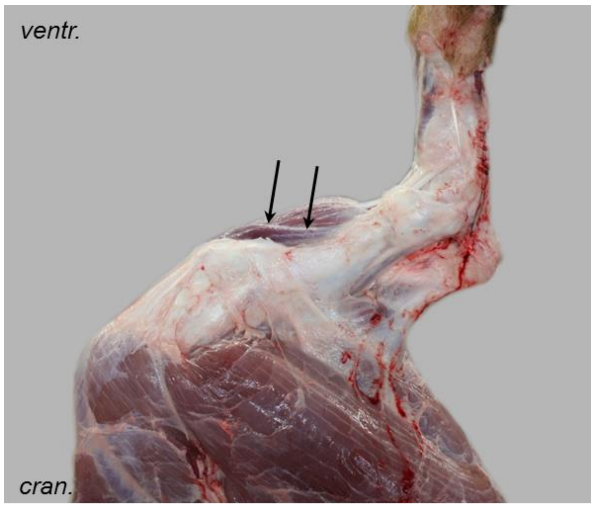


Fig. S58. Triceps brachii muscle *(left side). Samples are taken from the middle of the muscle belly of the medial head of the muscle.*



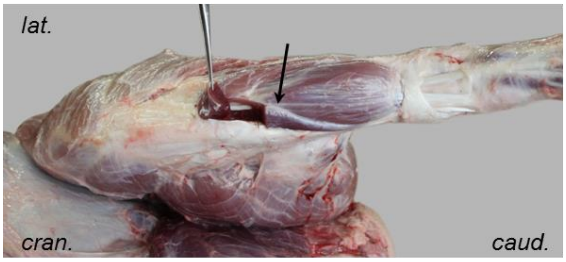


Fig. S59 (←) and Fig. S60 (↑). Tibialis cranialis muscle *(arrows)**.** Medial (S59) and dorsal (S60) aspect of the lower thigh of the left hindlimb. Samples are collected from the middle of the muscle belly.*

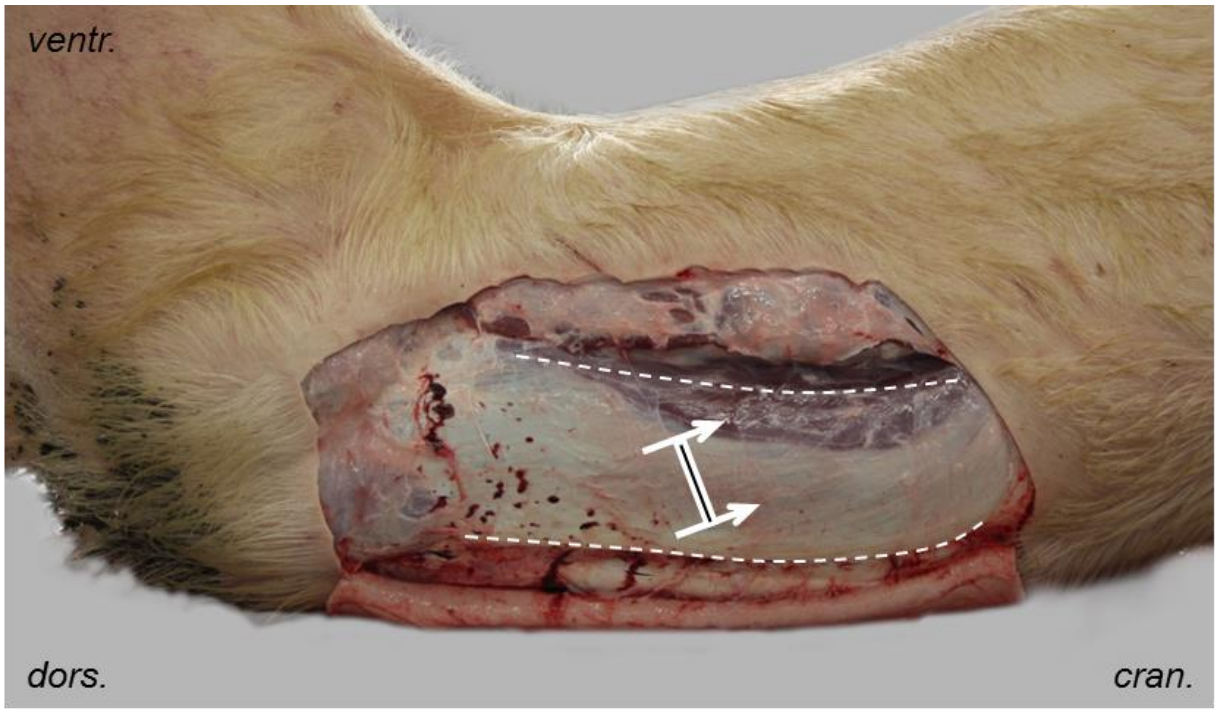


Fig. S61. *Longissimus lumborum muscle (left side). The muscle is sampled caudal to the last rib.*

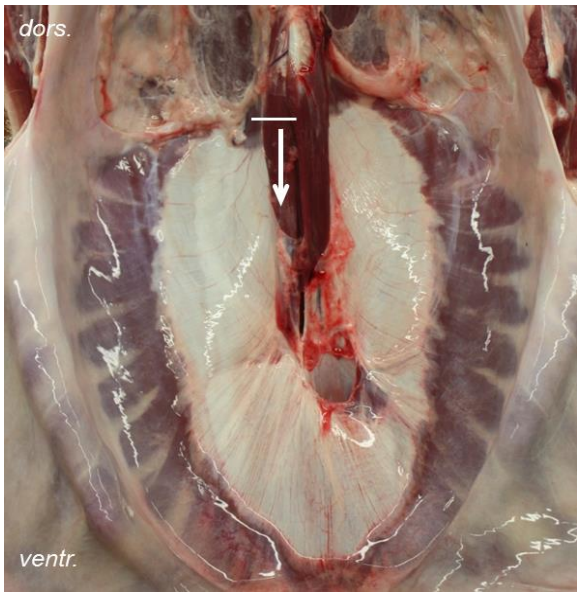


***Fig. S62. Left crus of the diaphragm**. Samples are taken from the middle of the muscle belly.*

General examination parameters

The left tibialis cranialis muscle can be excised completely and weighed if required for quantitative stereological analyses (Type-III studies), such as the estimation of the total number of muscle fibers in a muscle. Muscles are examined for symmetry and pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, parasitology and molecular analyses are taken from the altered site(s) if required.

2.6.1.1. Skeletal muscle samples in Type-I studies

In Type-I studies, skeletal muscle samples are consistently collected from the left gluteobiceps muscle and left longissimus lumborum muscle. The samples are taken from the middle of the muscle belly, as schematically shown below.

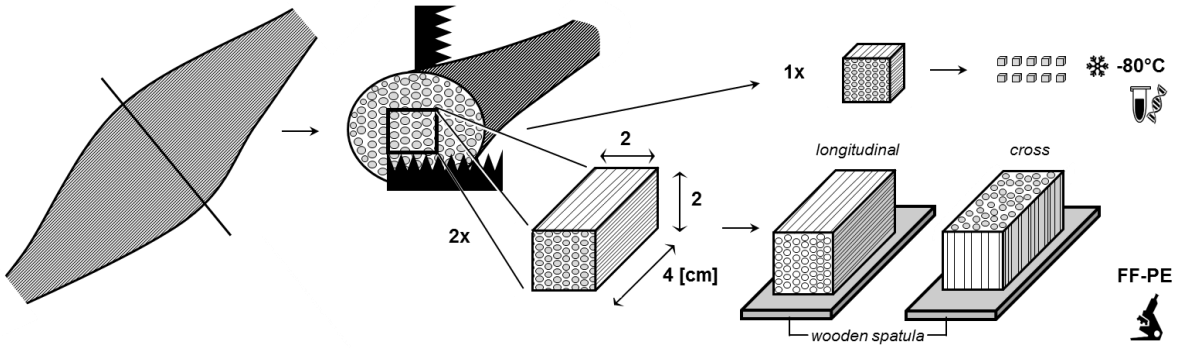


***Fig. S63.** A schematic illustration of skeletal muscle sampling in Type-I studies.*

Samples for molecular analyses

Location:	Left gluteobiceps muscle and left longissimus lumborum muscle. The sampling locations are indicated in Fig. S57 and Fig. S61.
Number of samples per muscle:	3-4 samples of ~2x2x2 mm.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS-profiling).

Histology samples

Location, number and orientation of sections:	Left gluteobiceps muscle and left longissimus lumborum muscle. The sampling locations are indicated in Fig. S57 and Fig. S61. One cross section and one longitudinal section are prepared for each muscle.
Fixation/Embedding:	FF-PE (Fig. S64).

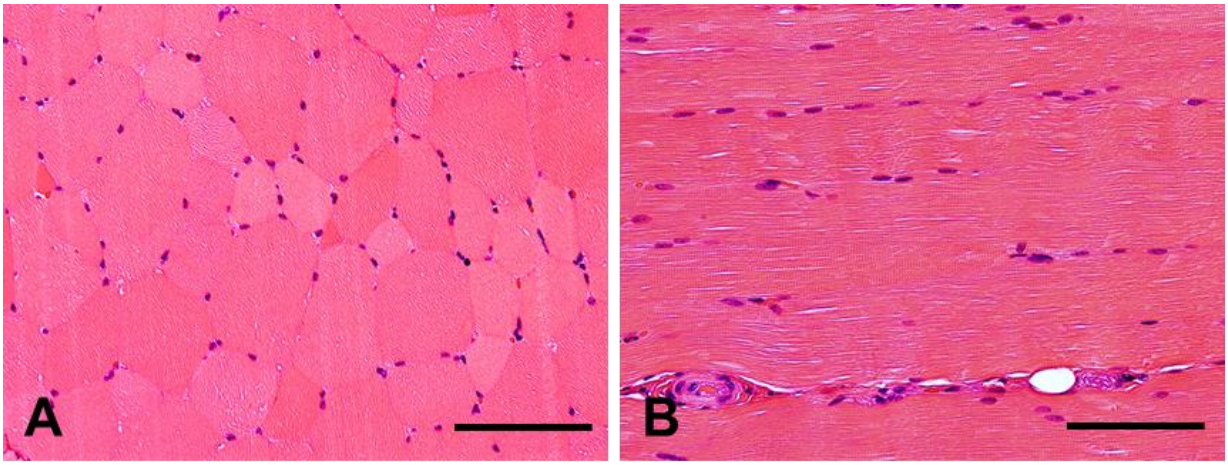


Fig. S64. Skeletal muscle histology *(longissimus lumborum muscle).* ***A****: Cross section.* ***B****: Longitudinal section. FF-PE. HE. Scale bars = 100 µm.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one longitudinal and one transverse section taken from the middle of the biceps femoris muscle (Morawietz et al., 2004). Considering the size of pigs, the examination of samples from two large skeletal muscles should be sufficient for an adequate evaluation of the skeletal musculature in routine toxicity studies. If gross alterations of the musculature are present at necropsy (Type-I study), the sampling of additional muscles should be considered.

2.6.1.2. Skeletal muscle samples in Type-II and Type-III studies

General sampling strategy

It is not possible or quite labor-intensive and difficult to determine the exact weight or volume of the sampled muscles. However, if absolute quantitative stereological analysis data are required, such as the total myocyte volume in a given muscle of interest, the total volume of the muscle should be determined and a volume-weighted sampling procedure should be applied. The muscle volume can be determined by weighing the dissected muscle without its tendinous part. This technique works reasonably well in muscles with one or a few origin and insertion points, such as the tibialis cranialis muscle; however, it is difficult for muscles with multiple insertion points, such as the longissimus lumborum muscle. The muscle volume is calculated from the weight and density of the muscle. Alternatively, the muscle volume can be determined using non-invasive computed tomography with a known inter-image-section thicknesses and applying the Cavalieri principle: Muscle volume = mean muscle sectional area x number of sections x mean section thickness. For the triceps brachii muscle, these sampling guidelines recommend the sampling and weighing of only the medial head of the muscle.

In Type-II and -III studies, samples are generally taken from either the left or right side of the body, and the sampled side is randomly chosen for each sampled muscle. The sampling positions and muscle size-dependent sample numbers are determined by systematic random sampling. For

systematic random sampling, the length of the excised muscle is measured and the muscle is cut into parallel equidistant slabs, approximately 20-40 mm thick, orthogonal to the longitudinal axis of the muscle. The first section is randomly positioned between 0 and 20/40 mm from the margin of the muscle (Fig. S65). The tissue slabs are counted and placed on the same side. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-3 cm, depending on the size and number of slabs). The systematic random sampling of muscle tissue locations is performed as described section 1.4. The sampled locations can be marked by placing pieces of confetti paper on the respective positions. An approximately 2x2x2-4-cm tissue piece is excised from each of the sampled locations. Each excised tissue specimen is then further subdivided into a cross section and longitudinal section for downstream analyses (Fig. S65). Thus, sections with a defined orientation are generated (cross and longitudinal sections). This procedure is advantageous for most qualitative and quantitative histopathological analyses but implies a significant source of bias compared with distinct quantitative stereological analysis parameters. For these special analyses, IUR-(ORTRIP), or VUR sections must be prepared from PRE samples (Mattfeldt et al., 1990).

Remarks

If it is not possible to take the entire muscle for sampling, the samples are obtained from a thick (~4 cm) cross section of muscle tissue taken from the middle of the muscle belly.

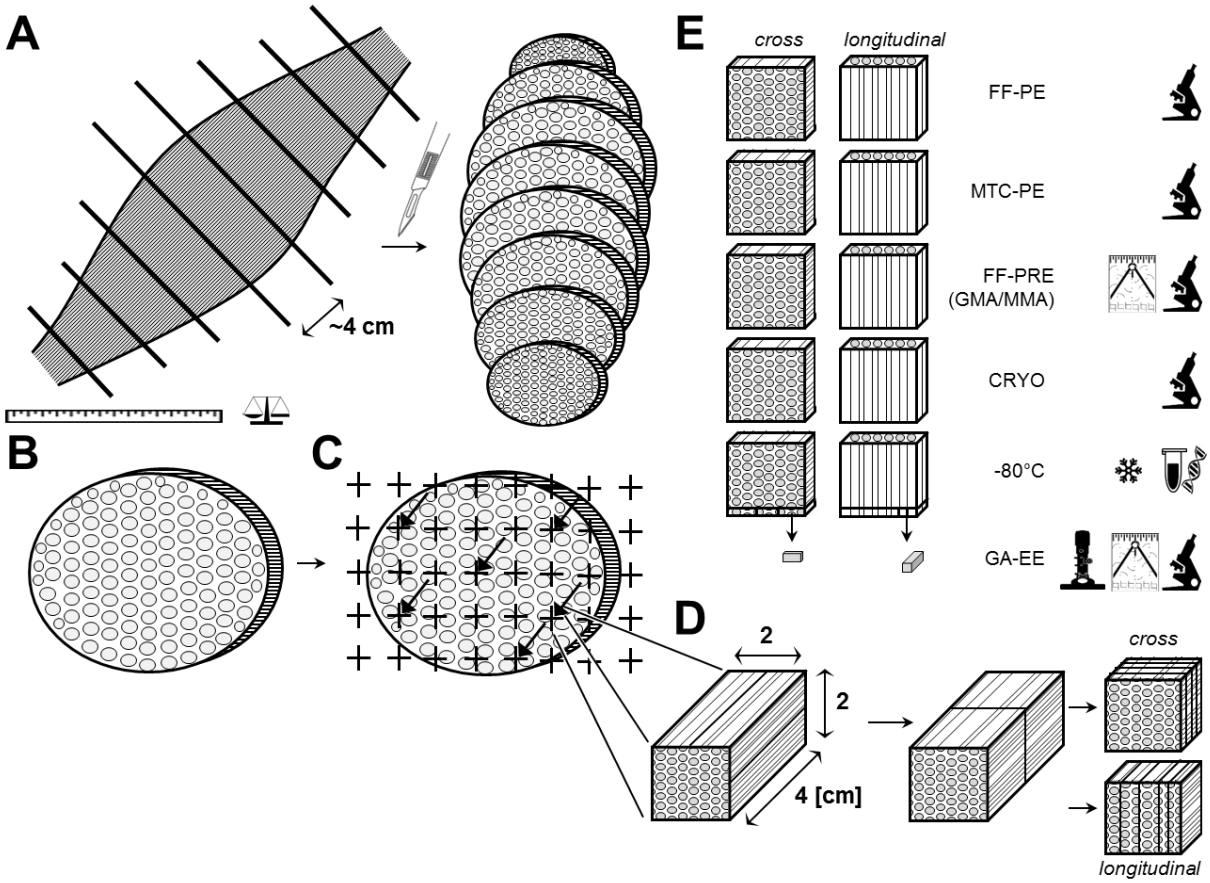


Fig. S65. A schematic illustration of skeletal muscle sampling in Type-II/III studies. A*: After the muscle is weighed and measured, approximately 4-cm-thick, parallel complete cross sections are cut from the muscle orthogonal to the main fiber orientation.* ***B****: Systematic random sampling is shown on a single excised cross section.* ***C****: Sampling locations are determined by systematic random sampling.* ***D****: One tissue specimen (2x2x4 cm) is excised from each sampled position and divided into a cross section and longitudinal section (each ~2x2x2 cm).* ***E****: Further subsamples (~0.5x2x2 cm) are cut while maintaining the cross and longitudinal section orientations. During fixation, the specimens for FF-PE, FF-PRE (GMA/MMA) and MTC-PE are placed on a wooden spatula or similar surface. For EE, rectangularly shaped samples (~4x2x2 mm) are carefully cut using a sharp razor-blade to distinguish the fiber orientation and then fixed in GA.*

Table S12. The type and number of skeletal muscle* samples in Type-II and Type-III studies.

	Number of locations/samples per muscle*	
Sample type	**Type-II studies**	**Type-III studies**
FF-PE	5 (5)	10 (5)
MTC-PE	-	1 from every 2nd location (-)
FF-PRE (GMA/MMA)	5 (5)	10 (5)
CRYO	5 (-)	1 from every 2nd location (-)
Molecular analyses (-80°C)	*5 (2)*	10 (2)
GA-EE	*5 (5)*	10 (5)

**Longissimus lumborum muscle, medial head of the triceps brachii muscle, and gluteobiceps muscle. Restricted sample numbers for the tibialis cranialis muscle and for the left crus of the diaphragm are given in brackets.*

Time and personnel requirements

At least 35-45 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one or two assistants. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Berridge et al., 2013, Greaves et al., 2013, Klymiuk et al., 2013, Morawietz et al., 2004, Valentine and McGavin, 2012)

2.6.2. Bones

The examination of bones, including functional analyses, measurements of mineralization and bone density, radiological examination, histology, and quantitative histopathological analyses, is a broad field with many special techniques and potential pitfalls. Therefore, these guidelines provide only a proposal for a broad spectrum of analyses for widespread analytical questions. In studies using porcine models of bone disease, specific sampling protocols adapted to the respective experimental task must be developed and used. If undecalcified bone sections are needed for special purposes, such as ground sections for differentiation of osteoid and mineralized bone, special tissue-processing protocols must be followed (Carlson and Weisbrode, 2012). These specialized protocols are not provided in these sampling guidelines.

Relevant anatomical features/preparation

Bones are usually harvested at the end of the necropsy after the skeletal muscle samples have been taken and the joints have been examined macroscopically. If synovial fluid or membrane sampling (section 2.6.3.) is recommended in the experimental design of a study, these samples should be harvested before the bones are removed. For bilateral bones, such as the femur or ribs, it is recommended to consistently sample the bones from one side (left) of the body for standardization and the reduction of workload (Type-I studies). Prior to bone excision, the bone's joint surfaces are examined macroscopically for pathological alterations. After removal of the bone, the adjacent muscle and connective tissues are removed carefully without damaging the periosteal tissue. After determination of the bone length and diameter (in Type-II/III studies only), the excised bones are cut using a saw. The cutting directions are specified in Table S13 and Fig. S66. If an electric saw is required, a clean and sharp saw blade should be used at low speed with an appropriate water-based lubricant to avoid contamination of the section with lubricating grease and the frictional heat associated with tissue combustion. The joint, growth plate tissue or spongiosa samples are cut from the excised disc of bone using a sharp scalpel blade and hammer but not a saw.

General examination parameters

At a minimum, the mineralization of the skeleton should be examined during the necropsy by evaluating the breaking strength of the ribs(Carlson and Weisbrode, 2012). If present, pathological alterations of the bones or of the bone's joint surfaces are described and photographed as necessary. Additional samples for histopathology, microbiology, and molecular analyses are taken from the altered site(s) if required. In Type-II/III studies but not Type-I studies, the femurs are weighed and the bone lengths are measured. The widths of bones are measured at the widest point of the diaphysis using calipers.

Sampling of bones in different study types

In routine Type-I studies, samples for molecular analyses and histopathological examination are generated from the (left) femur, left seventh rib, and the sternum. The sampling of the sternum was previously described (section 2.5.4., Sternal bone and bone marrow).

In advanced studies (Type-II and Type-III), samples for qualitative histopathological evaluation using light microscopy (FF-DECAL-PE sections) are prepared from the additional bones listed in Table S13. However, samples for advanced quantitative histopathological analyses, ultrastructural analyses, and molecular analyses in Type-II and Type-III studies are prepared from the femurs only (section 2.6.2.2.).

Table S13. Bone samples for histopathological examination in Type-I-III studies.

Study type	Bone	Histology section(s)/section orientation*
I, II III	Femur (left) Femurs (both sides)	Type-I study: One midsagittal longitudinal section in the latero-medial orientation through the head of the femur. The section should contain synovial membrane, articular cartilage, spongiosa of the epiphysis, growth plate, and spongiosa of the metaphysis. One section of the medial condyle of the femur with articular cartilage. Additionally, one cross section through the cortical bone of the diaphysis of the femur including the periosteum and the medullary cavity (section 2.6.2.1.). Type-II/III studies: Section 2.6.2.2.
II, III	Tibia (left)	One midsagittal longitudinal section in the dorso-plantar orientation through the tibial tuberosity. The section should contain synovial membrane, articular cartilage, spongiosa of the epiphysis, tibial growth plate, and spongiosa of the metaphysis.
III	Humerus (left)	One midsagittal longitudinal section in the cranio-caudal orientation through the head of the humerus. The section should contain synovial membrane, articular cartilage, spongiosa of the epiphysis, growth plate, and spongiosa of the metaphysis.
III	Ulna (left)	One midsagittal longitudinal section in the dorso-palmar orientation through the olecranon. The section should contain spongiosa of the epiphysis, the olecranon growth plate, and spongiosa of the metaphysis.
II III	Metatarsal bone II (left) Metatarsal bone II and III (left)	One midsagittal longitudinal section in the dorso-plantar orientation. The section should contain synovial membrane, articular cartilage, spongiosa of the epiphysis, growth plate, spongiosa of the metaphysis, and the medullary cavity of the diaphysis.
I-III	7^{tn} Rib (left)	One cross section and one midsagittal longitudinal section in the latero-medial orientation through the body of the rib, including the costochondral junction.

**Also indicated in Fig. S66-S68.*

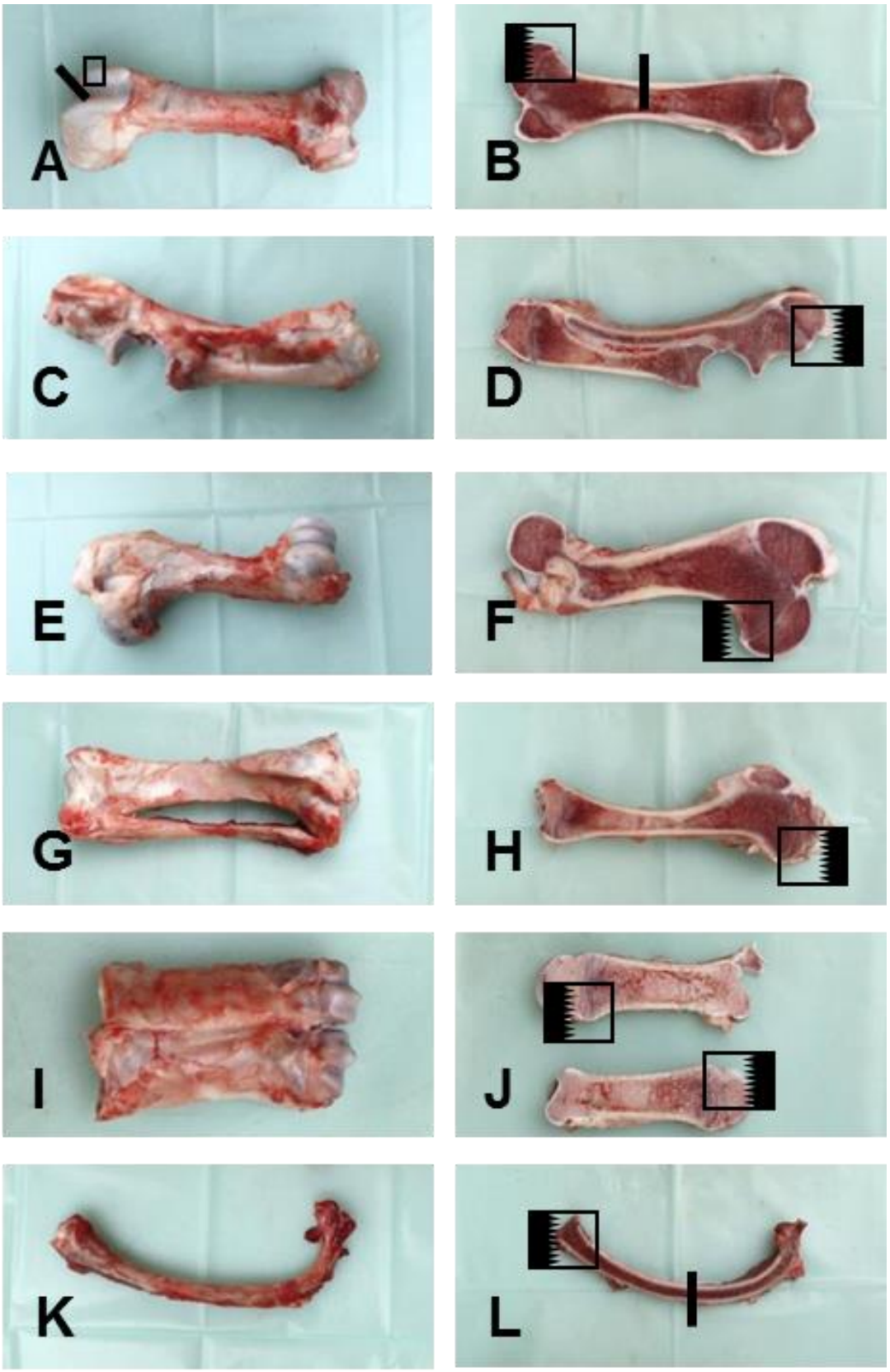


Fig. S66. Excised and sectioned bones. *The locations and orientations of the histology (black lines) and molecular analysis samples (black rectangles) in Type-I studies are indicated.* ***A***, ***B****: Femur. In* ***A****, the position of the sample for histopathological examination and molecular analyses of the articular cartilage of the medial condyle of the femur is indicated.* ***C***, ***D****: Radius and ulna.* ***E***, ***F****: Humerus.* ***G***, ***H****: Tibia and fibula.* ***I***, ***J****: Second and third metatarsal bone.* ***K***, ***L****: Rib.*

2.6.2.1. Bone samples in Type-I studies

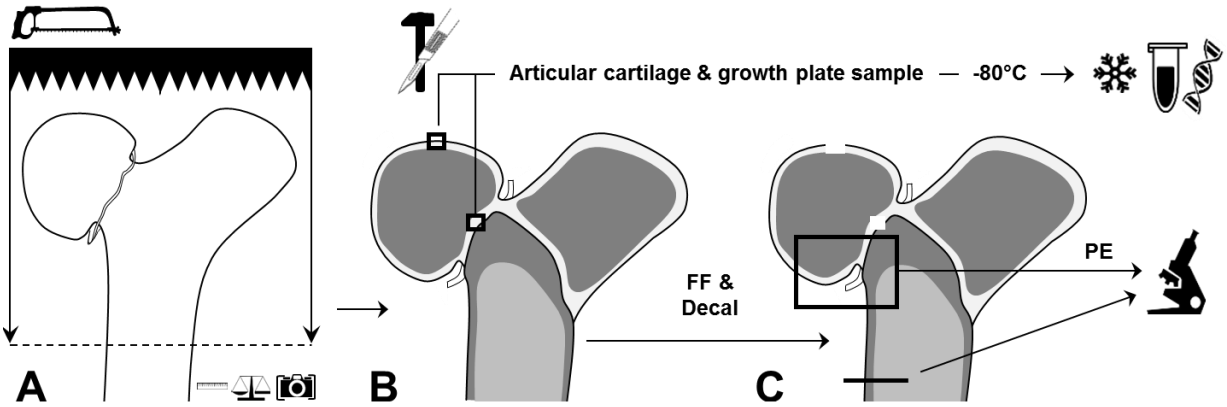


Fig. S67. *A schematic illustration of femur sampling in Type-I studies. The locations and orientations of the histology (black lines) and molecular analysis (black rectangles) samples are indicated.* ***A****: The femur (right or left side) is weighed and measured (length and width). Next, the head of the femur is sectioned (midsagittal longitudinal section in the medio-lateral orientation) into an approximately 5-mm-thick slab using a saw.* ***B****: Samples of articular cartilage and growth plate tissues are excised for molecular analyses using a scalpel and hammer. The remaining tissue is fixed with a formaldehyde solution and subsequently decalcified.* ***C****: For PE-histology, a sample of the head of the femur with articular cartilage, epiphyseal spongiosa, growth plate, and metaphyseal spongiosa and a cross section of the cortical bone of the diaphysis of the femur, including the periosteum and the medullary cavity are obtained.*

Samples for molecular analyses

Location and number of samples:	One sample of articular cartilage, and one sample of growth plate tissue from the head of the femur are obtained as indicated in Fig. S67. Additionally, one sample of cartilage is taken from the medial condyle of the femur (Fig. S66A).
Remarks:	A scalpel and hammer but not a saw are used to excise the samples for molecular analyses.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:

In Type-I studies without suggestion or macroscopic evidence of bone disease, histological sections of the left head of the femur, including the synovial membrane, articular cartilage, growth plate and epi/metaphyseal spongiosa, are collected. One diaphyseal cross section of the cortical bone of the diaphysis of the femur with periosteal tissue and the medullary cavity and a section of the cartilage of the medial condyle of the femur are obtained (Fig. S66, S67, S68 and Table S13). One section of the left seventh rib through the body and the costochondral junction is prepared (Fig. S66 and Table S13). Multiple sections are prepared if the size of the bone section is too large for a single embedding cassette or histological section containing all structures of interest.

Fixation: 4% formaldehyde solution. The maximal section thickness should not exceed 5 mm for adequate penetration of the fixative. Bouin's fixative or alcohol-formalin solution (1:10 4% formaldehyde solution:70% ethanol) may be used instead of the formalin fixative to provide better cell morphology and maintenance of fluorescent bone labels.

Decalcification: Although EDTA-based decalcification (10% EDTA in distilled water at pH 7.4) is considered to be the "gold-standard", decalcification can be achieved quicker using acid-based slow-acting decalcification solutions, such as 5% formic acid, or commercially available solutions, such as DC (Labonord, Germany). This method is recommended for standard histology analyses and allows for the application of several different immunohistochemical analyses (section 2.7.2., Ears). Detailed information on different decalcification procedures and their application times, advantages and limitations regarding different analyses are provided in Brown et al. (2002). The necessary time for decalcification can be estimated using the radiographic determination of decalcification end-points (Carlson and Weisbrode, 2012).

Embedding: Paraffin (Fig. S68 and Fig. S69).

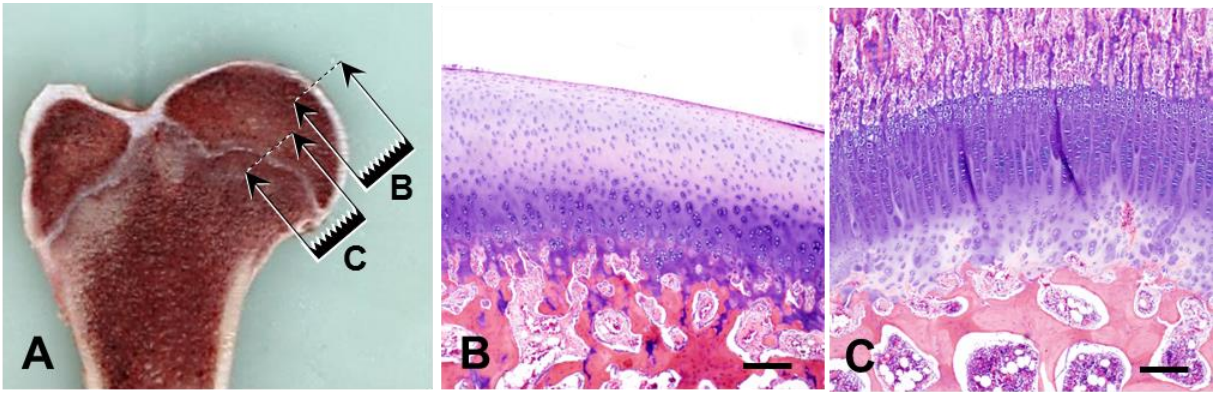


Fig. S68. Bone histology of the head of the femur *of a six-month-old pig.* ***A****: Longitudinally sectioned bone with histology section orientations indicated (B and C refer to the histology images).* ***B****: Articular cartilage.* ***C****: Growth plate. FF-DECAL-PE. HE. Scale bars = 100 µm.*

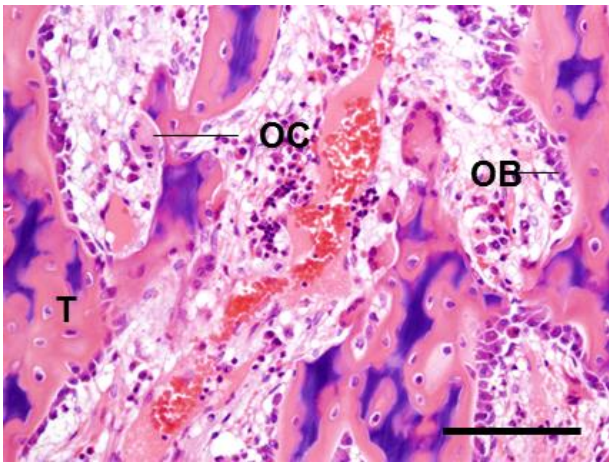


Fig. S69. Histology of the epiphyseal spongiosa of the head of the femur *(six-month-old pig). T: Trabecular bone. OC: Osteoclast, OB: Osteoblast. FF-DECAL-PE. HE. Scale bar = 100 µm.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one longitudinal decalcified bone section, including the distal femur, knee joint, and proximal tibia (Morawietz et al., 2004). Examination of the samples from the femur, sternum and rib should be sufficient for an adequate evaluation of the bones and articular cartilage in routine toxicity studies involving pigs.

2.6.2.2. Bone samples in Type-II and Type-III studies

General sampling strategy, types and numbers of samples

In Type-II and Type-III studies, standard FF-DECAL-PE histology samples are generated from additional bones (Table S13). These guidelines recommend advanced sampling procedures for the generation of specimens from the right and left femur for qualitative and quantitative histopathological analyses, ultrastructural analyses, and molecular analyses (Figure S70 and Fig. S71). In specific studies, the sampling design can be adapted to other bones and the desired sample types.

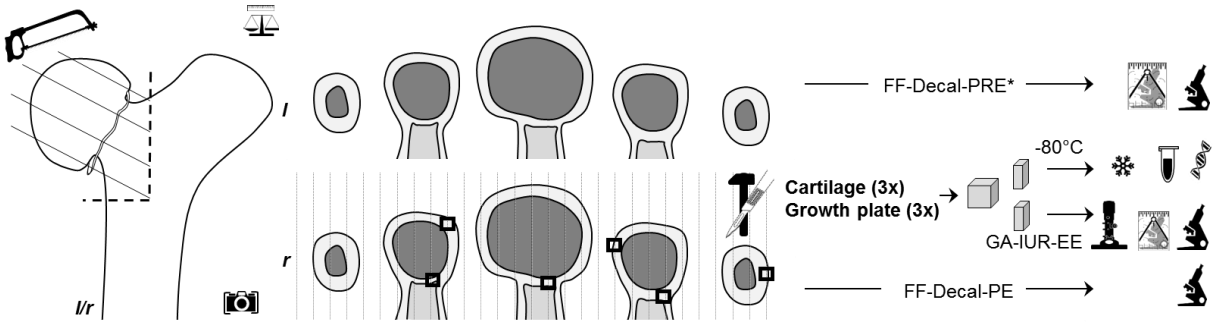


Fig. S70. *A schematic illustration of sampling of the heads of the femurs in Type-II/III studies.*PR = GMA/MMA.*

After determination of the weight, length and width of both femurs, one head of the femur is randomly chosen, completely sectioned in parallel equidistant slabs (~5 mm thick), fixed in a formaldehyde solution, decalcified and embedded in plastic resin (GMA/MMA). These samples can be used for qualitative and quantitative histopathological examination. For histomorphometry and quantitative stereological analyses, PR embedding is advantageous compared with paraffin embedding because of the limited and uniform embedding-related tissue shrinkage. The second head of the femur is sawed into equidistant slabs, and specimens of the articular cartilage and growth plate are systematically randomly sampled.

The tissue slabs are laid on a plain surface and overlaid with a plastic transparency with printed equidistant (1-2 cm) parallel test lines. Three locations are systematically selected from the test lines that hit the articular cartilage and growth plate (Fig. S70). From each of the sampled locations, two approximately 2x2x2 mm pieces of tissue are excised using a sharp scalpel and hammer for a clean and quick cut. For semithin section histology, ultrastructural and quantitative stereological analyses, one of the two samples is further processed for GA-IUR-EE (undecalcified tissue; ORIENTATOR or ISECTOR sectioning). The other sample is frozen at -80°C for subsequent molecular analyses. The remaining tissue is fixed in a formaldehyde solution, decalcified and embedded in paraffin for qualitative and quantitative histopathological examination and IHC. Additionally, four cross-sectioned FF-DECAL-PE samples, three FF-DECAL-PRE (GMA/MMA) samples, three GA-IUR-EE samples and two molecular analyses samples are generated from the cortical bone of the diaphysis of the femur (Fig. S71).

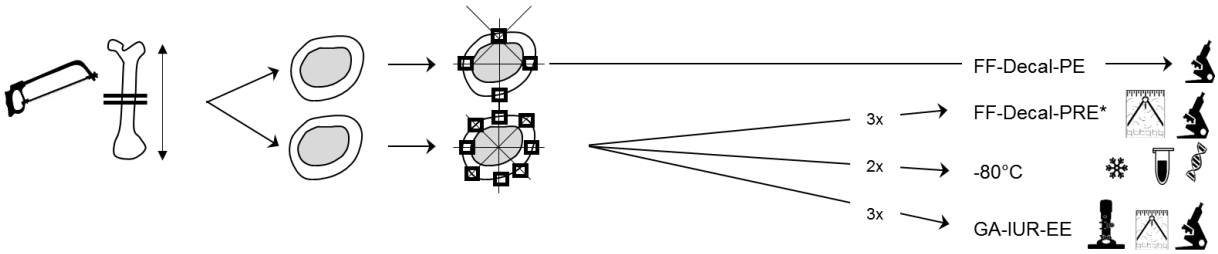


Fig. S71. *A schematic illustration of the sampling of the cortical bone of the diaphysis of the femur, including the periosteum and the medullary cavity in Type-II/III studies.*

Time and personnel requirements
At least 30 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature
(Brown et al., 2002, Dalle Carbonare et al., 2005, Gunson et al., 2013, Morawietz et al., 2004, Turner et al., 2001)

2.6.3. Joints

Relevant anatomical features/preparation
Prior to joint tissue sampling, synovial fluid can be harvested (Fig. S72) from one or two macroscopically unaltered large joints, such as the knee, tarsal, or elbow joint. Sterile joint fluid aspiration is easier if the overlying skin is removed in advance at necropsy. For visual inspection and tissue sampling, the joints should be incised carefully by an experienced pathologist to avoid the introduction of artificial alterations, such as knife-cuts, in the articular cartilage. These guidelines recommend the generation of articular cartilage and synovial membrane samples. The sampling of articular cartilage for histology and molecular analyses was previously described (section 2.6.2., Bones). For histopathological examination of the synovial membrane and generation of specimens for molecular analyses, the Type-I-III-study sampling guidelines recommend the sampling of the dorsal recess of the left knee joint (Fig. S73-S75) due to its large size. Additional samples of other joint structures, such as the cruciate ligaments or the menisci of the knee, can be obtained but are not recommended in these sampling guidelines.

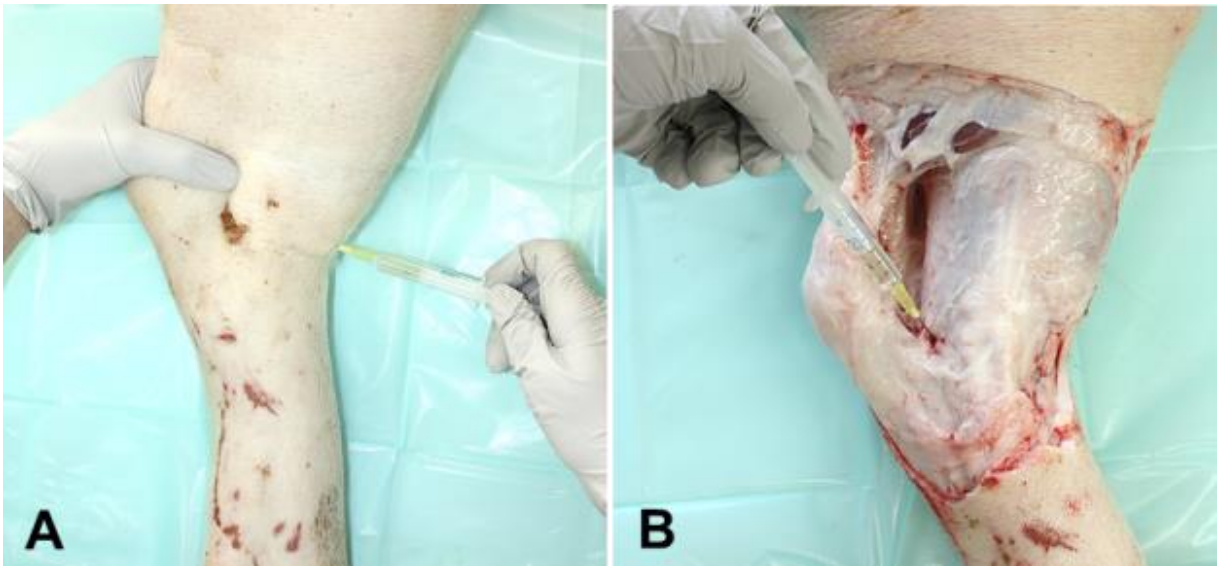


Fig. S72. *Collection of synovial fluid from the right tarsal joint (lateral aspect).* ***A****: Dorsal approach (with intact skin).* ***B****: Plantar approach after dissection of the overlying skin.*

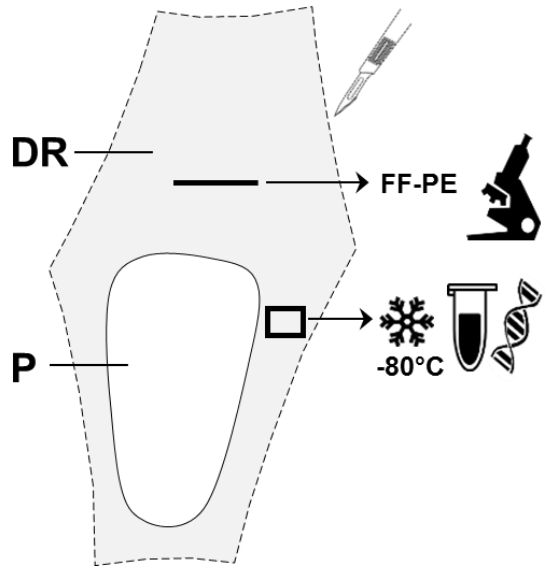


Fig. S73. A schematic illustration of sampling of the dorsal recess of the knee joint*. Synovial membrane of the (opened and excised) dorsal recess (DR) of the knee joint. P: Patellar bone. The locations and orientations of the histology (black line) and molecular analysis samples (black rectangle) for Type-I studies are indicated.*

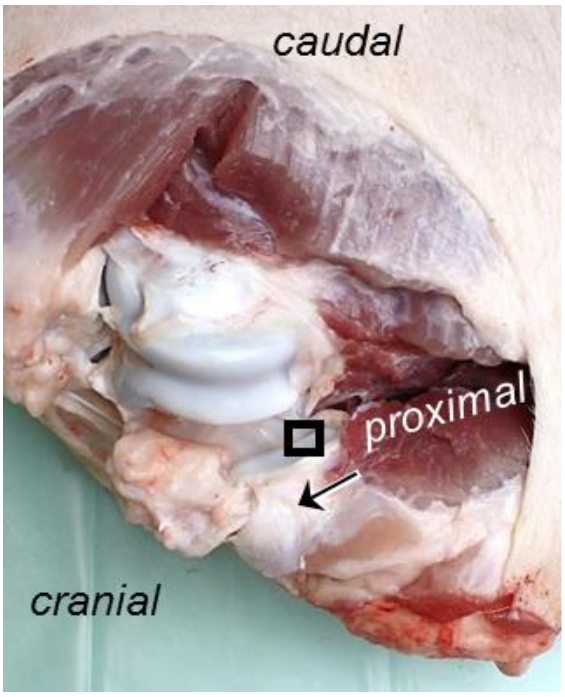


Fig. S74. Opened knee joint*. The arrow marks the synovial membrane of the joint capsule of the distal part of the dorsal recess of the knee joint. The location and orientation of the sample for molecular analyses (black rectangle) in Type-I studies are indicated.*

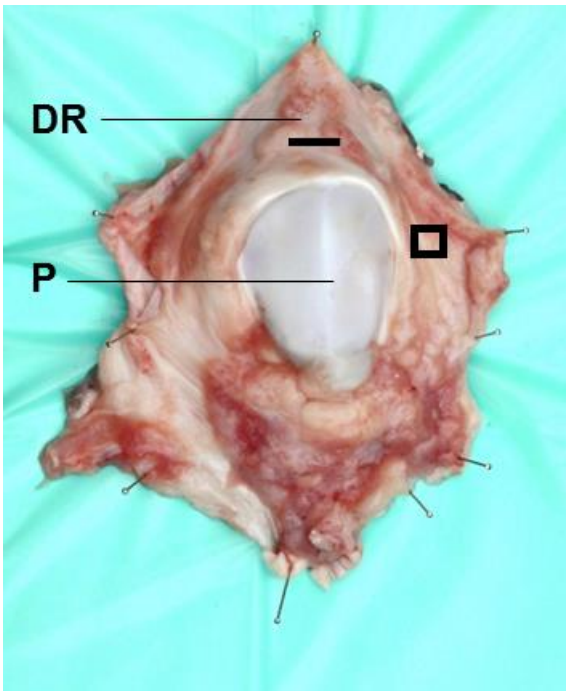


Fig. S75. Excised and opened dorsal recess (DR) of the knee joint. *P: Patellar bone. The locations and orientations of the histology (black line) and molecular analyses samples (black rectangle) for Type-I studies are indicated.*

General examination parameters

At least six major joints (hip joints, knee joints, tarsometatarsal joints, carpometacarpal joints, elbow joints, and toe joints) should be routinely examined macroscopically during the necropsy (Jackson and Cockcroft, 2007). The filling of the joint, synovial fluid, synovial membrane, joint capsule, articular cartilage, ligaments, menisci, and the bony rims of the joint should be evaluated. The medial condyle of the femur and the head of the humerus should be specifically examined for pathological alterations (Jackson and Cockcroft, 2007). If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, and molecular analyses are taken from the altered joint(s) if required.

2.6.3.1. Joint capsule and synovial membrane samples in Type-I studies

Samples for molecular analyses

Location:	Dorsal recess of the left knee joint as indicated in Fig. S73-S75.
Number of samples:	One.
Remarks:	A sample of synovial membrane is collected.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Dorsal recess of the left knee joint next to the location of the sample for molecular analyses. The section should contain the joint capsule and synovial membrane. Sampling locations are indicated in Fig. S73-S75.
Fixation/Embedding:	FF-PE (Fig. S76).

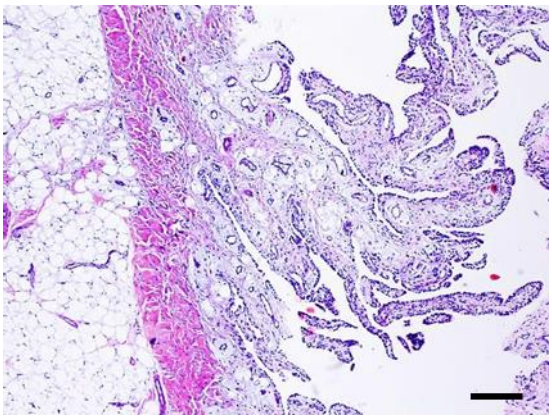


Fig. S76. Histology of synovial membrane. *Dorsal recess of the knee joint. FF-PE. HE. Scale bar = 100 µm.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one parasagittal decalcified section of the knee joint, including the distal femur and the proximal tibia (Morawietz et al., 2004). The synovial membrane samples from the knee joint that are generated according to the Type-I-study sampling protocols will allow for an adequate evaluation of porcine synovial membrane in routine studies.

2.6.3.2. Joint capsule and synovial membrane samples in Type-II and Type-III studies

General systematic random sampling strategy

The dorsal recesses of both knee joints are excised and placed on a planar sterile surface (Fig. S75). To determine the area of the joint recesses (reference compartment), the tissue is photographed with a scale (Fig. S77). The sampling positions are determined by systematic random sampling. The tissue is overlaid with a cross grid printed on a transparency (grid size: 3-5 mm). Three sampling locations per side are systematically randomly sampled (Fig. S77). An approximately 1x1-cm tissue piece is excised from each of the sampled locations. The excised tissue specimens are further subdivided for downstream analyses (Fig. S77): One CRYO-section cut at a vertical orientation to the inner surface of

the joint capsule, one GA-fixed sample, and one specimen of the synovial membrane (remove joint capsule tissue) for molecular analyses (-80°C). After GA fixation, a VUR section is prepared and embedded in Epon resin for semithin section histology, ultrastructural analysis and quantitative stereological analyses. Additionally, a tissue specimen for a FF-PE sample for histology, ISH and IHC and one tissue specimen for a FF-VUR-PRE (GMA/MMA) section are taken from the systematically randomly sampled locations of the joint capsule recess (Fig. S77 and Table S14).

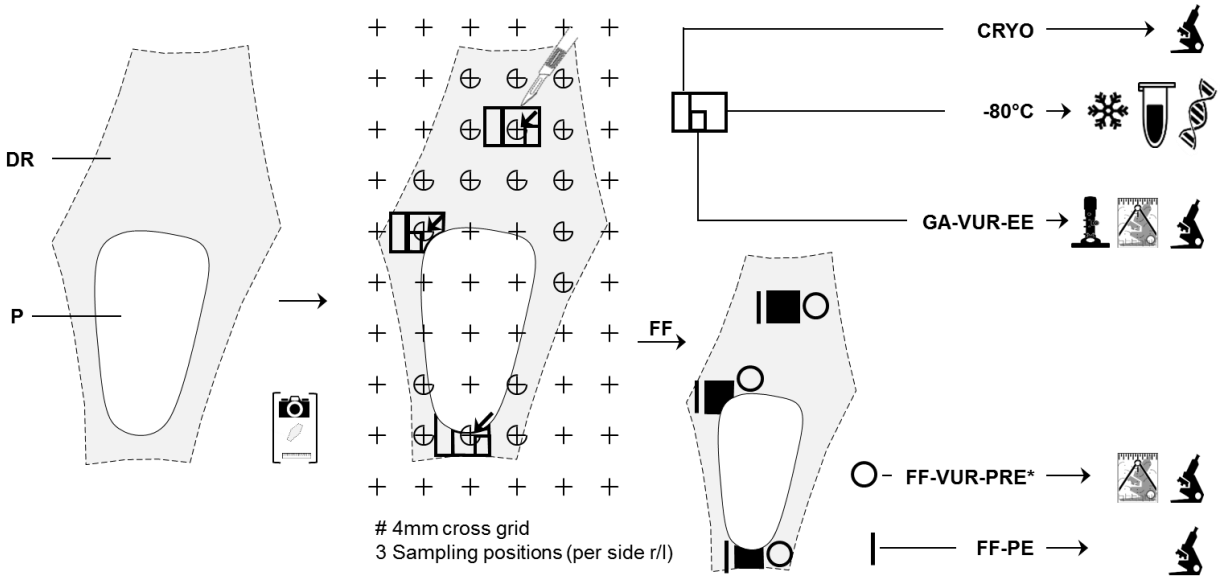


Fig. S77. *A schematic illustration of sampling of the joint capsule and synovial membrane of the dorsal recess (DR) of the knee joint and preparation of subsamples for downstream analyses in Type-II/III studies. P: Patellar bone. *PR = GMA/MMA. Refer to the text above for details.*

Table S14. The type and number of joint capsule and synovial membrane samples (dorsal recess of the knee joint) in Type-II and Type-III studies.

	Number of locations/samples	
Sample type	**Type-II studies**	**Type-III studies**
FF-PE	1 per sampled location	1 per sampled location
CRYO	1 out of every 3rd location	1 per sampled location
GA-VUR-EE	1 per sampled location	1 per sampled location
FF-VUR-PRE	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 out of every 2nd location	1 per sampled location

Time and personnel requirements

At least 20 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Gunson et al., 2013, Hartlev et al., 2013, Hunziker, 2007, Morawietz et al., 2004)

2.6.4. Tendons

Relevant anatomic features/preparation

Porcine tendons, including the deep and superficial flexor tendons of the forelimbs and hindlimbs, have been widely used in translational medical research (Duenwald-Kuehl et al., 2012, Feitosa et al., 2006). These sampling guidelines recommend the generation of tendon tissue specimens from the easily accessible deep digital flexor tendons of the hindlimb. For standardization and a reduction in workload for routine Type-I studies, it is recommended that tendon samples are consistently collected from the same (left) side of the body as the skeletal muscle, joint, and bone samples. Tendon sampling is performed prior to the metatarsal bone sampling and after the collection of the joint and skeletal muscle samples. The tendons of the flexor digitorum profundus muscle are located directly to the plantar side of metatarsal bones III and IV (Fig. S78 and Fig. S79). The tendons are excised proximal to their insertion at the distal phalanx (hoof bone) of the third and fourth digit and at the level of the tarsal joint. For excision, a sharp blade, such as a scalpel or a microtome blade, is used.

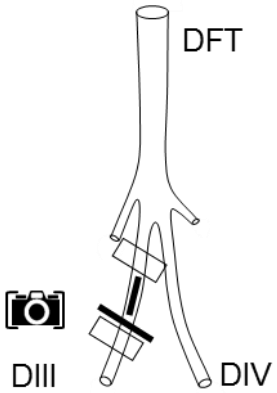


Fig. S78. *A schematic illustration of the deep digital flexor tendon (DFT) at its insertion at the third (DIII) and the fourth digit (DIV). The locations and orientations of the histology (black line) and molecular analyses (black rectangle) samples for Type-I studies are indicated.*

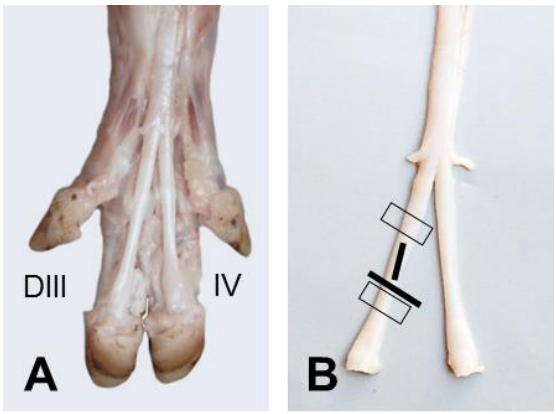


Fig. S79. A*: Ventral aspect of the metatarsus that demonstrates the anatomical location of the deep digital flexor tendon. Third (D III) and fourth digit (D IV).* ***B****: Excised deep digital flexor tendon. The locations and orientations of the histology (black lines) and molecular analyses (black rectangle) samples for Type-I studies are indicated.*

General examination parameters

The tendons and their sheaths and insertion points are inspected for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, and molecular analyses are taken from the altered site(s) if required.

2.6.4.1. Tendon samples in Type-I studies

Samples for molecular analyses

Location:	Deep digital flexor tendon of the third digit of the left hindlimb. The samples are taken proximal and distal to the locations of the histology specimen as indicated in Fig. S80.
Number of samples:	Two (Fig. S78-S80).
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Deep digital flexor tendon of the third digit of the left hindlimb. One cross section and one longitudinal section are taken. The sampling locations are indicated in Fig. S78, S79, and S80.
Fixation/Embedding:	FF-PE (Fig. S81).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies

Tendon tissue is not commonly evaluated by histopathology in regulatory toxicity studies in rodents. A selective generation of specimens from macroscopically unaltered tendon tissue for histopathological examination in regulatory toxicity studies is not necessary in studies involving pigs. However, intact tendon morphology is crucial for undisturbed locomotion, especially in large heavy pigs; thus, tendon tissues should be routinely included in the histopathological evaluation of the musculoskeletal system. This method is included in the porcine Type-I-study sampling protocols. The necropsy of "newly developed" genetically-modified pig models, particularly models displaying alterations in the locomotor system, should involve the collection and examination of tendon tissue during the initial pathological examination.

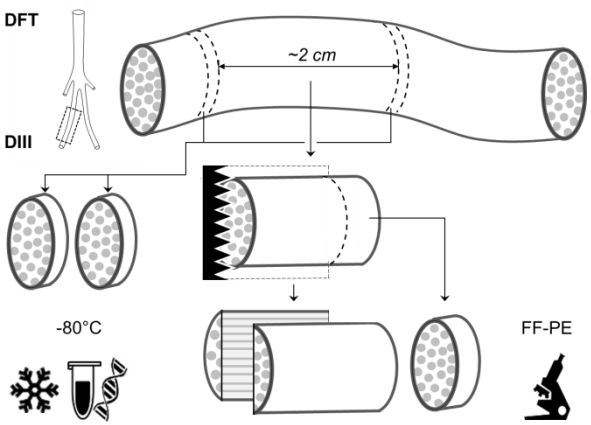


Fig. S80. *A schematic illustration of the sampling of the deep digital flexor tendon (DFT) of the third digit (DIII). The locations and orientations of the histology and molecular analyses samples for Type-I studies are indicated.*

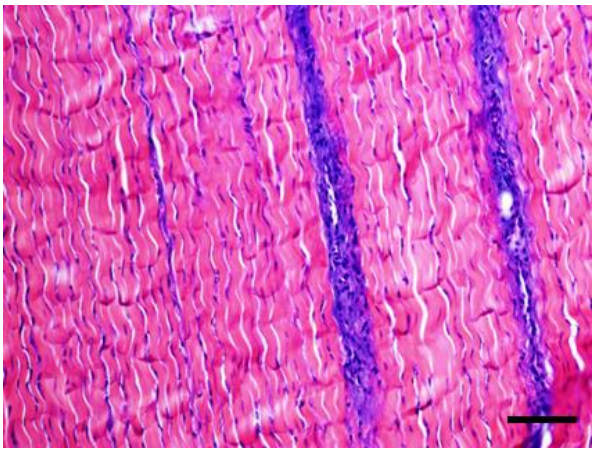


Fig. S81. Tendon histology. *Longitudinal section of the deep digital flexor tendon of the third digit of the hindlimb. FF-PE. HE. Scale bar = 100 µm.*

2.6.4.2. Tendon samples in Type-II and Type-III studies

For tendons, these guidelines do not recommend different sampling protocols for Type-II and Type-III studies. In Type-II/III studies, the deep digital flexor tendons (DFT) of both the third and fourth digit of the right or left (randomly allocated) hindlimb are sampled (Fig. S82 and Fig. S83). IUR-sectionable samples should be processed for quantitative stereological analyses because tendon tissue is highly anisotropic. Samples for molecular analyses, as well as longitudinally sectioned and cross-sectioned FF-PE and CRYO specimens are prepared from the DFT III (Fig. S82). Four cross-sectioned GA-VUR-EE specimens, four longitudinally sectioned GA-VUR-EE specimens and four FF-IUR(ORTRIP)-PRE (GMA/MMA) samples are prepared from the DFT IV (Fig. S82 and Fig. S83).

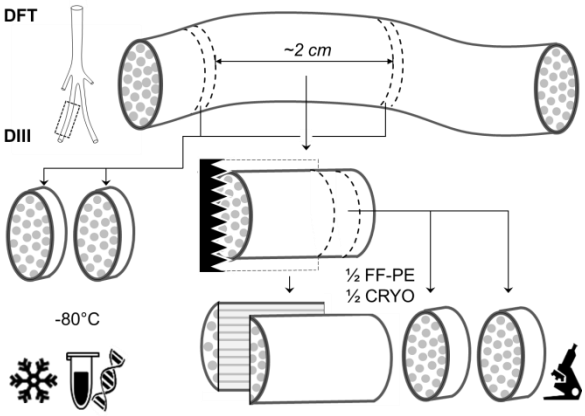


Fig. S82. *A schematic illustration of sampling of the DFT of DIII in Type-II/III studies. *PR = GMA/MMA.*

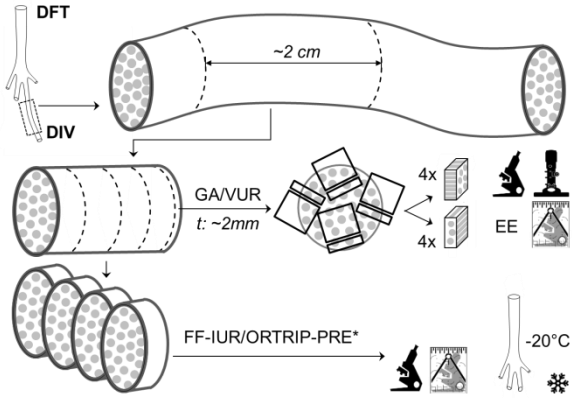


Fig. S83. *A schematic illustration of sampling of the DFT of DIV in Type-II/III studies. *PR = GMA/MMA.*

Time and personnel requirements

At least 25 minutes must be scheduled for the execution of the complete Type-II/III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Duenwald-Kuehl et al., 2012, Feitosa et al., 2006)

2.7. Head and neck

2.7.1. Eyes and adnexa

Relevant anatomical features/preparation

This guide recommends the generation of samples from the eyes, eyelids, lacrimal glands (*glandulae lacrimales*), third eyelids/nictitating membranes with superficial accessory lacrimal glands (*glandulae superficiales*, nictitating glands), Harderian glands (*glandulae profundae palpebrarum tertiarum*), and optic nerves. The lacrimal gland is located dorsal and caudal to the eye in the orbital cavity as indicated in Fig. S84, S86-S88 and S90. The nictitating gland is closely associated with the third eyelid. Both the lacrimal and nictitating glands are thin, pale structures that are difficult to distinguish from the surrounding connective tissue. The Harderian gland is well-developed and is larger than the lacrimal gland in pigs (Munkeby et al., 2006). The Harderian gland is located medio-rostro-ventral to the eye in the orbital cavity and is pale brown in color, with a lobulated structure (Fig. S84, S86-S88 and S90). The eye(s) should be harvested quickly (within ~5 minutes) after the animal's death to avoid retinal RNA/protein degradation. First, the dorsal and ventral eyelids are removed. Next, the globe with the lacrimal gland, third eyelid with the nictitating gland, Harderian gland, and optic nerve are removed from the orbita using tweezers and scissors.

Fig. S84. *Location of the lacrimal gland (LG) and Harderian gland (HG).*

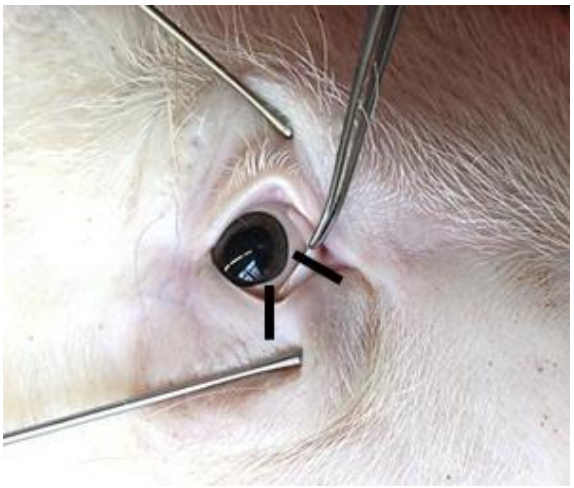


Fig. S85. *Right eye with eyelid section directions indicated.*

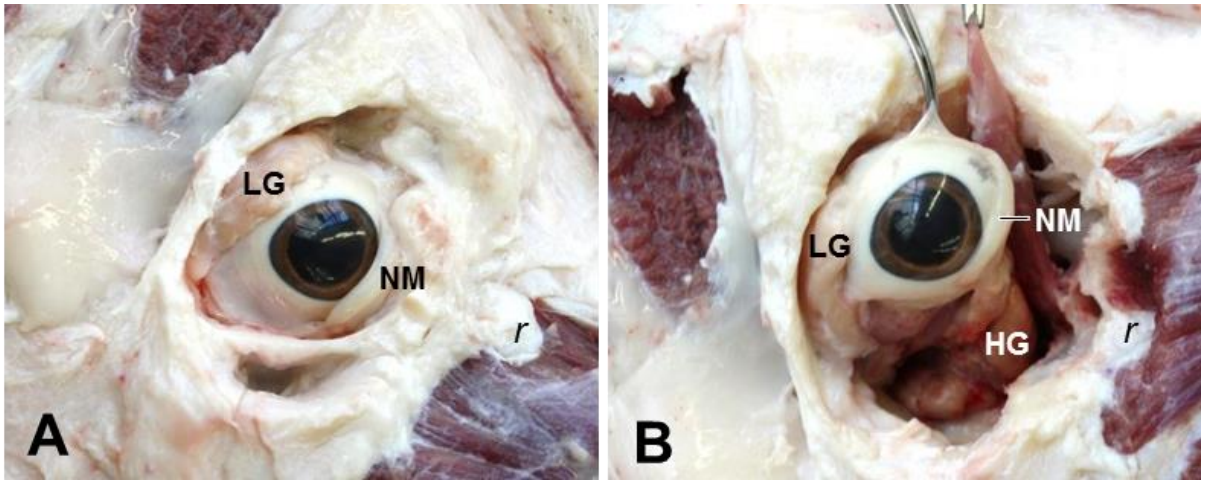


Fig. S86 (↑). *Right eye. The rostral side is indicated (r).* ***A****: Location of the lacrimal gland (LG) dorso-caudal to the globe. NM: Nictitating membrane.* ***B****: Location of the Harderian gland (HG) rostro-medio-ventral to the globe.*

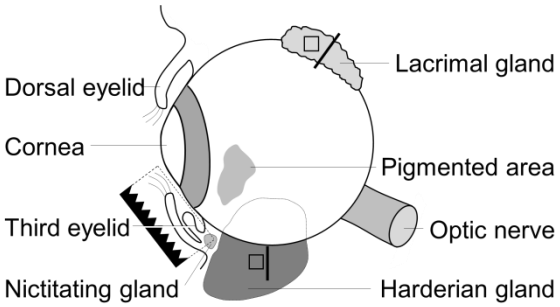


Fig. S87. *A schematic illustration of the globe (rostral aspect of the right eye) with eyelids, lacrimal gland, nictitating gland and Harderian gland. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. For histology, a sagittal section of the third eyelid and nictitating gland is taken.*

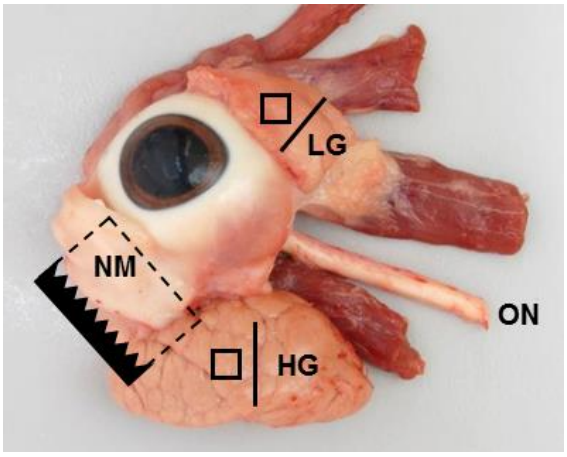


Fig. S88. *Enucleated (left) globe with lacrimal gland (LG), nictitating membrane (NM), Harderian gland (HG), and optic nerve (ON). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. A sagittal section of the third eyelid and the nictitating gland is taken.*

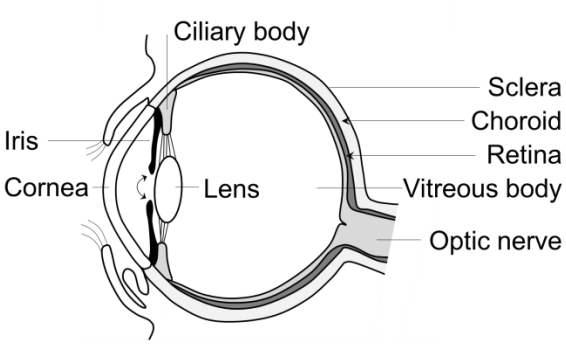


Fig. S89*. A schematic illustration of a sagittal section through the globe. The pupil is indicated by a bent arrow.*

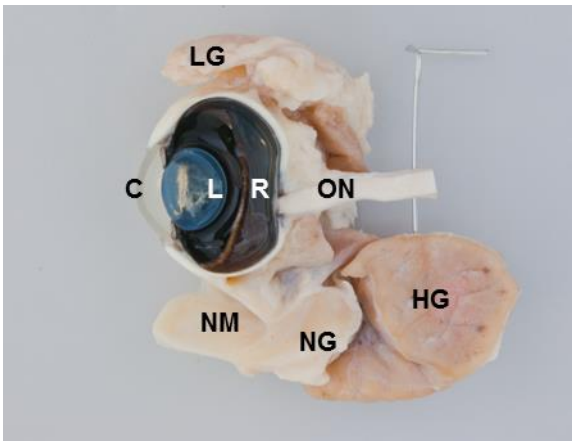


Fig. S90. *Parasagittal section through the fixed globe (for demonstration purposes). Lacrimal gland (LG); cornea (C); lens (C); retina (R); optic nerve (ON); nictitating membrane (NM); nictitating gland (NG); Harderian gland (HG).*

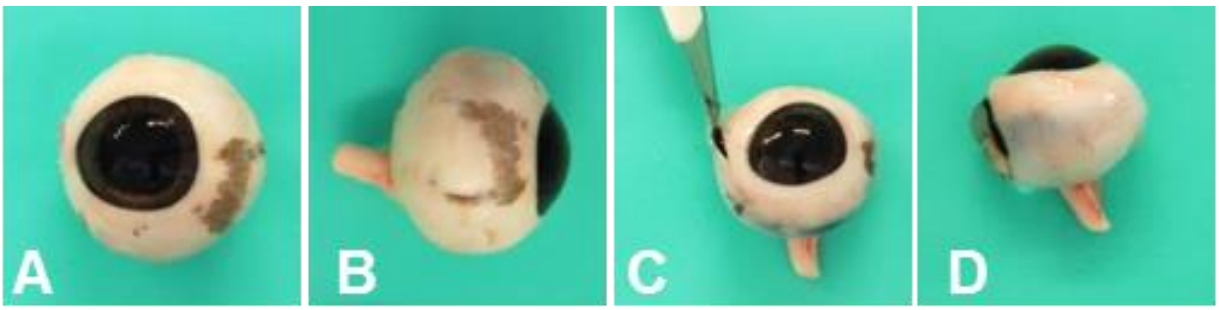


***Fig. S91 (↑). Pig eye preparation for histopathological examination in Type-I and Type-II studies**. **A**: Frontal aspect of a left globe. Note the typical pigmented area at the nasal angle of the sclera. **B**: Lateral aspect of the same globe. Note that the optic nerve exits the globe from the ventromedial quadrant. **C**, **D**: A small cap is cut from the globe to facilitate fixation of the inner eye structures. For paraffin histology, a sagittal section of the globe is cut through the optic disc after fixation. The lens is embedded in an extra cassette.*

General examination parameters
The size (dimensions) and weight of each globe are determined. The eyes and their adnexa are inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

2.7.1.1. Eye samples in Type-I and Type-II studies

In Type-I and Type-II studies, the same samples are taken for molecular analyses and histopathological examination.

Samples for molecular analyses (eye, lacrimal gland, nictitating gland, and Harderian gland)
In Type-I and II studies, samples for molecular analyses of the lacrimal and Harderian gland, retina (*in toto*), cornea, lens (in total), and vitreous body are collected from the left eye.

Location:	The sampling locations are indicated in Fig. S87, S88, and S92.
Remarks:	The entire retina, cornea, and lens are collected for molecular analyses (Fig. S92).
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

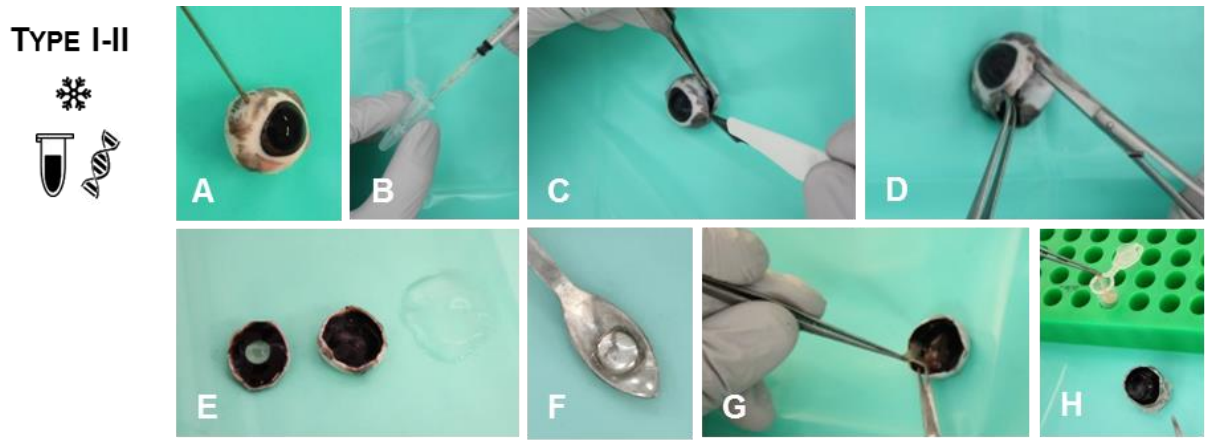


***Fig. S92.* Pig eye preparation** *for the generation of molecular analyses samples. **A**: A sample of the vitreous body (max. 1 ml) is taken using a 1 ml syringe adapted to a 20-Gauge cannula and transferred into a centrifuge tube (**B**) prior to freezing. The globe is then carefully cut into an anterior and posterior portion as indicated in **C**-**E**. **F**: The lens is then removed from the anterior portion using a sharp spoon. Additionally, the cornea can be excised for molecular analyses (not shown). **G**, **H**: The complete retina is carefully removed using tweezers and scissors.*

Histology samples

Location, number and orientation of sections: Histology samples are generated from the right eye. The sampling locations and orientations at the eyelids, lacrimal gland, and Harderian gland are indicated in Fig. S85, S87, S88, and S95. The globe is processed as shown in Fig. S91. After fixation, a sagittal (mid-axial) section is cut through the globe, including the attachment point of the optic nerve (compare to Fig. S89 and Fig. S90). The optic nerve should be present in both resulting sections.

Fixation/Embedding: Modified Davidson's (30% of a 37-40% solution of formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled H_2O) is the standard fixation solution for eye histopathology (Latendresse et al., 2002). Alternative fixation solutions, such as 4% formalin, Bouin's fixative (75 ml of saturated picric acid, 25 ml of 40% formaldehyde and 5 ml of acetic acid), or Rossman's fixative (90 ml of absolute ethyl alcohol saturated with picric acid (8-9%) and 10 ml of 40% formaldehyde), may be used according to the recommended downstream analyses (Culling, 1974). After sectioning, the samples are transferred into extra-deep embedding cassettes for processing and paraffin embedding (Fig. S93-S95).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

For histopathological examination of rodent and non-rodent eyes in nonclinical general toxicity studies, the Working Group on Nervous System Sampling of the Society of Toxicologic Pathology recommends a species-dependent trimming of eyes to obtain a sagittal section of the retina (Bolon et al., 2013b). The Type-I-study sampling protocols provided for porcine models principally recommend the generation of samples with comparable section levels and orientations to allow for the histopathological examination of the same eye structures, including the optic nerve. Similarly, the revised guides for organ sampling and trimming in rats and mice in regulatory toxicity studies recommend the preparation of two (one per side) longitudinal vertical sections through the eye in the plane of the optic nerve after fixation in Davidson's fixative. Sections cut in the described orientation also contain tissue from the Harderian gland (Morawietz et al., 2004). The porcine Type-I study and Type-II sampling protocols recommend the generation of representative samples of all relevant structures of the globe and eye's adnexa; thus, this method should be sufficient for an adequate evaluation of the eye in routine studies.

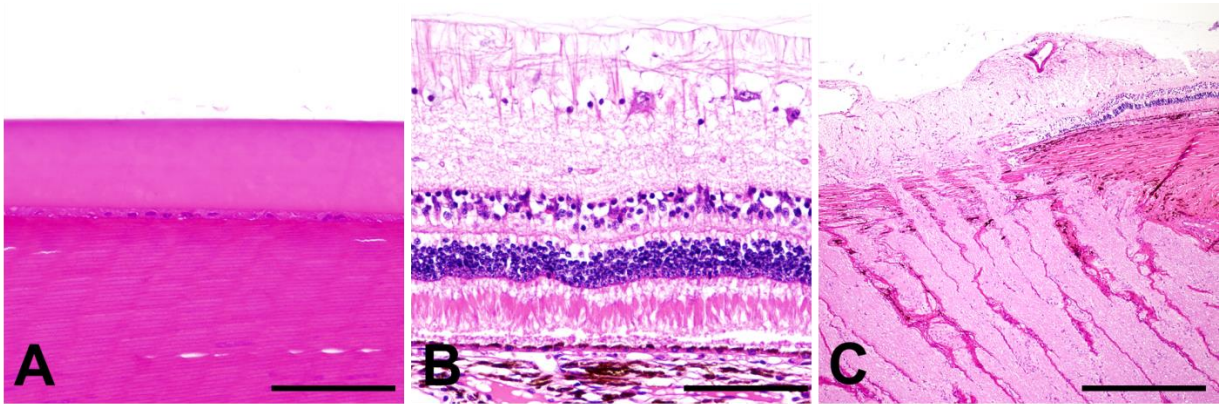


***Fig. S93. Histology of sensitive eye structures. A**: Lens. **B**: Retina. **C**: Optic disc and optic nerve. Fixation: Modified Davidson's fixative. Paraffin sections. HE. Scale bars = 100 µm.*

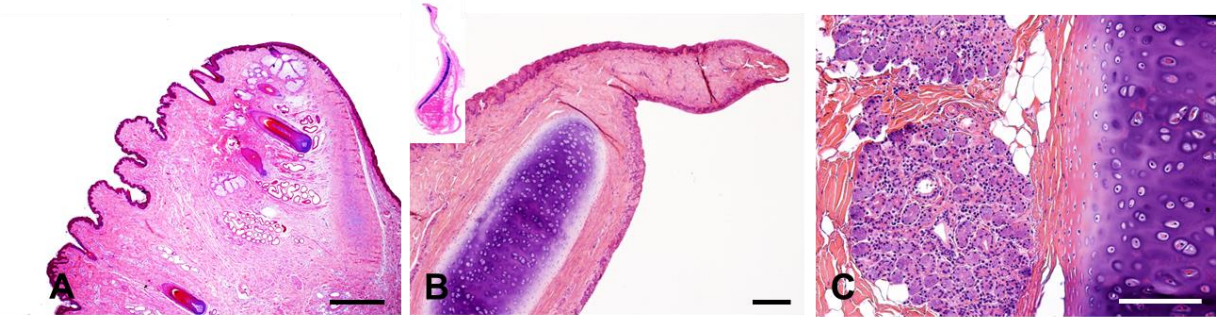


***Fig. S94. Eyelid histology. A**: Lower eyelid. **B**: Tip of the third eyelid (nictitating membrane). Inset: Histological slide with a section of the third eyelid. **C**: Lobules of the nictitating gland adjacent to the cartilage of the nictitating membrane. FF-PE. HE. Scale bars = 1 mm.*

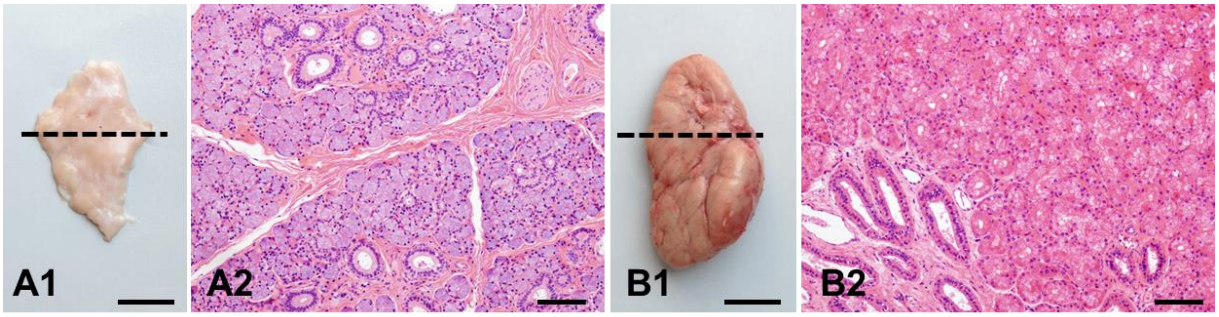


***Fig. S95. Lacrimal gland (A) and Harderian gland (B). A1**, **B1:** Freshly dissected glands. Dotted lines indicate the orientation of the histology samples. Scale bars = 1 cm. **A2**, **B2:** Histology. FF-PE. HE. Scale bars = 100 µm.*

2.7.1.2. Eye samples in Type-III studies

In Type-III studies, samples for histopathological and molecular analyses are prepared as indicated and described in Fig. S96. This sampling guide does not recommend the generation of samples for ultrastructural or quantitative histopathological analyses because these would require a sampling regime not compatible with the generation of larger numbers or different types of samples suitable for other analyses. If ultrastructural or quantitative histopathological analyses are of particular interest in an experiment, the sampling design must be adapted appropriately.

Alternative to the fixation of samples in various fixation solutions, specimens can be processed for cryosectioning (not shown). Admittedly, the advantage of the generation of a maximum of differentially processed specimens for various different downstream analyses requires a high sampling effort (time) and a potential impairment of the morphological sample quality by introduction of technical artifacts due to manipulation of the unfixed globe. For the eyelids, lacrimal gland, and Harderian gland, advanced sampling procedures different from the ones described above for Type-I studies are not recommended in this guide.

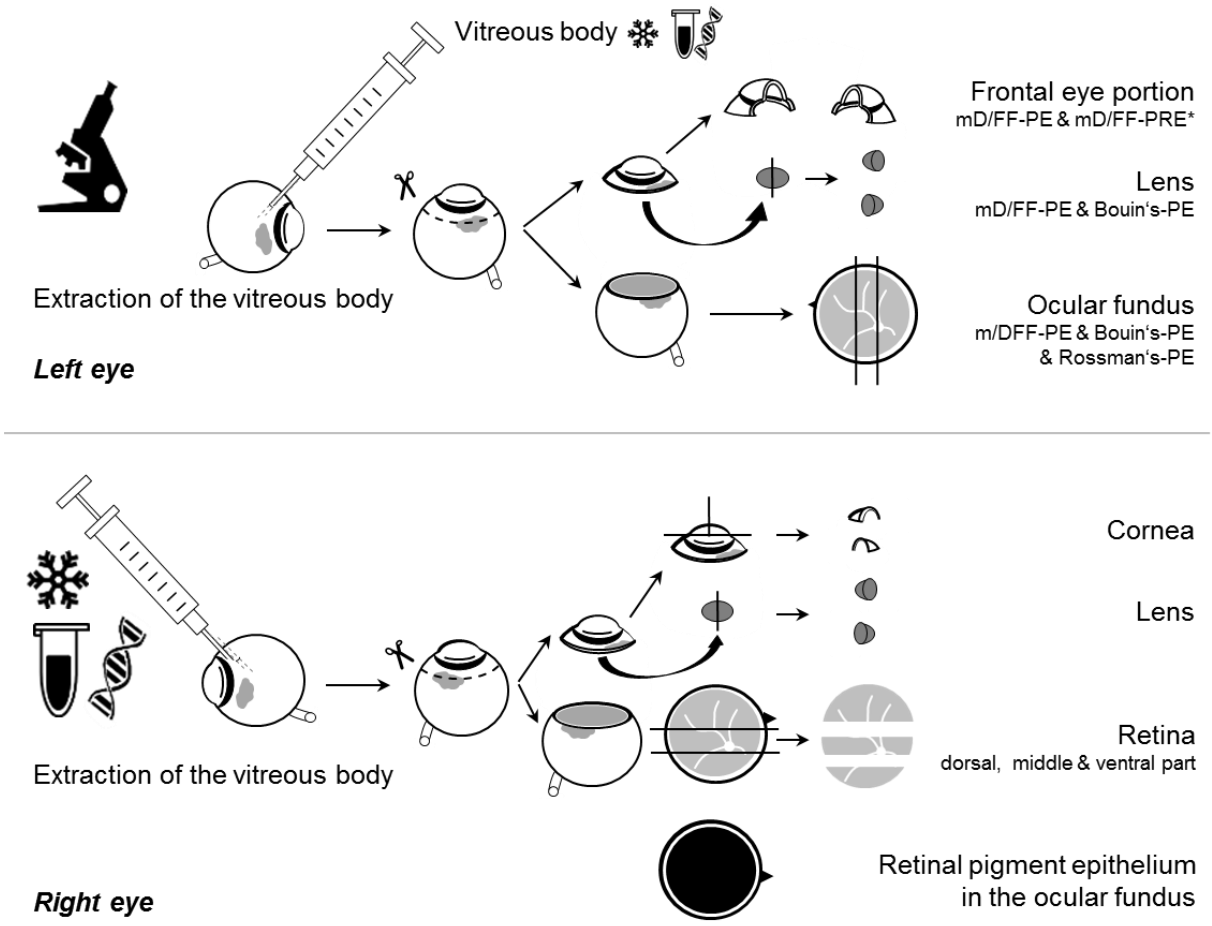


Fig. S96. A schematic illustration of eye sampling in Type-III studies. *The right or left eye are randomly assigned to sampling for histopathological or molecular analyses. The vitreous body is extracted from both eyes as also shown in Fig. S92 and frozen at -80 or -20°C for further molecular analyses. Next, the globe is cut open as also shown in Fig. S92. From the resulting frontal portions, the lenses are removed and divided into two halves, which are either frozen at -80°C for subsequent molecular analyses or fixed in modified Davidson's fixative (mD), formalin (FF), or Bouin's fixative for PE. Cornea samples for molecular analyses are taken from one of the remaining frontal globe hemispheres. The other globe hemisphere is cut in half as indicated (vertical section orientation) and fixed in modified Davidson's fixative (mD), formalin (FF), or Bouin's fixative for PE. One ocular fundus is cut in a dorso-ventral direction through the optic disc for an additional parallel rostral section. The rostral side is indicated by a black triangle. The three segments are fixed in modified Davidson's fixative (mD) or formalin (FF) (temporal segment), Bouin's fixative (medial segment), and Rossman's fixative (rostral segment) for PE and preparation of orthogonal sections of the ocular fundus. The applied sectioning directions ensure that the resulting sections contain retinal areas of both high and low ganglion cell-density (Garca et al., 2005). From the ocular fundus of the other eye, three strips of retina are carefully removed and frozen at -80°C for molecular analyses, including one rostro-caudal strip around the optic disc displaying the highest density of retinal ganglion cells and one dorsal and one ventral strip with a lower retinal ganglion cell-density. After removal of the retina, the ocular fundus with its exposed retinal pigment epithelium can be subjected to further analyses, such as biotin-labeling of the surface proteins of the retinal pigment epithelium (Uhl et al., 2014).*

Table S15. The type and number of eye samples for histopathological examination and molecular analyses in Type-III studies.

Ocular structure	Fixation/processing	Number of samples
Cornea	Molecular analyses (-80°C)	*2*
Frontal portion of the eye	mD*/FF-PE	*1*
	mD*/FF-PRE**	*1*
Lens	mD*/FF-PE	*1*
	Bouin's fixation-PE	*1*
	Molecular analyses (-80°C)	*2*
Ocular fundus *(with retina)*	mD*/FF-PE	*1*
	Bouin's fixation-PE	*1*
	Rossman's fixation-PE	*1*
Retina - dorsal portion	Molecular analyses (-80°C)	*1*
Retina - middle portion	Molecular analyses (-80°C)	*1*
Retina - ventral portion	Molecular analyses (-80°C)	
Ocular fundus *(without retina)*	Biotin-labeling of retinal pigment epithelium surface proteins	*1*

**modified Davidson's fixation (mD) or formalin fixation (FF). **PR = GMA/MMA.*

Time and personnel requirements

At least 30 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the receipt of the eyes to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Bolon et al., 2013b, Garca et al., 2005, Mao et al., 2014, Morawietz et al., 2004, Munkeby et al., 2006, Uhl et al., 2015, Uhl et al., 2014)

2.7.2. Ears

Relevant anatomical features/preparation

In pigs, preparation of the middle and inner ears is more complicated than from species with large tympanic bullae. The middle ear cavity can be accessed by preparation along the external acoustic meatus using a knife, scalpel, bone forceps and tweezers. Fig. S97 shows the stepwise ablation of tissue structures to expose the middle ear cavity for visual inspection and harvesting of the petrous part of the temporal bone with the inner ear for light microscopic examination.

For a basic histopathological examination of the inner ear structures, the petrosal part of the temporal bone can be fixed in a formalin solution and decalcified prior to embedding in paraffin. For better infiltration of fixative into the labyrinth, the membranous cover of the foramen rotundum is carefully punctured using a thin cannula.

Advanced histopathological analyses, including the use of acid-free decalcification solutions, PRE-histology, TEM and REM, as well as the generation of samples for molecular analyses are very time- and labor-intensive. These procedures are normally restricted to studies specifically investigating the inner ear (Parzefall et al., 2009, Lovell and Harper, 2007). Therefore, this guide features only standard FF-PE-histology techniques for overview studies of the inner ear.

General examination parameters

The middle-ear cavities of both ears should be examined by visual inspection because otitis media is not uncommon in pigs and may induce abnormal results on neurologic examination. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, and molecular analyses are taken from the altered site(s) if required.

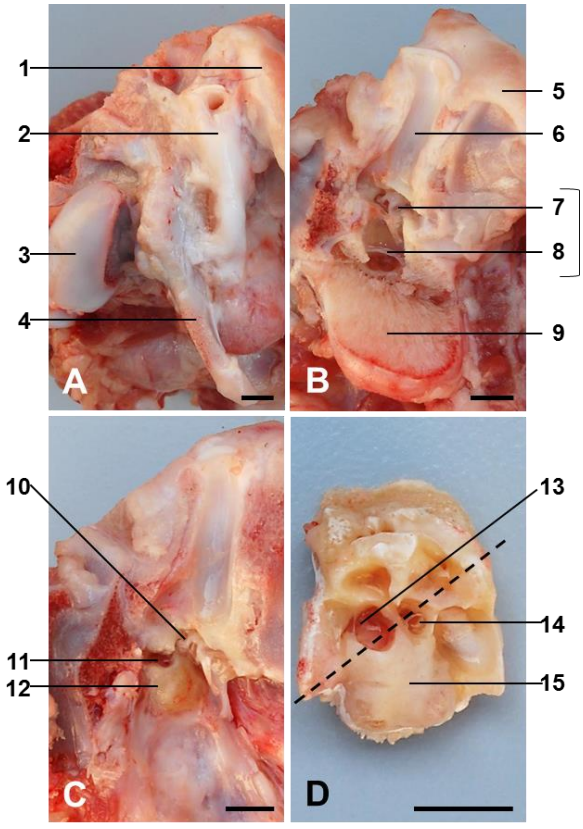


Fig. S97. Stepwise (A→C) preparation of the external, middle and inner ear. *Latero-ventral aspect of a right ear.* ***D****: Isolated petrous part of the temporal bone. Lateral aspect. The dotted line indicates the sectioning direction for preparation of histological sections.* ***1****: Zygomatic bone,* ***2****: External acoustic meatus,* ***3****: Occipital condyle,* ***4****: Paracondylar process,* ***5****: Zygomatic bone,* ***6****: External acoustic meatus (opened),* ***7****,* ***8****: Middle ear cavity,* ***7****: Auditory ossicles,* ***8****: Tympanic membrane,* ***9****: Tympanic part of the temporal bone (partially removed),* ***10****: Auditory ossicles,* ***11****: Foramen rotundum,* ***12****: Promontory containing the cochlea and the vestibular system,* ***13****: Foramen rotundum,* ***14****: Stapes sitting in the foramen ovale,* ***15****: Promontory. Scale bars = 5 mm.*

2.7.2.1. Standard histology of the petrous part of the temporal bone in Type-I studies

Location, and number of samples	The petrous parts of both temporal bones (right and left) are excised as indicated in Fig. S97.
Fixation: 4% *FA.*	For better infiltration of fixative into the labyrinth, the membranous cover of the foramen rotundum is carefully punctured using a thin cannula.
Decalcification:	D2 (acid-based) slow acting decalcification solution. Decalcification might take up to several weeks.
Section direction:	After decalcification, the petrous bone is sectioned following a line connecting the foramen ovale and the foramen rotundum, which will provide a section through the most relevant structures, including the cochlea, vestibular system, promontory, middle ear mucosa, facial nerve and the cochlear ganglion and nerve.
Embedding:	Paraffin (Fig. S98-S101).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies

The middle and inner ear are not commonly evaluated by histopathology in regulatory toxicity studies in rodents. Without macroscopic or clinical findings that indicate hearing or equilibrium disturbances, a selective generation of inner ear specimens for histopathological examination in regulatory toxicity studies involving pigs is not necessary. However, the collection and examination of middle and inner ear samples should be completed during the initial pathological examination of newly developed, genetically modified pig models.

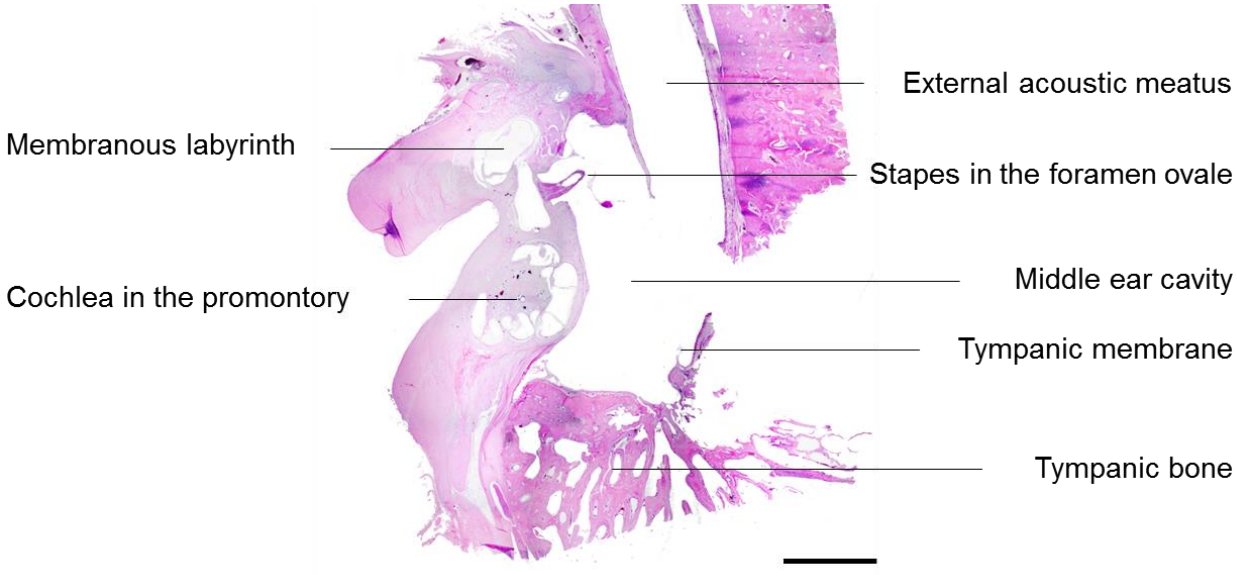
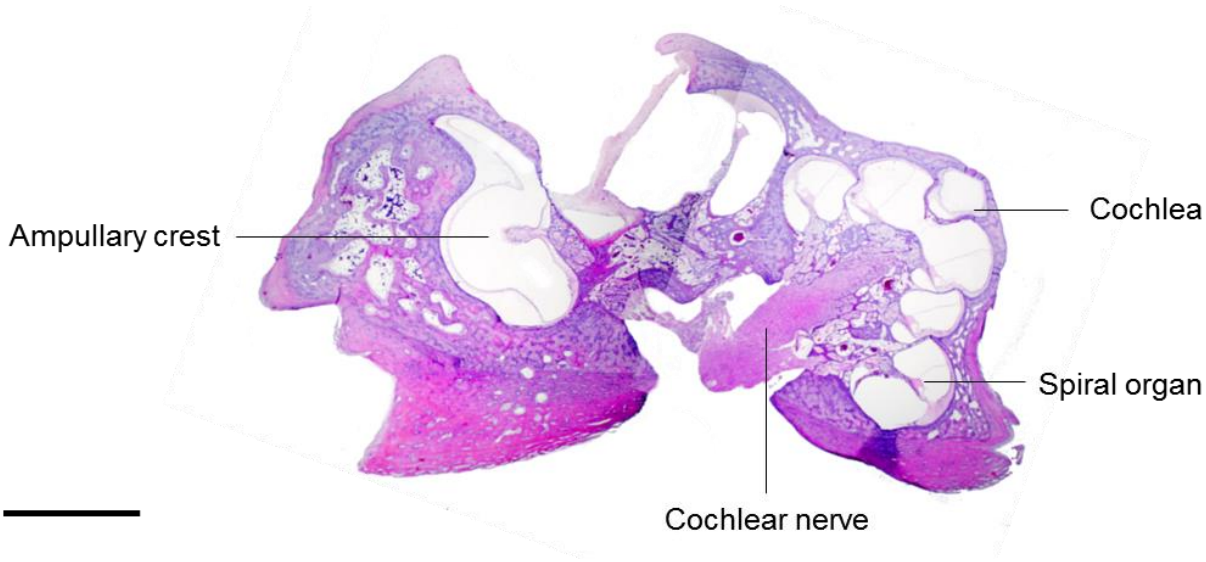


Fig. S98. *Histology of the external acoustic meatus, the middle ear, and the inner ear (right side). The section plane orientation refers to Fig. S97D. FF-DECAL-PE. Photomontage. Scale bar = 5 mm.*



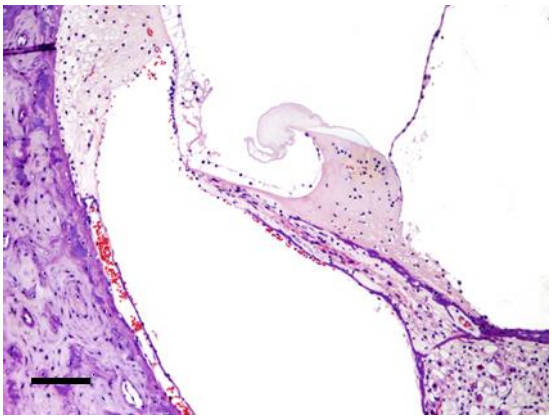


Fig. S99 (↑). *Histology of the petrous part of the right temporal bone with the inner ear. FF-DECAL-PE. Photomontage. Scale bar = 5 mm.*

Fig. S100 (←). *Histology of the spiral organ. FF-DECAL-PE. Photomontage. Scale bar = 100 µm.*

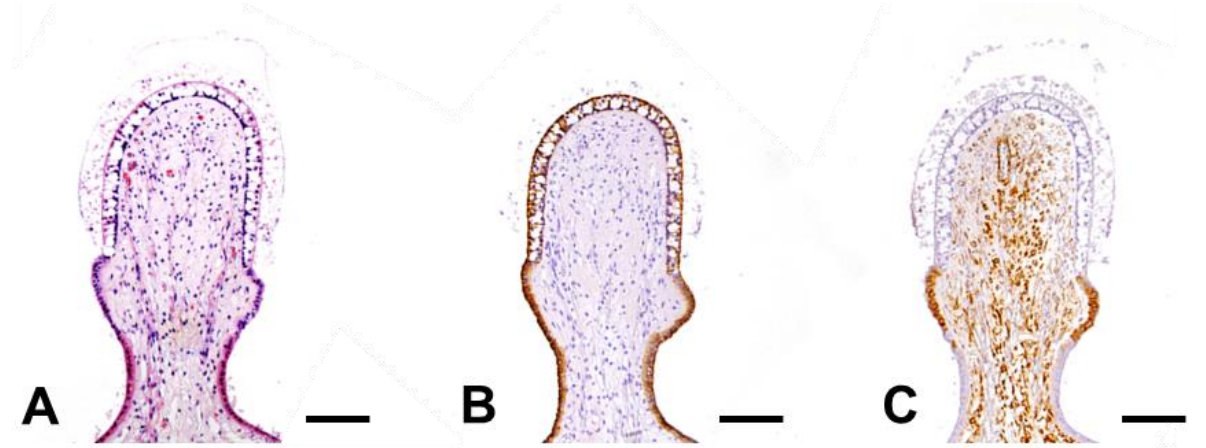


Fig. S101. *The histology and IHC of the ampullary crest demonstrate good preservation of morphological details and the applicability of IHC in FF-DECAL-PE sections.* ***A****: HE stained section.* ***B****: IHC for detection of cytokeratin.* ***C****: IHC for detection of vimentin. [Cytokeratin and vimentin immunohistochemistry was performed using standard protocols. Primary antibodies: Monoclonal Mouse Anti-Vimentin (Clone V9, 1:300, Code No. M0725); Monoclonal Mouse Anti-Human Cytokeratin (Clones AE1/AE3, 1:50, Code No. M3515, DakoCytomation, Denmark); secondary antibodies: Peroxidase Conjugated Rabbit Anti-Mouse Immunoglobulins (1:100, Code No. P0161), Polyclonal Swine Anti-Rabbit Immunoglobulins/HRP (1:100, Code No. P0217, DakoCytomation, Denmark). Diaminobenzidine was used as the final chromogen, and hemalaun was used as a nuclear counterstain]. Paraffin sections. Scale bars = 100 µm.*

Related literature

(Parzefall et al., 2009, Lovell and Harper, 2007, Madsen et al., 2001)

2.7.3. Sampling of tongue, esophagus, teeth, nasal septum, turbinates, ethmoidal labyrinth, larynx, and parathyroid glands.

For the tongue, esophagus, nasal cavities with the nasal septum, turbinates, olfactory mucosa, larynx, and parathyroid glands, this guide recommends the generation of standard histology samples (Type-I studies) and retention samples for molecular analyses if necessary. Advanced sampling protocols (Type-II/III studies) are not recommended for these organs. If required for a Type-II/III study, the sampling of the esophagus can be performed using the study Type-II/III sampling procedures described for the duodenum (section 2.10.2.2.). In oral and inhalation toxicity studies, different sampling protocols are recommended for the nasal cavities with the nasal septum, turbinates, and larynx (Table S16). The routine sampling of teeth is not recommended in these guidelines. As a retention sample for subsequent molecular analyses, 1-2 incisors and molar teeth may be extracted and frozen at -80°C (section 2.7.3.2.). Sampling of other neighboring tissues/structures of the head/pharynx, such as tonsils (section 2.5.3.), salivary glands (section 2.7.4.), thyroid gland (section 2.7.5.), or distinct vessels of the neck (section 2.4.), are described in separate sections.

Table S16. Samples of the tongue, esophagus, teeth, larynx, nasal septum, turbinates, olfactory mucosa, and parathyroid glands for histology and molecular analyses in Type-I studies.

Organ/tissue	Histopathological samples[1] N°, orientation, location of sections	Molecular analysis samples[2]
Tongue	3: 1, cross, left middle of tongue[3]	1
	1, longitudinal, left middle of tongue[3]	-
	1, cross, left side of tongue base[3]	-
Esophagus	4: 1, cross, proximal third (c/l)[4]	
	1, longitudinal, proximal third (c/l)[4]	1
	1, cross, distal third (c/l)[4]	1
	1, longitudinal, distal third (c/l)[4]	
Teeth	-	Left I2, P2[8]
Larynx (oral study)	1: frontal, left aryepiglottic fold[5]	1 (right)
Larynx (inhalation study)	4: 1, frontal, base of epiglottis, left side[5]	1 (right)
	1, frontal, left aryepiglottic fold[5]	1 (right)
	1, horizontal, left vocal cord[5]	1 (right)
	1, frontal, cricoid cartilage, left side[5]	1 (right)
Nasal cavities		
Nasal septum (oral study)	1: frontal, level of the first premolar[6]	-
Nasal septum (inhalation study)	3: 1, frontal, caudal to the first incisors[6]	-
	1, frontal, level of the first premolar[6]	-
	1, frontal, caudal end of the nasal septum[6]	-
Turbinates (oral study)	1: frontal, left side, level of the first premolar[7]	-
Turbinates (inhalation study)	3: 1, frontal, left side, caudal to the first incisors[7]	-
	1, frontal, left side, level of the first premolar[7]	-
	1, frontal, left side, caudal end of turbinates[7]	-
Olfactory mucosa	1: parasagittal, left side[7]	-
Parathyroid glands[5]	1-2	1

[1]FF-(DECAL)-PE. [2]Freeze and store at -80°C. [3-7] Section locations and orientations are indicated in: [3]Fig. S102, [4]Fig. S105, [5]Fig. S108, [6]Fig. S109, [7]Fig. S110. [8]Teeth of lower jaw.

2.7.3.1. Tongue and esophagus

Relevant anatomical features/preparation
The tongue is excised with the larynx, trachea and its adjacent structures and viscera of the thoracic cavity. The soft palate is incised longitudinally to expose the larynx. The esophagus is longitudinally opened at its full length and dissected from the trachea. The tongue is cut off at the base of the epiglottis, weighed and measured (length). The weight and length of the esophagus are determined.

General examination parameters
The tongue and esophagus are inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

Tongue samples for molecular analyses and histopathological examination in Type-I studies, including general toxicity studies of orally administered drugs

Sample for molecular analyses

Number and location of samples:	A specimen of the the proper lingual muscles is obtained as indicated in Fig. S102 and Table S16. In large tongues, only the left side of the organ is sampled.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One cross section and one longitudinal section are prepared from the middle of the tongue as indicated in Fig. S102 and Table S16. In large tongues, only the left side of the organ is sampled. For histological samples of the taste buds, a cross section at the level of the vallate papillae is prepared.
Fixation/Embedding:	FF-PE (Fig. S103).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents
For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one longitudinal vertical tongue section and one optional transverse section through the mid-portion of the tongue (Ruehl-Fehlert et al., 2003). The porcine Type-I study protocols recommend the same sample generation to adequately evaluate the tongue in routine studies.

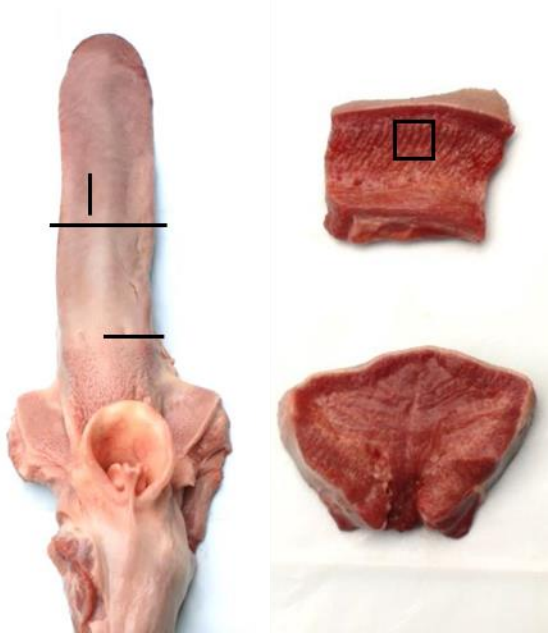


***Fig. S102. Tongue**. The locations and orientations of the histology (black lines) and molecular analysis (black rectangle) samples for Type-I studies are indicated. A cross section and longitudinal section from the middle of the tongue are prepared for FF-PE histology. A sample for molecular analyses is excised from the proper lingual muscles. For the histopathological examination of taste buds, an additional cross section at the level of a vallate papilla from the base of the tongue can be prepared (Table S16).*

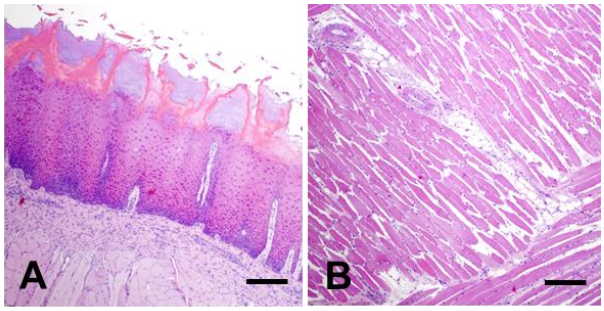


***Fig. S103. Histology of the tongue**. **A**: Surface of the tongue. Longitudinal section. **B**: Deep tongue musculature (proper lingual muscles and hyoglossus muscle). Cross section. FF-PE. HE. Scale bars = 100 µm.*

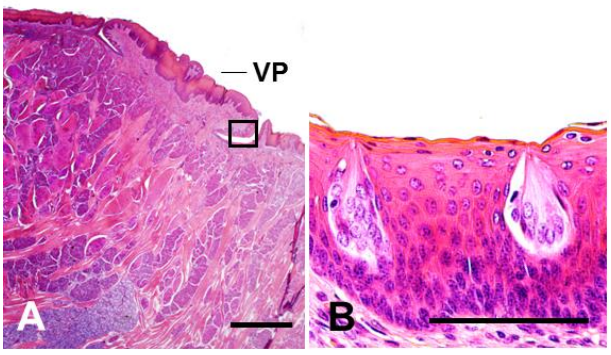


***Fig. S104. Taste bud histology. A**: A cross section through the base of the tongue at the level of a vallate papilla (VP). The location of the taste buds is indicated by a rectangle. **B**: Histology of taste buds. FF-PE. HE. Scale bars = 100 µm.*

Esophagus samples for molecular analyses and histopathological examination in Type-I studies, including general toxicity studies of orally administered drugs

Samples for molecular analyses

Number and location of samples:	Two samples of the esophageal mucosa are taken from the middle of the proximal third and from the middle of the distal third of the esophagus as indicated in Fig. S105 and in Table S16.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One cross section and one longitudinal section are prepared from the proximal third and from the distal third of the esophagus as indicated in Fig. S105 and in Table S16.
Fixation/Embedding:	FF-PE (Fig. S106).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory (oral) toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one transverse section through the esophagus and the trachea at the level of the thyroid gland (Kittel et al., 2004, Ruehl-Fehlert et al., 2003).

Considering the length of the porcine esophagus, the examination of samples taken from two locations of the esophagus is recommended in the Type-I-study sampling protocols to ensure an adequate evaluation of the porcine esophagus in routine studies.

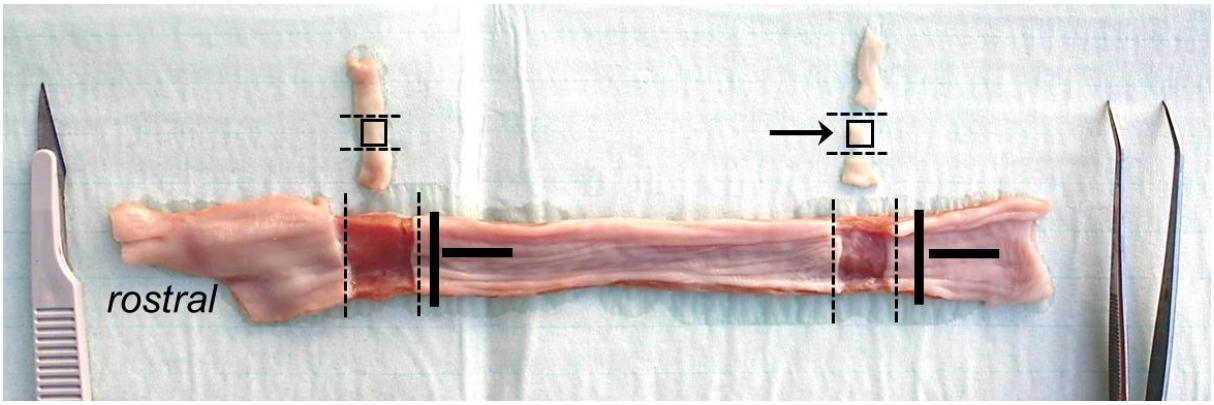


Fig. S105. Esophagus *(longitudinally opened). The locations and orientations of the histology samples (black lines), and the incision lines (black dotted lines) in the esophageal mucosa for collection of the the samples for molecular analyses (black rectangle) for Type-I studies are indicated.*

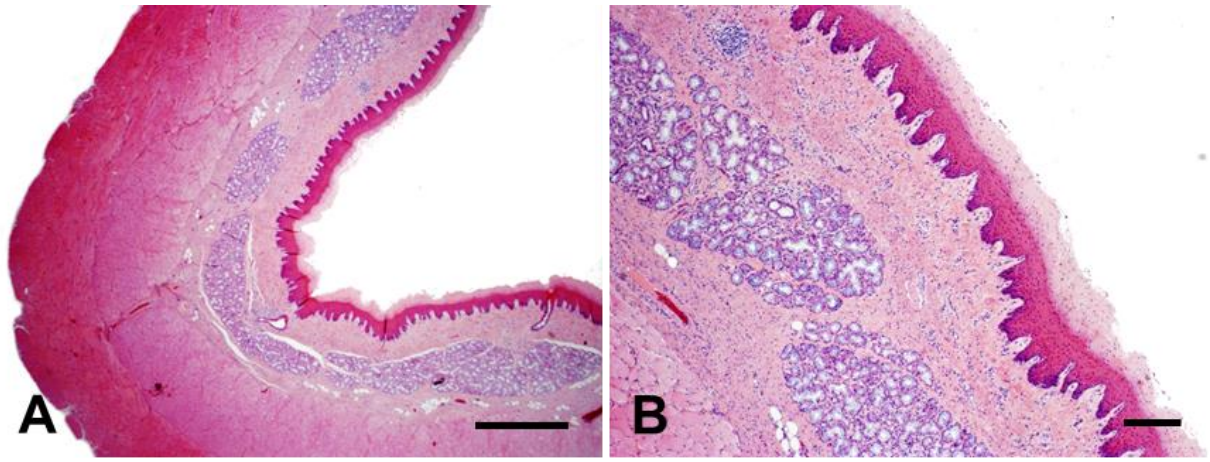


***Fig. S106. Histology of the esophagus (proximal third). A**: Overview. **B**: Detailed enlargement. Note the submucosal glands and the stratified epithelium. FF-PE. HE. Scale bars = 100 µm.*

Related literature
(Bertram et al., 2013, Gelberg, 2012, Juhl et al., 1995, Perkins et al., 2008, Ruehl-Fehlert et al., 2003)

2.7.3.2. Teeth

The preparation of standard histology samples of teeth is not recommended in this guide. For histopathological examination of teeth in pigs, the interested reader is referred to the 2012 review of B. L. Foster, which recommends fixation of teeth samples in Bouin's fixative or formalin, followed by long-term decalcification. In Type-III studies of porcine models without suspected or evident alteration of teeth, extracted teeth may be obtained and stored at -80°C as a retained sample for subsequent molecular analyses. An extraction without damaging the teeth is a challenging task, especially in large, adult pigs. If an extraction is not attainable, the teeth can be removed with adjacent jawbone tissue using a saw (Fig. S107). In porcine biobank projects, the author's laboratory routinely collects two second incisors (I2) and two second molar teeth (P2) of the lower jaw to sample different types of teeth (Table S16).

***Fig. S107. Dissected teeth.** Left: Extracted second incisor of the lower jaw (I2). Right: Sawed out second back tooth (P2) of the lower jaw. The teeth. are frozen and stored at -80°C as retention samples for Type-III studies.*

Related literature
(Foster, 2012a, Gelberg, 2012, Wang et al., 2007)

2.7.3.3. Larynx

Relevant anatomical features/preparation

After removal of the esophagus, the larynx is longitudinally incised at its dorsal side. This incision is then extended into the trachea and the main bronchi. After removal of the tongue, the larynx can be isolated or samples of the larynx can be collected while the larynx remains attached to the trachea. In small pigs, the larynx can be sagittally sectioned into two halves. Frontal hemisections are prepared for histopathological examination from one half of the larynx and samples for molecular analyses are prepared from the other half of the specimen. In larger animals, separate histology samples must be generated to fit into the standard embedding cassettes.

General examination parameters

The larynx is inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, mycology, virology and molecular analyses are taken from the altered site(s) if required.

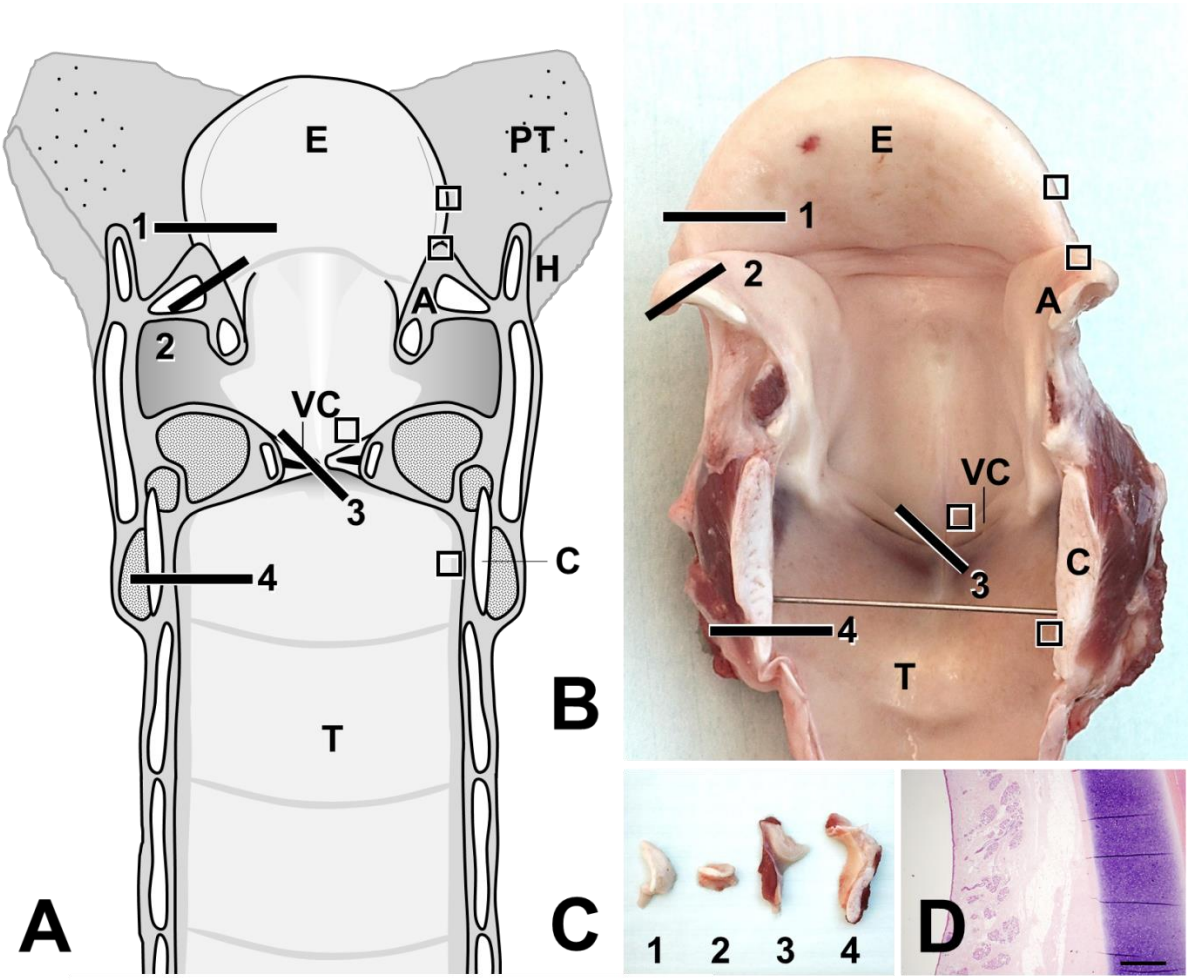


***Fig. S108. Sampling of the larynx. A**: Schematic illustration. E: Epiglottis. PT: Palatine tonsil. A: Arytenoid cartilage. H: Hyoid bone. VC: Vocal cord. C: Cricoid cartilage. T: Trachea. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. **B, C:** Images of the larynx and excised samples. **D:** Larynx histology (location N°2). Note the cartilage and submucosal glands. Scale bar = 100 µm. Histology samples are taken from the left side of the larynx, and samples for molecular analyses are taken from the right side of the larynx (or vice versa). In oral toxicity studies, the N°2 location is sampled. In inhalation toxicity studies, locations N°1-4 are sampled (Table S16).*

Larynx samples for molecular analyses and histopathological examination in Type-I studies, including oral and inhalation toxicity studies

Different sampling protocols are applied for the generation of laryngeal samples in oral and inhalation toxicity studies. Samples for histopathological examination are routinely taken from the left side of the larynx, and samples for molecular analyses are obtained from the right side of the larynx (or *vice versa*).

Samples for molecular analyses (oral study)

Number and location of samples:	One sample of the mucosa is taken from the right aryepiglottic fold as indicated in Fig. S108 (Location 2) and in Table S16.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Samples for molecular analyses (inhalation study)

Number and location of samples:	Four samples of the laryngeal mucosa are generated as indicated in Fig. S108 and Table S16. One sample is taken from the right lateral margin of the epiglottis (location 1 in Fig. S108), one sample is taken from the right aryepiglottic fold (location 2 in Fig. S108), one sample is taken from the lateroventral laryngeal mucosa cranial to the vocal cord (location 3 in Fig. S108), and one sample is taken from the lateroventral laryngeal mucosa of the cricoid cartilage (location 4 in Fig. S108).
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples (oral study)

Location, number and orientation of sections:	One cross section of the left aryepiglottic fold is prepared as indicated in Fig. S108 and in Table S16.
Fixation/Embedding:	FF-PE (Fig. S108D).

Histology samples (inhalation study)

Location, number and orientation of sections:	Four samples of the larynx are prepared, each including laryngeal mucosa, sub-mucosa and cartilage as indicated in Fig. S108 and in Table S16. One frontal (cross) section is taken from the right lateral margin of the epiglottis (location 1 in Fig. S108), one frontal (cross) section is taken from the right aryepiglottic fold (location 2 in Fig. S108), one oblique-horizontal section is taken from the vocal cord and the ventral pouch (location 3 in Fig. S108), and one frontal (cross) section is taken from the lateroventral laryngeal mucosa of the cricoid cartilage (location 4 in Fig. S108).
Fixation/Embedding:	FF-PE.

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory oral toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Ruehl-Fehlert et al., 2003) recommend the collection of one transverse section through the middle of the larynx. For inhalation studies, three transverse sections are prepared from the base of the epiglottis, the ventral pouch, and the cricoid cartilage (in rats only). Considering the size of the porcine larynx, samples from one (oral studies) or four locations (inhalation studies) of the larynx is recommended in the Type-I-study sampling protocols to ensure an adequate evaluation of the porcine larynx in routine studies.

Related literature

(Gorti et al., 1999, Kittel et al., 2004, Renne et al., 2009, Ruehl-Fehlert et al., 2003)

2.7.3.4. Nasal septum, turbinates and ethmoidal labyrinth

Relevant anatomical features/preparation

The surfaces of the nasal septum, turbinates and ethmoidal labyrinth are covered by four different types of epithelia: Squamous epithelium (vestibulum), transitional epithelium (dorsal meatus), respiratory epithelium (middle and ventral meatus, sinus, nasal septum), and olfactory epithelium (ethmoturbinates) (Kuper et al., 2012). Samples for histopathological examination are routinely taken from the left nasal cavity. After removal of the brain (section 2.8.1.1.), pituitary gland (section 2.8.1.2.), eyes (section 2.7.1.), petrosal part of the temporal bone with the middle and inner ear (section 2.7.2.), tongue and larynx (section 2.7.3.), and skin (section 2.2.1.), the head is sawed sagittally slightly right paramedian of the nasal septum. After collection of samples of the nasal septum (Fig. S109), the rest of the cartilaginous portion of the septum is removed and samples of the turbinates and the ethmoidal labyrinth are excised using a sharp scalpel and hammer if necessary. After fixation in a 4% formaldehyde solution, the specimens are decalcified prior to trimming and embedding in paraffin. The generation of samples from the paranasal sinus is not recommended in these sampling guidelines.

General examination parameters

The nasal septum, turbinates and ethmoidal labyrinth are inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, mycology, virology and molecular analyses are taken from the altered site(s) if required.

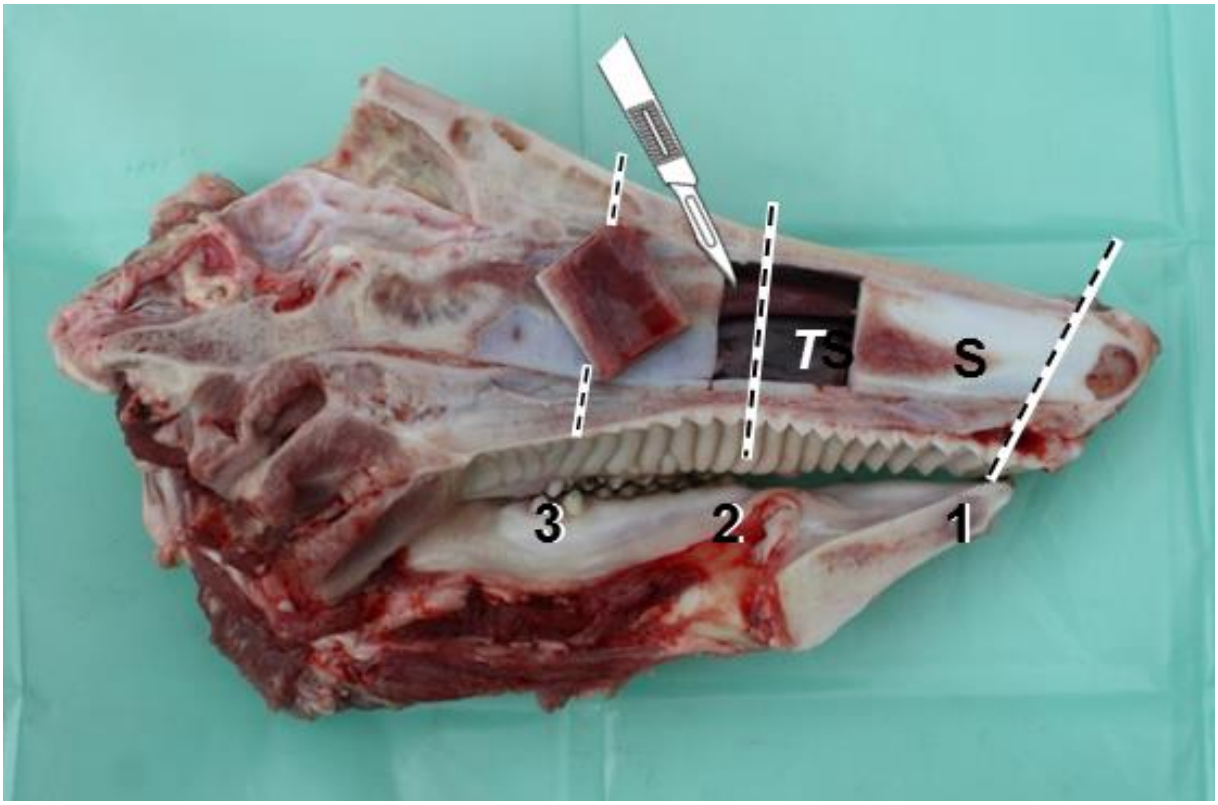
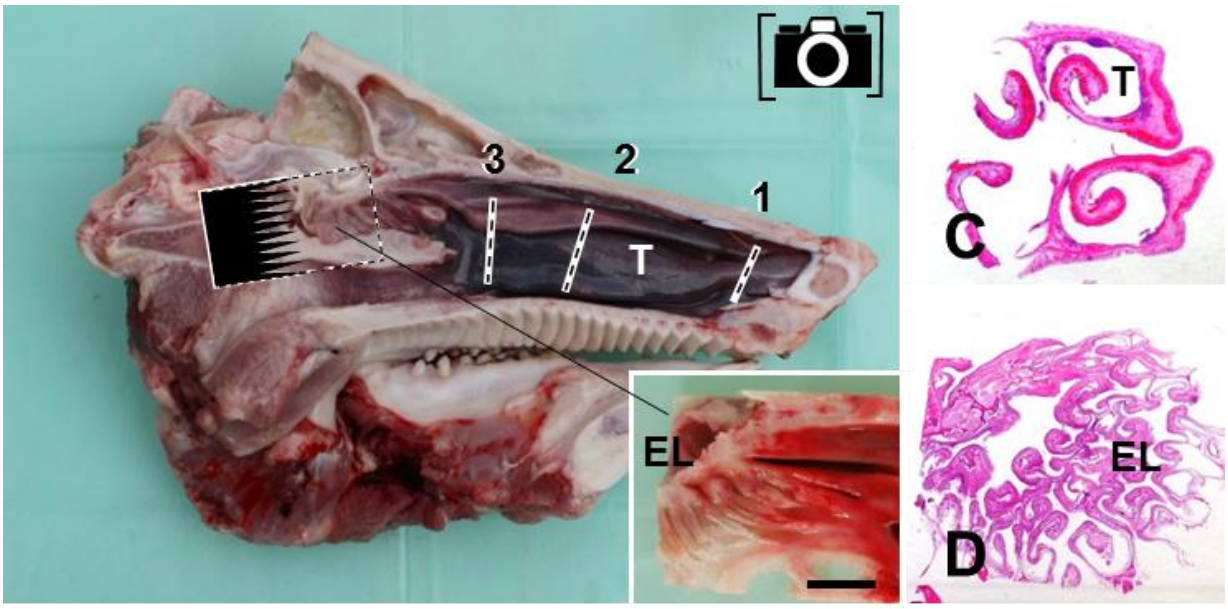


***Fig. S109. Excision of samples from the nasal septum (S)**. T: Nasal turbinate. The section orientations are indicated by dotted lines. In Type-I studies, including oral toxicity studies, the N°2 location is sampled. In inhalation toxicity studies, samples are taken from locations N° 1-3.*

***Fig. S110 (↓). A: Turbinates (T) and left ethmoidal labyrinth (EL, olfactory mucosa) sampling.** Cutting directions/section orientations are indicated by dotted lines. In Type-I studies, including oral toxicity studies, a section from the turbinates at location N° 2 is collected. In inhalation toxicity studies,* turbinate *samples are taken from locations N°1-3. Scale bar in inset = 1 cm. **B**, **C**: Photo of a histological slide of a turbinate (**B**) and ethmoidal labyrinth (**C**). FF-PE. HE.*



Histology samples of the nasal septum, turbinates, and the ethmoidal labyrinth in Type-I studies, including oral and inhalation toxicity studies

Different sampling protocols are applied for generation of histology samples in oral and in inhalation toxicity studies. Generation of samples for molecular analyses of the nasal septum, the turbinates and the ethmoidal labyrinth is not scheduled in study Type-I sampling protocols (Table S16).

Histology samples (oral study)

Location, number and orientation of sections:

Nasal septum. One frontal section is taken at the level of the first premolar as indicated in Fig. S109 (location N° 2) and in Table S16.
Turbinates. One frontal section is taken from the left nasal turbinates at the level of the first premolar as indicated in Fig. S110 (location N° 2) and in Table S16.
Ethmoidal labyrinth. A parasagittal section of the left ethmoidal labyrinth is taken as shown in Fig. S110.

Histology samples (inhalation study)

Location, number and orientation of sections:

Nasal septum and the turbinates. Three frontal sections of the nasal septum and the turbinates are taken at defined levels. One frontal section is taken caudal to the first incisors as indicated in Fig. S109 and in Fig. S110 (location N° 1), one frontal section is taken at the level of the first premolar (location N° 2) and one frontal section is taken at the caudal/aboral end of the turbinates (location N° 3).
Ethmoidal labyrinth. A parasagittal section of the left ethmoidal labyrinth is taken as shown in Fig. S110.

In location 1, the turbinates are mainly covered by transitional epithelium. Squamous epithelium is present at the rostral tip of the turbinates. In location 2, the turbinates are covered by transitional epithelium (dorsal meatus) and respiratory epithelium (middle and ventral meatus). In location 3, the turbinates are covered by respiratory epithelium (Kuper et al., 2012). The nasal septum is covered by

respiratory epithelium (Fig. S111). Sections of the ethmoidal labyrinth contain olfactory epithelium and respiratory epithelium (Fig. S112).

Fixation/Embedding: After fixation in formaldehyde solution, the samples of the turbinates and ethmoidal labyrinth are decalcified then embedded in paraffin. Decalcification protocols are provided in section 2.6.2, Bones. No decalcification is needed for the sample of the nasal septum (Fig. S111 and Fig. S112).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For evaluation of the nasal cavity, nasopharynx, and paranasal sinus in regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one (oral toxicity studies, level 3) or four (inhalation studies) samples, including transverse sections through the fixed skull at the level of the posterior part of the upper incisors (level 1), incisive papilla (level 2), second palatine crest (level 3), and first molar teeth (level 4). Sections are decalcified prior to paraffin embedding (Kittel et al., 2004). In studies involving pigs, these sampling guidelines recommend to sample one location (oral studies) or three locations (inhalation studies) of the nasal septum and turbinates and one location of the ethmoidal labyrinth to ensure an adequate evaluation of the different types of epithelia in the nasal cavity.

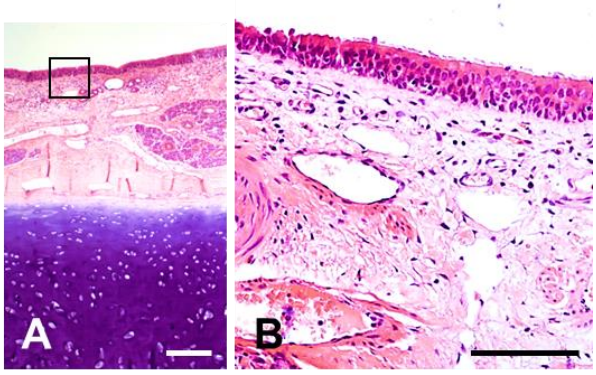


Fig. S111. Histology of the nasal septum.
A*: Overview. Note the cartilage and submucosal glands. The location of the nasal mucosa shown in B is indicated by a black rectangle.* ***B****: Histology of the nasal mucosa with respiratory epithelium. FF-PE. HE. Scale bars = 100 µm.*

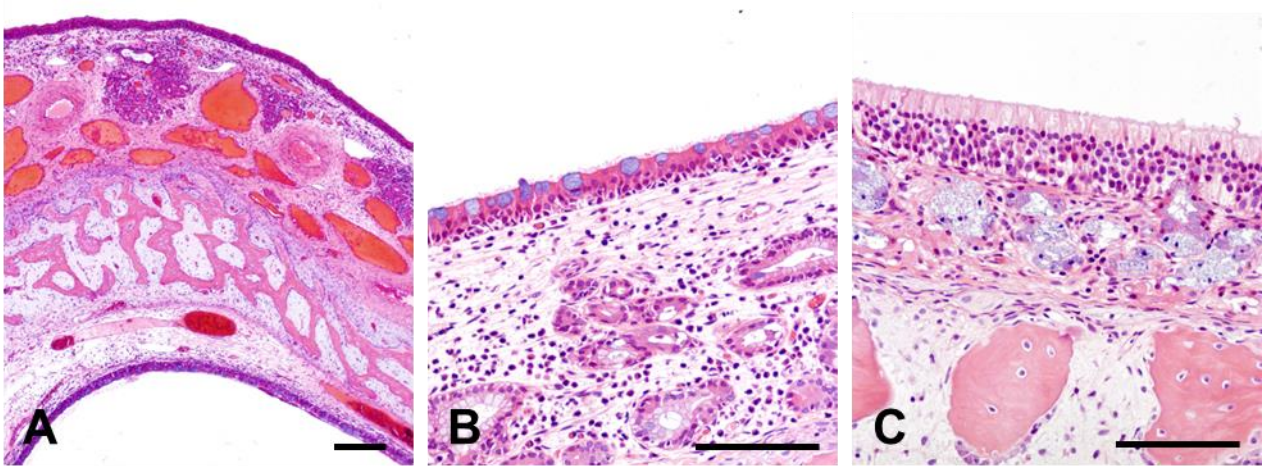


Fig. S112. Histology of the ethmoidal labyrinth. ***A****: Overview. Note the bony framework beneath the well-vascularized submucosa with abundant glands.* ***B****: Nasal mucosa with respiratory epithelium consisting of ciliated cells and abundant goblet cells.* ***C****: Olfactory mucosa. FF-PE. HE. Scale bars = 100 µm.*

Related literature

(Glorieux et al., 2007, Hennig-Pauka et al., 2012, Kittel et al., 2004, Kuper et al., 2012, Renne et al., 2009)

2.7.3.5. Parathyroid glands

Relevant anatomical features/preparation

In pigs, the parathyroid glands are separate from the thyroid gland and can be found near the cranial part of the cervical portion of the thymus apposed to a branch of the carotid artery at the approximate level of the larynx (Fig. S113 and Fig. S114). The glands have a bean-like shape and are often embedded in thymus tissue. In adult pigs, each gland is approximately 10x8x5 mm. Their color varies from tan-beige to violet. The parathyroid glands have a slightly harder consistency than the thymus. However, the parathyroid glands can often not be identified due to the similar appearance of the adjacent adipose tissue, lymph nodes and thymus. If the glands cannot be identified, larger pieces of thymus tissue are taken from the regions where the parathyroid glands are presumably located (Fig. S113), fixed in formaldehyde solution, and embedded in paraffin. The parathyroid glands may then be identified in the histological sections of these tissue samples (Soshin et al., 2010).

These sampling guidelines recommend the collection of samples for standard histology and molecular analyses, but not for advanced studies (Type-II and -III) because the parathyroid glands are difficult to identify. The locations for histology and molecular analysis samples are indicated in Fig. S113.

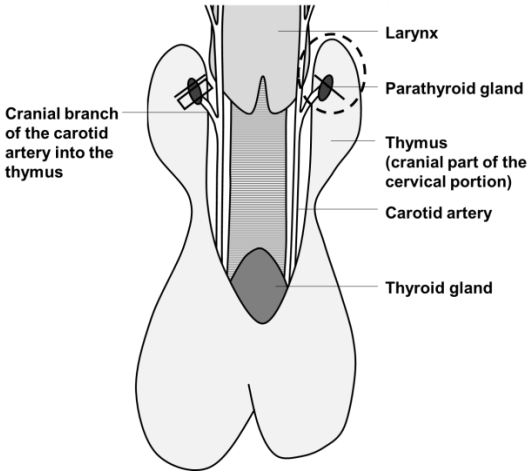


Fig. S113. A schematic illustration of the topographic anatomy of the parathyroid gland. *Ventral aspect of the neck (adapted from Soshin et al., 2010). The dotted line marks the approximate position of the left parathyroid gland that may be difficult to macroscopically identify. The Type-I-study sampling location for molecular analyses (black rectangle in the right gland) and the location/section orientations for histology samples (black lines in the left and the right gland) are indicated.*

General examination parameters

If the parathyroid glands can be identified macroscopically, both glands are measured, weighed, and inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, and microbiological analysis are taken from the altered site if required.

Samples for molecular analyses

Location:	If macroscopically identifiable, one sample is taken from the middle of the left or right gland parenchyma.
Remarks:	The sample is split into two halves. Histology of one half is performed to confirm that the tissue is parathyroid gland tissue.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Number and orientation of sections:	Cross sections from both glands are prepared as indicated in Fig. S113.
Fixation/Embedding:	FF-PE (Fig. S115).

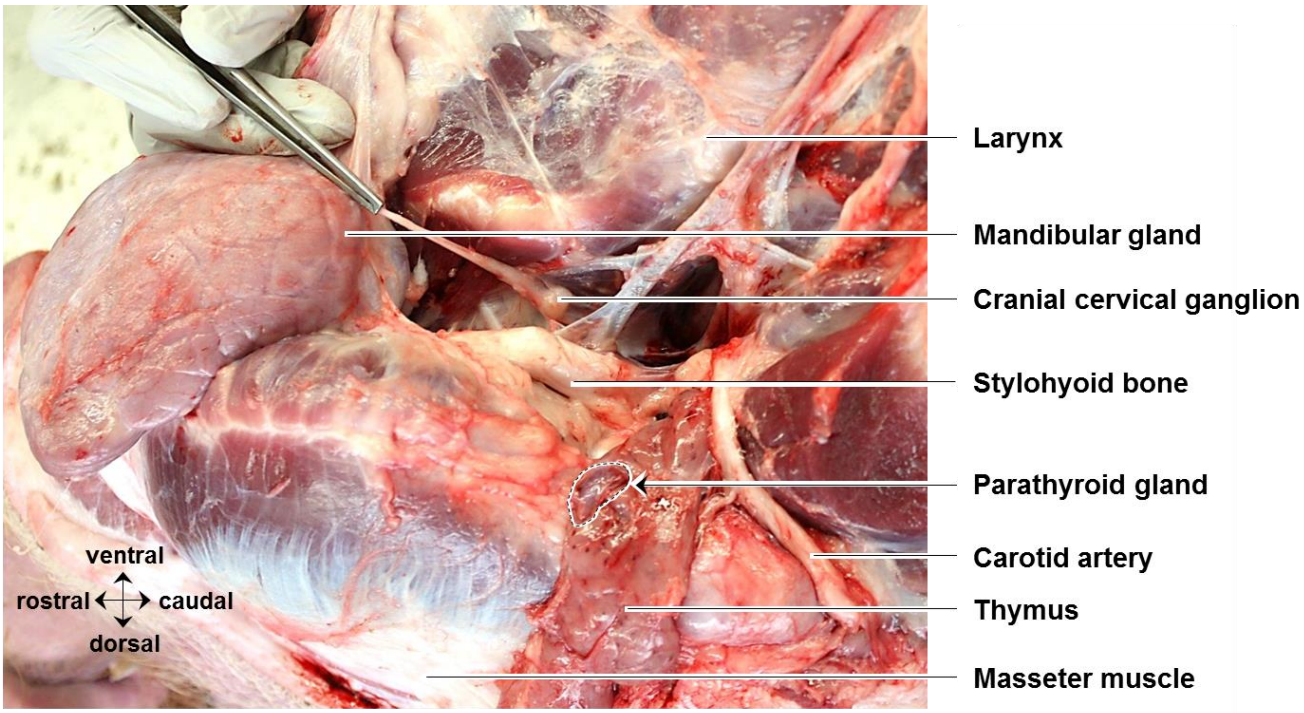


Fig. S114. *Topographic anatomy of the parathyroid gland in a nine-month-old boar. Note that the cranial part of the cervical portion of the thymus with the parathyroid gland has been reflected back.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For histopathological examination of the parathyroid glands in regulatory (oral) toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one transverse section through the esophagus and the trachea at the level of the thyroid gland. Alternatively, longitudinal horizontal sections through the isolated thyroid glands are prepared. If necessary, re-cuts are made to include the parathyroid in the sections (Kittel et al., 2004, Ruehl-Fehlert et al., 2003). In comparison, the samples generated according to the Type-I-study sampling protocols allow for the adequate evaluation of the porcine parathyroid gland in routine studies.

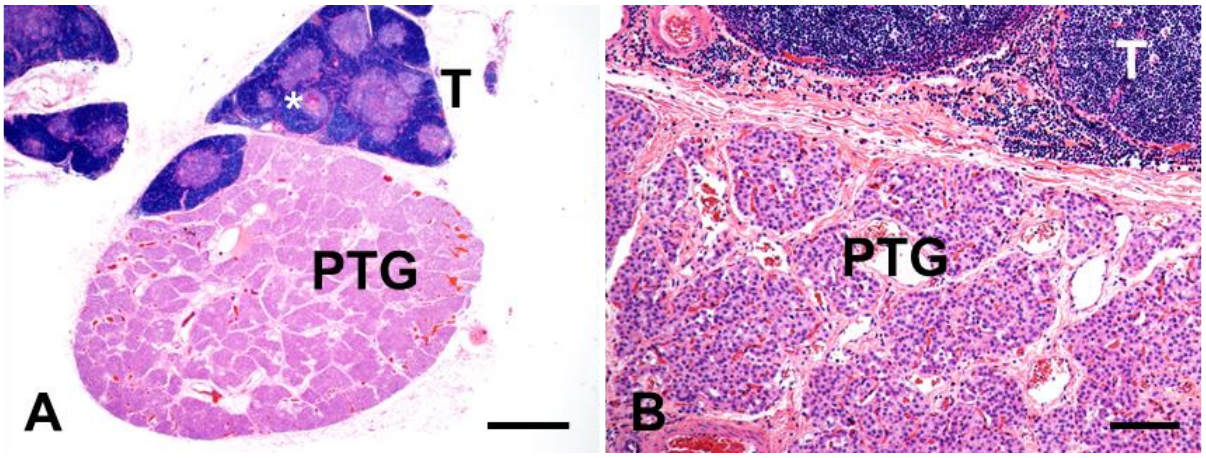


Fig. S115. Histology of the parathyroid gland (PTG)*.* ***A****: Overview. Note the adjacent thymus tissue (T) with Hassall's corpuscles (thymic body, *).* ***B****: Detailed enlargement. FF-PE. HE. Scale bars = 100 µm.*

Related literature

(Soshin et al., 2010, Kittel et al., 2004, Ruehl-Fehlert et al., 2003)

2.7.4. Salivary glands

Relevant anatomical features/preparation

From the salivary glands (buccal, sublingual, parotid and mandibular glands), the mandibular gland and the parotid gland are sampled. For standardization and a reduction in workload, it is recommended to consistently sample the salivary glands from one side (left) of the body. After removal of the tongue and trachea with its adjacent structures, the parotid gland is harvested. This glad is triangular, lobulated, light brown in color and located under the skin directly caudal to the mandibular bone. Next, the mandibular gland, located medial and slightly cranial to the parotid gland, can be accessed. The topographic anatomy of the mandibular gland and the parotid gland are indicated in Fig. S116 and Fig. S117.

General examination parameters

The size (dimensions) and weight of the salivary glands are determined. The salivary glands are inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

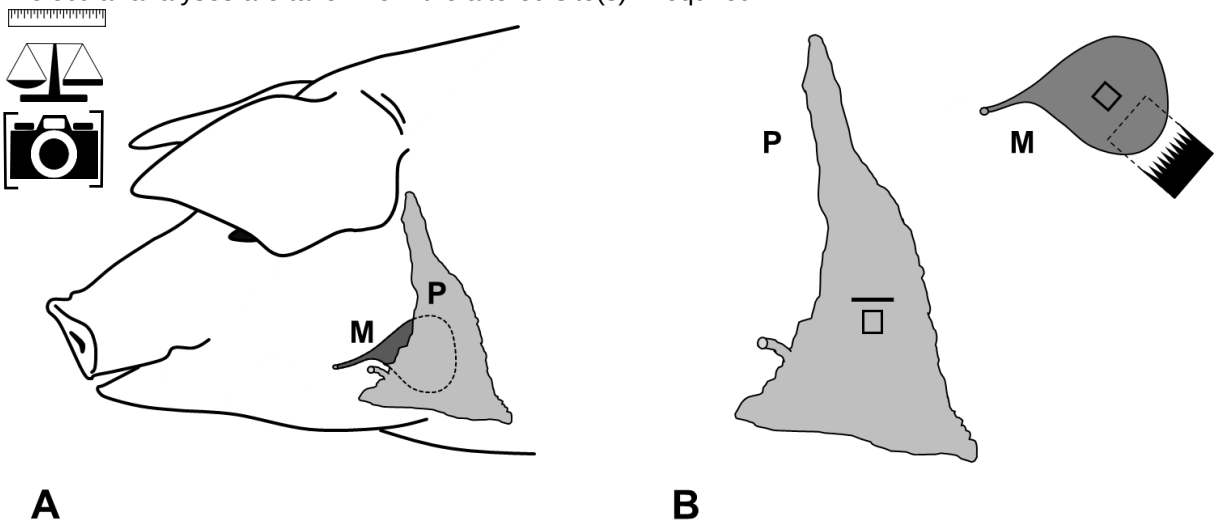


Fig. S116. *A schematic illustration of the anatomical location (**A**) and preparation of the parotid gland (P) and mandibular gland (M).* ***B:*** *The locations and orientations of the samples for histology (black line) and molecular analyses (black rectangles) in Type-I studies are indicated.*

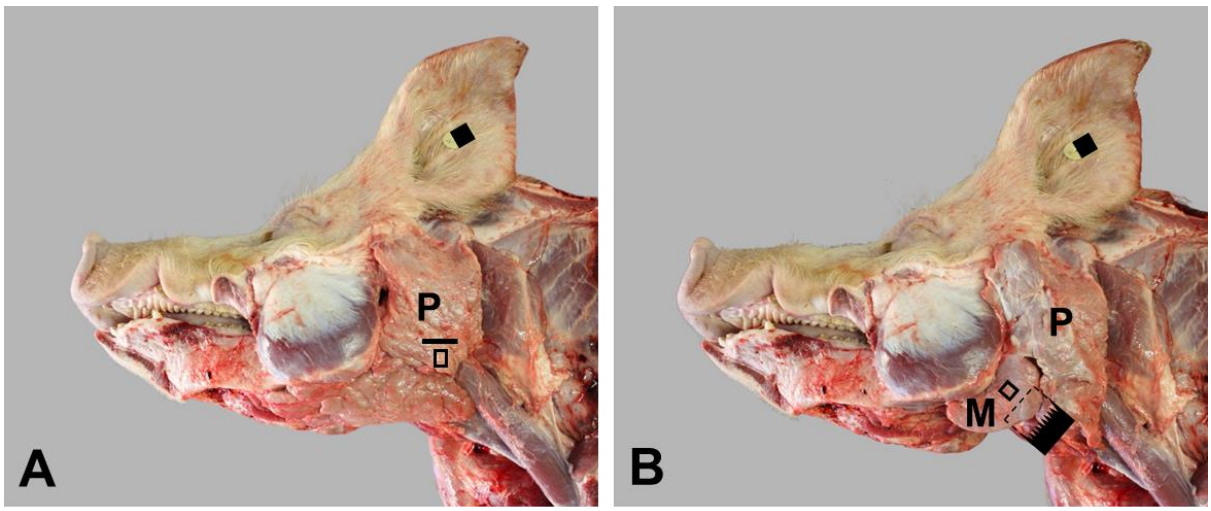


Fig. S117. A*: Anatomical location and preparation of the left parotid (P) and left mandibular gland (M). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.* ***B****: For presentation purposes, the parotid gland was partially removed to demonstrate the relative position of the mandibular gland.*

2.7.4.1. Salivary gland samples in Type-I studies

Samples for molecular analyses

Location:	One sample is taken from the left parotid gland, and one sample is taken from the left mandibular gland. The sampling locations are indicated in Fig. S116 and Fig. S117.
Number of samples:	One per salivary gland.
Remarks:	Homogeneous samples are cut from the parenchyma of the glands while avoiding samples with a nonhomogenous composition due to large glandular ducts.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One section is prepared from the left parotid gland, and one sample is taken from the left mandibular gland next to the location of the samples for molecular analyses. The sampling locations and section directions are indicated in Fig. S116 and Fig. S117. Sections should contain the capsule and parenchyma of the glands.
Fixation/Embedding:	FF-PE (Fig. S118).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one longitudinal horizontal section through the largest surface of the mandibular gland, including the mandibular, parotid, and sublingual glands (Ruehl-Fehlert et al., 2003). The samples generated according to the Type-I-study sampling protocols allow for an adequate evaluation of porcine salivary glands in routine studies.

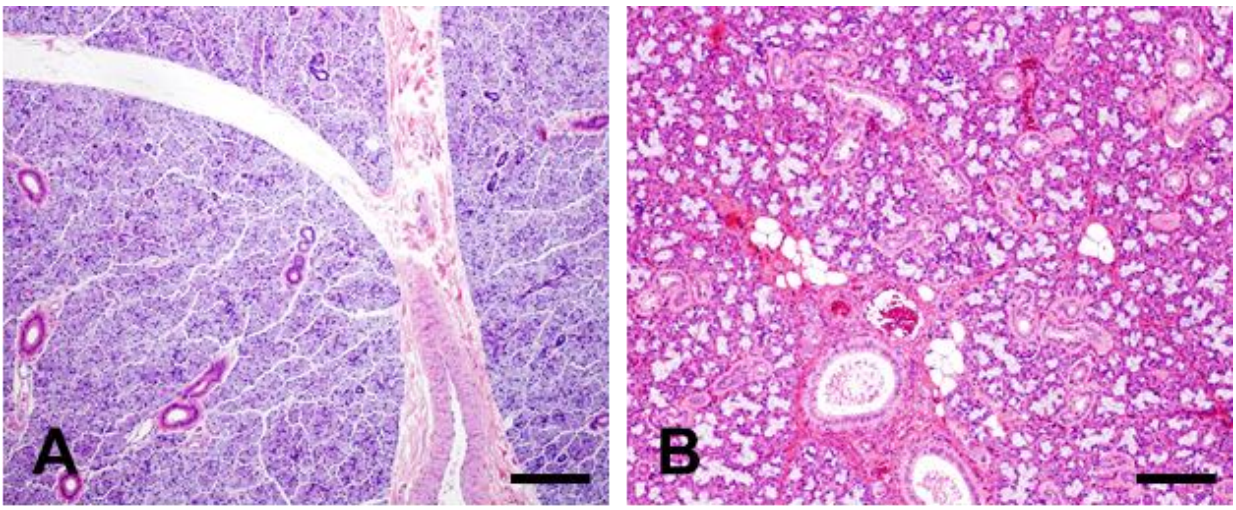


Fig. S118. *Histology of the parotid gland (left) and mandibular gland (right). FF-PE. HE. Scale bars = 100 µm.*

2.7.4.2. Salivary gland samples in Type-II and Type-III studies

General systematic random sampling strategy

In Type-II and -III studies, the left or right parotid and mandibular glands are sampled (randomly allocated). The sampling positions and organ-size-dependent sample numbers are determined by systematic random sampling. The weight and length of each gland is determined. Each gland is then cut into parallel equidistant slabs of 10-20 mm thickness orthogonal to the longitudinal axis of the gland. The first section is randomly positioned between 0 and 10/20 mm from the margin of the gland (Fig. S119). The organ slabs are then placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-4 cm, depending on the gland size and number and area of slabs). The systematic random sampling of six tissue locations is performed as described in section 1.4. The sampled positions can be marked by placing pieces of confetti paper on the respective positions. An approximately 1.5x1.5x2-cm tissue piece is excised from each of the sampled locations regardless of which structures are present. If the sampled location contains large vascular structures or ducts, these structures must be included in the rest of the sample to maintain representativity. The excised tissue specimens are subdivided into slabs for downstream analyses (Fig. S120).

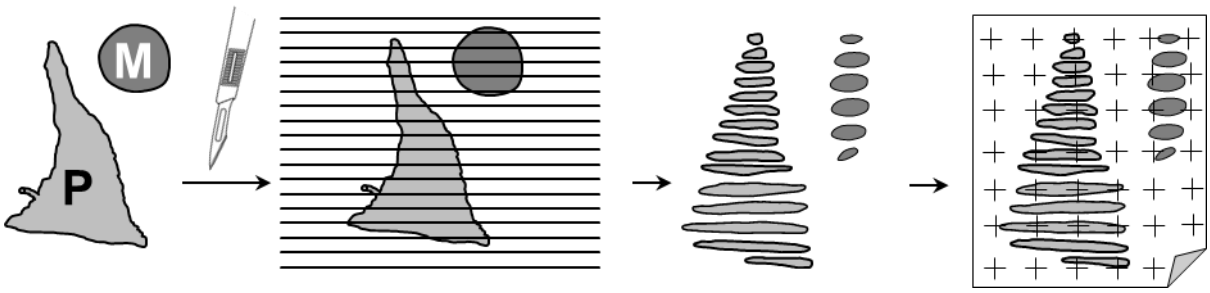


Fig. S119. *A schematic illustration of the systematic random sampling of the parotid gland (P) and the mandibular gland (M) in Type-II/III studies.*

Table S17. The type and number of salivary gland samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

	Number of sampled locations per side and samples per location	
Sample type	**Type-II studies**	**Type-III studies**
Sampled locations/side	*3*	*3*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 out of every 3rd location
CRYO	1 out of every 3rd location	1 out of every 3rd location
GA-EE	1 per sampled location	1 per sampled location
FF-PRE (IUR)	-	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

Remarks

The subsamples for paraffin histology and PRE consist of ~5-mm-thick slabs taken from each of the systematically randomly sampled tissue samples (Fig. S120). An additional, thinner (~2 mm) slab is taken from each of the systematically randomly sampled tissue samples. From this slab, the subsamples for molecular analyses and GA fixation and Epon embedding (GA-EE sample “a” in Fig. S120) are excised from areas of homogeneous gland parenchyma.

For qualitative histopathological and ultrastructural analyses of glandular duct epithelium, additional samples (GA-EE sample “b” in Fig. S120) are taken from the systematically randomly sampled locations in the tissue (Table S18). If duct section profiles are present in more locations than needed for acquisition of the recommended number of GA-EE samples, the tissue slabs to be sampled are chosen by systematic random sampling.

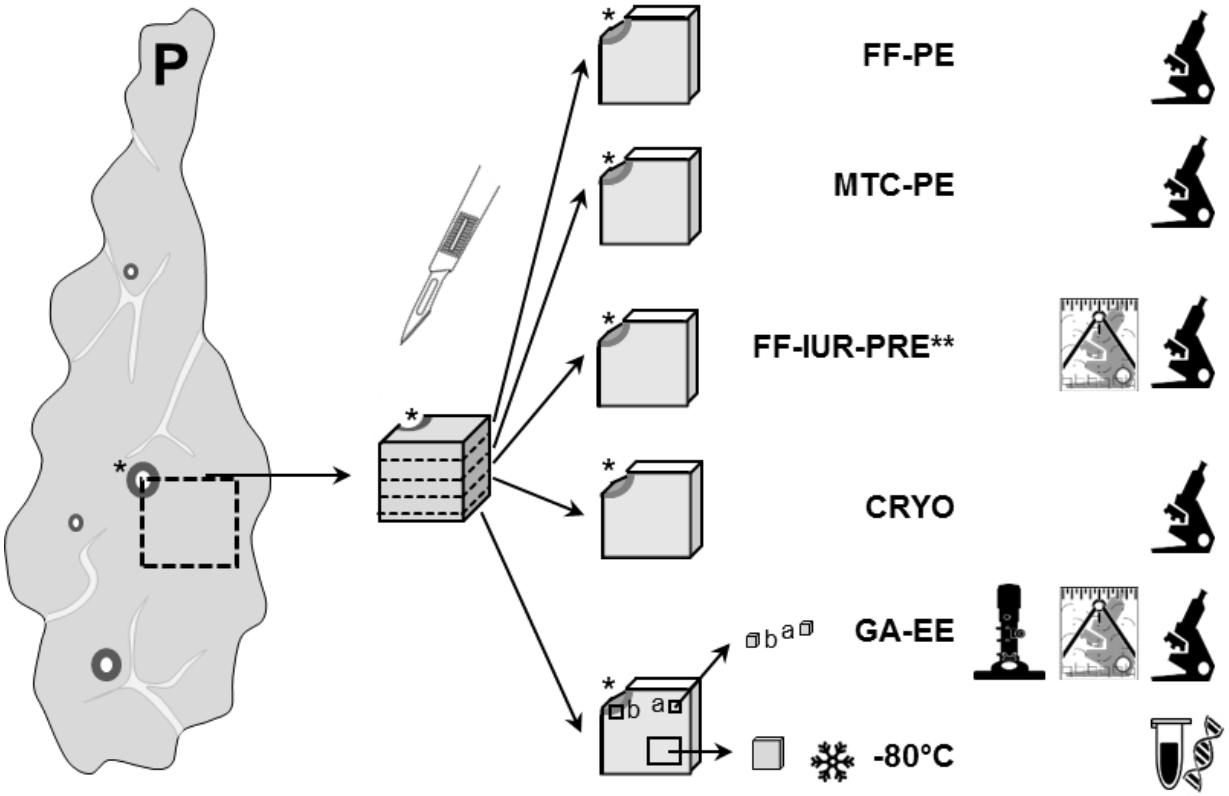


Fig. S120. *A schematic illustration of subsample preparation from one systematically randomly sampled location of parotid gland (P) tissue for downstream analyses (Type-II/III studies). *Glandular duct;**PR = GMA/MMA. The mandibular gland is sampled in the same manner.*

Table S18. Additional samples for molecular analyses and GA fixation with Epon embedding.

		Total number of samples	
Location	**Sample type**	**Type-II studies**	**Type-III studies**
Glandular duct (epithelium)	**GA-EE**	*6 (3/side)*	*10 (5/side)*

Time and personnel requirements

At least 25 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Gelberg, 2012, Ruehl-Fehlert et al., 2003, Wang et al., 2007)

2.7.5. Thyroid gland

Relevant anatomical features/preparation

In pigs, the thyroid glands are almost completely fused and lack inner parathyroid gland bodies. The thyroid gland is located ventral to the trachea just proximal to the thoracic inlet. The thyroid gland is removed from the body with the tongue, pharynx, larynx, trachea, lung, and heart (Fig. S121 and Fig. S122).

General examination parameters

The weight and the dimensions (longitudinal length) of the gland are determined. The thyroid gland is inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

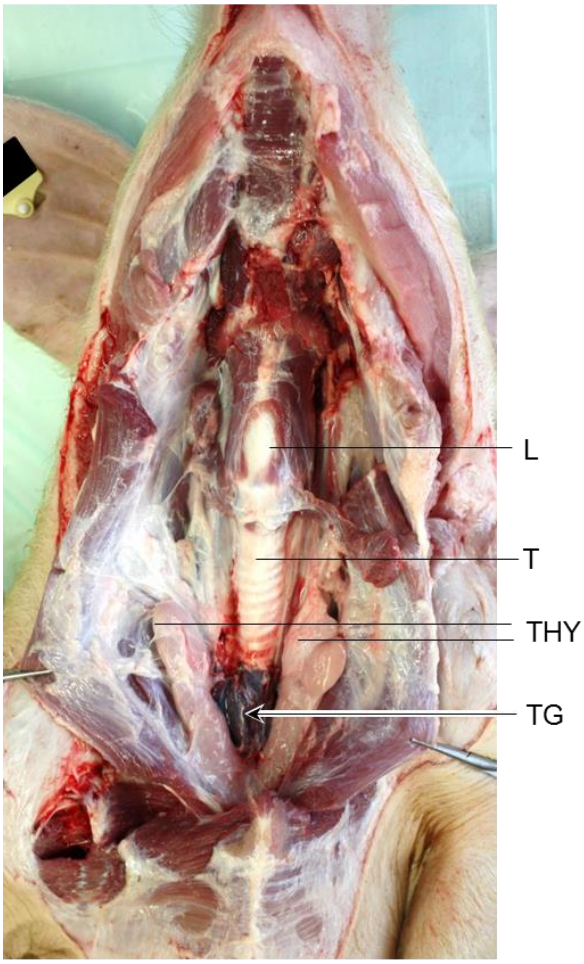


Fig. S121. *Ventral aspect of the neck. Larynx (L), trachea (T), thymus (THY), and thyroid gland (TG).*

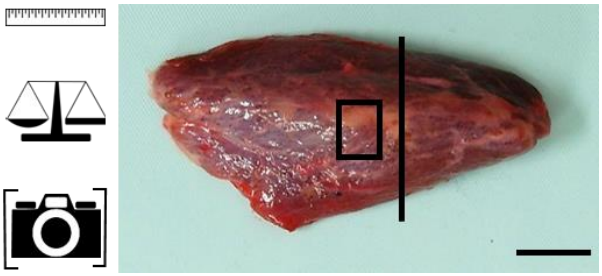


Fig. S122. Thyroid gland. *The location and orientation of the samples for histology (black line) and molecular analyses (black rectangle) in Type-I studies are indicated. Scale bar = 1 cm.*

2.7.5.1. Thyroid gland samples in Type-I studies

Samples for molecular analyses

Location:	The sampling location is indicated in Fig. S122.
Number of samples:	One.
Remarks:	A homogeneous sample is cut from the thyroid gland parenchyma.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One cross section containing the capsule and parenchyma as indicated in Fig. S122.
Fixation/Embedding:	FF-PE (Fig. S123).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For histopathological examination of the thyroid gland in regulatory (oral) toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one transverse section through the esophagus and trachea at the level of the thyroid gland. Alternatively, longitudinal horizontal sections through the isolated thyroid glands are prepared (Kittel et al., 2004, Ruehl-Fehlert et al., 2003). Similarly, the samples generated according to the Type-I-study sampling protocols allow for an adequate evaluation of the porcine thyroid gland in routine studies.

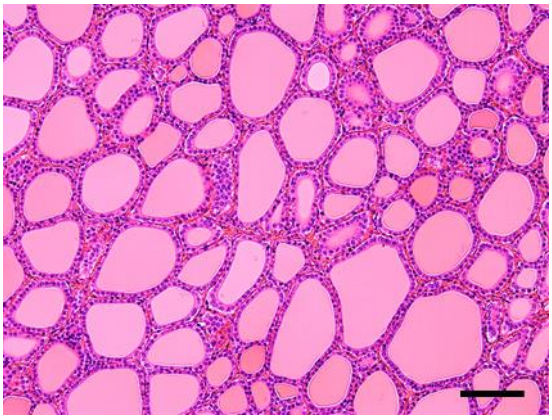


Fig. S123. Thyroid gland histology*. FF-PE. HE. Scale bar = 100 µm.*

2.7.5.2. Thyroid gland samples in Type-II and Type-III studies

General systematic random sampling strategy

The sampling positions and organ size-dependent sample numbers are determined by systematic random sampling. The thyroid gland weight and width is determined, and the organ is cut into parallel equidistant slabs orthogonal to the width axis of the organ and a maximum thickness of 10 mm. The first section is randomly positioned between 0 and 10 mm from the margin of the organ (Fig. S124 and Fig. S125). The organ slabs are placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 1-2 cm, depending on the organ size and number and area of slabs). The systematic random sampling of 5-6 tissue locations is performed as described in section 1.4. The sample positions can be marked with pieces of confetti paper (Fig. S124 and Fig. S125). An approximately 1x1x1-cm tissue piece is excised from each of the sampled locations and is further subdivided into slabs for downstream analyses (Fig. S126, Table S19).

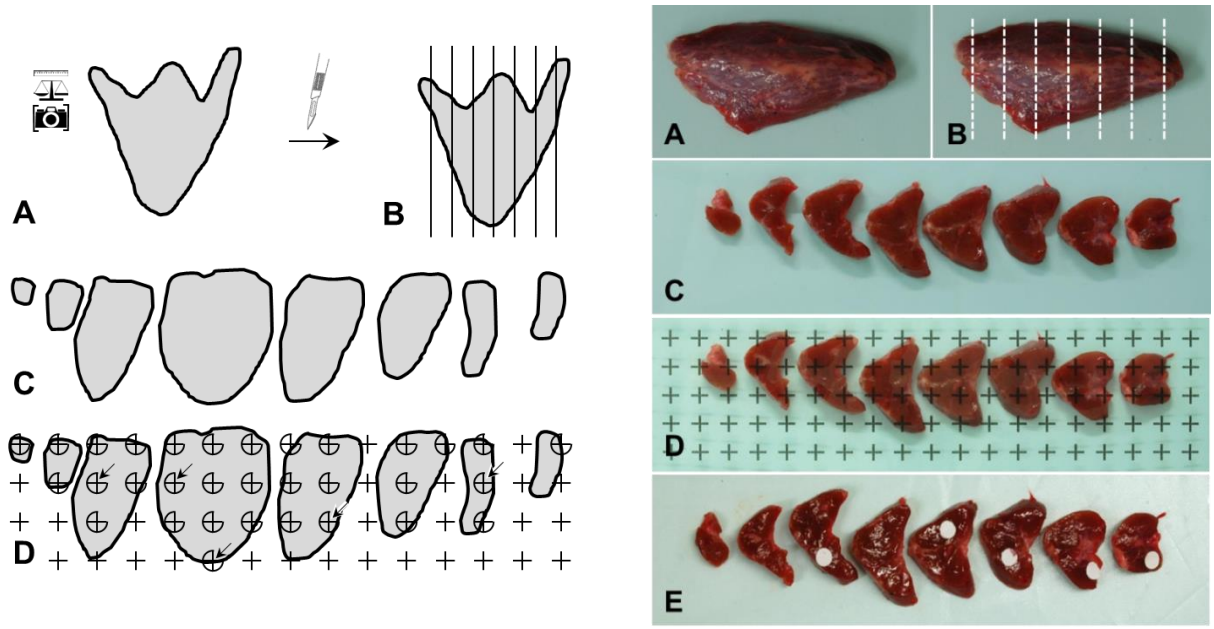


Fig. S124 (left) and S125 (right). A schematic illustration and images of thyroid gland processing for the systematic random sampling in advanced histopathological analyses (Type-II and Type-III studies). *The thyroid gland weight and length is determined (**A**). The organ is then cut into equidistant (max. ~1 cm thick) slices parallel to the longitudinal organ axis (**B**). The slices are placed on the same side (**C**) and overlaid with a cross grid (1-2 cm) printed on a plastic transparency (**D**). A total of 5-6 locations were used for systematic random sampling. The sampled positions can be marked by pieces of confetti paper (**E**).*

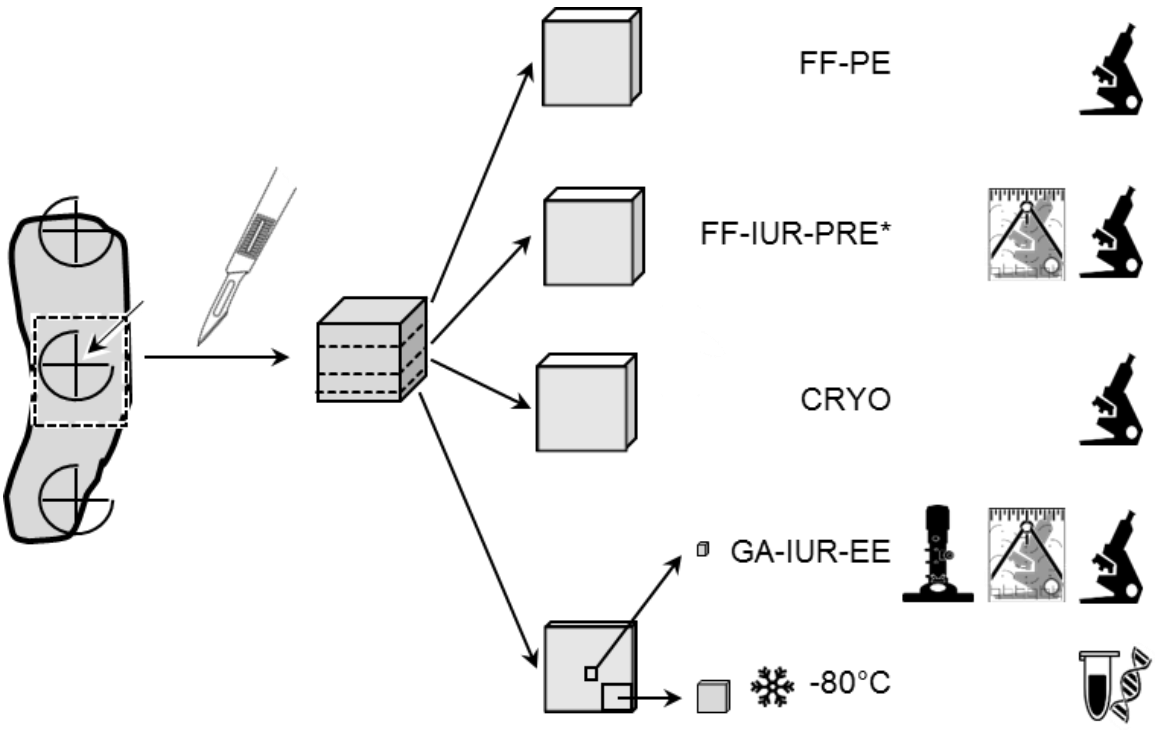


Fig. S126. *A schematic illustration of subsample preparation from one systematically randomly sampled thyroid gland for downstream analyses (Type-II/III studies). *PR = GMA/MMA.*

Table S19. The type and number of thyroid gland samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

	Number of locations/samples	
Sample type	**Type-II studies**	**Type-III studies**
Sampled locations	*5-6*	*5-6*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
CRYO	1 out of every 2nd location	1 per sampled location
GA-IUR-EE	1 per sampled location	1 per sampled location
FF-PRE (IUR)	-	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

Time and personnel requirements

At least 15 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Kittel et al., 2004, La Perle, 2012, Manojlovic-Stojanoski et al., 2014, Rosol et al., 2013, Ruehl-Fehlert et al., 2003)

2.8. Nervous system

2.8.1. Central nervous system

2.8.1.1. Brain

Relevant anatomical features/preparation

After removal of the tongue, pharynx, and larynx, the head is removed from the body at the atlanto-occipital joint. Prior to removal of the head, cerebrospinal fluid can be harvested using an 18-gauge cannula with a 5-10 ml syringe. The atlanto-occipital joint is accessed from the ventral side as shown in Fig. S127. The brain is removed after preparatory saw cuts through the skull bone starting dorsal to the occipital condyles and ending at the temporal angles of the ipsilateral eye on both sides. A connecting cut at the widest width of the neurocranium is made, the skullcap is removed, and the dura mater is dissected as outlined in Fig. S128.

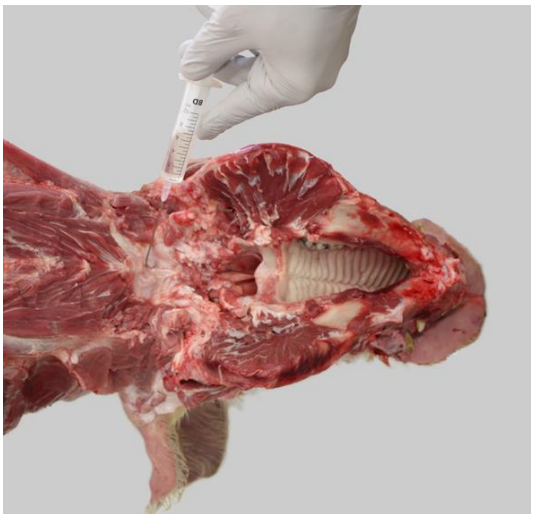
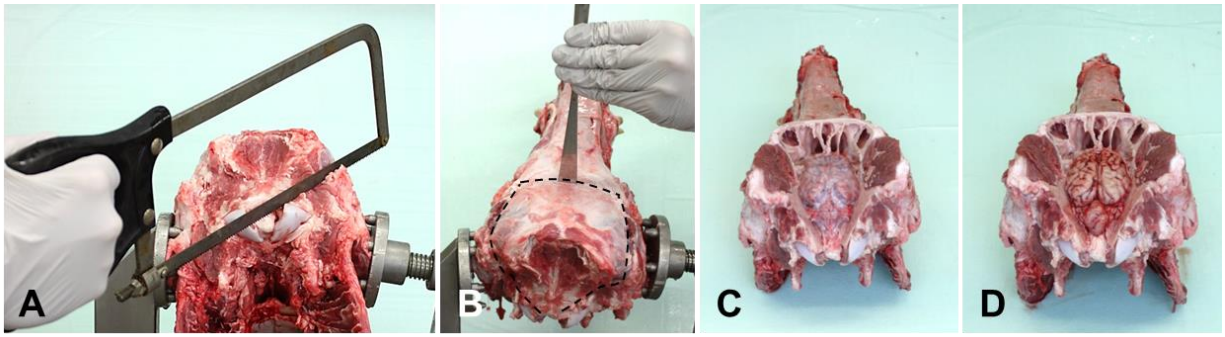


Fig. S127 (←). *Illustration of cerebrospinal fluid (CSF) collection after dissection of the atlanto-occipital joint (ventral view). Ideally, the CSF sample should be collected approximately 10 minutes after the animal's death/asystole. The sample is prone to blood contamination if the CSF is harvested directly after euthanasia. The cannula pierces through the atlanto-occipital membrane for the removal of CSF from the subarachnoid space.*

Fig. S128 (↓). *Illustration of the removal of the brain from the skull.* ***A***, ***B****: After the temporal muscle is removed, the skull is cut along the indicated lines using a hand saw, hammer and chisel* ***C****: View of the brain (covered by the dura mater) after removal of the skullcap.* ***D****: View of the brain after removal of the dura mater.*



General examination parameters

The size (dimensions) and weight of the brain are determined. The brain is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

2.8.1.1.1. Generation of brain samples in Type-I-III studies

General remarks

Type-I-study sampling protocols are applied for routine examination of the brain. If (central) neurological dysfunction is indicated in clinical, gross, or histopathological findings or if alterations of the nervous system are expected in a particular genetically modified pig model, advanced Type-II or Type-III-study sampling protocols should be applied. In all proposed brain sampling protocols, the fresh brain is longitudinally halved. Samples for molecular analyses are taken from one hemisphere, whereas the other hemisphere is fixed and processed for histopathological examination. This approach is considered to be advantageous because it enables the collection of samples for molecular analyses. However, histopathological examination of only one hemisphere can mask unilaterally occurring alterations. Therefore, if clinical signs, necropsy findings, or previous histopathological findings indicate the presence of unilateral lesions, the sampling protocols should be modified, and additional samples for histopathological examination should be collected from the other brain hemisphere.

2.8.1.1.2. Brain samples in Type-I studies

For the preparation of samples for molecular analyses and FF-PE sections for histological examination, the fresh unfixed brain is cut midsagittally following the longitudinal cerebral fissure as shown in Fig. S129. For standardization in Type-I studies, samples for molecular analyses should be consistently collected from the left hemisphere, and samples for histopathological examination should be collected from the right hemisphere.

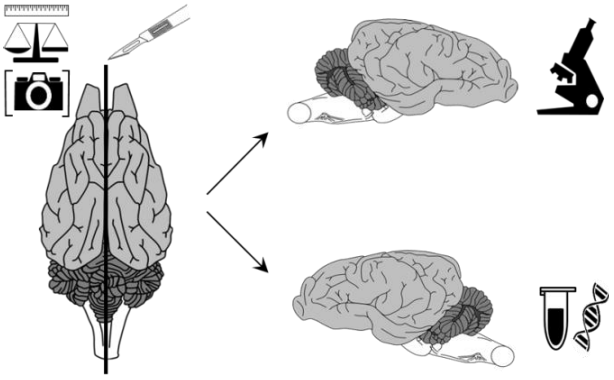


Fig. S129. *A schematic illustration of fresh brain processing in Type-I studies. The right half of brain is subjected to FF-PE-histology, and the samples for molecular analyses are prepared from the left half of the brain. When the brain hemispheres are dissected, the pineal gland can be harvested for examination (section 2.8.1.3.).*

Samples for molecular analyses

For the generation of samples from defined brain regions for molecular analyses (OMICS profiling, DNA, RNA, and protein analyses), the left brain half is laid on a glass slide and placed on a block of dry ice until the tissue slightly freezes (Fig. S130 and Fig. S131). This procedure allows for the cutting of an approximately 4-5 mm thick sagittal slab from the brain. The resulting tissue slab can be stored on the glass plate at -80°C until further investigation. The sampling locations (brain regions) for routine molecular analyses are shown in Fig. S130 and Fig. S131. The entire freezing-slicing-sampling process is repeated with the remaining brain tissue. In total, approximately 6-10 brain slices can be generated from one brain half of an adult pig using the described techniques. After sampling, the remaining tissue can be stored on glass plates at -80°C until further investigation.

Location:	Neocortex (1), cerebellar cortex (2), caudate nucleus (3), thalamus (4), hippocampus (5), hypothalamus (6), and pons (7). The numbers in parentheses refer to the brain locations indicated in Fig. S130 and Fig. S131.
Number of samples/location//hemisphere:	One.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

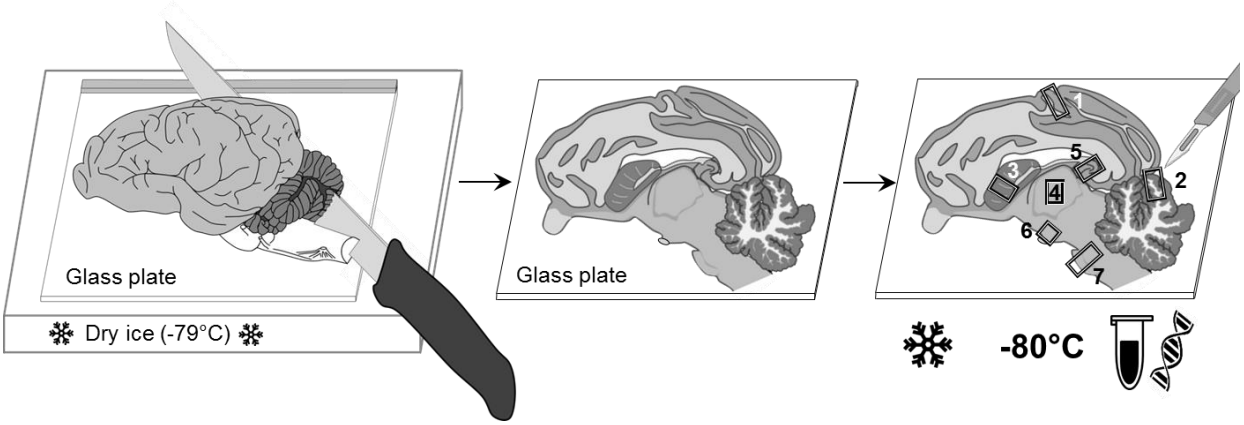


Fig. S130. *A schematic illustration of the sample collection from defined brain regions for molecular analyses in Type-I studies.*

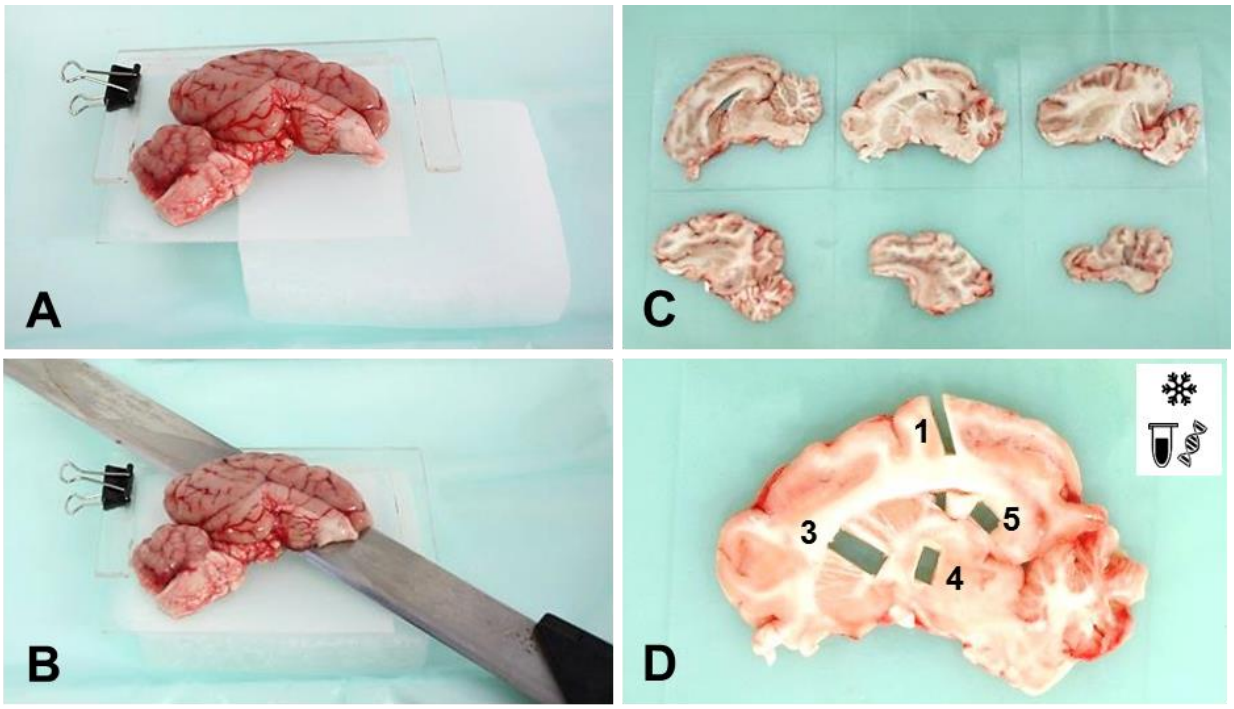


Fig. S131. *Sample collection from defined brain regions for molecular analyses in Type-I studies.* ***A****: Freezing of the halved brain on a glass plate on dry ice.* ***B****: The slicing of the brain when the parenchyma adjacent to the glass plate begins to freeze.* ***C****: Frozen brain slices.* ***D****: Excision of defined brain regions.*

Histology samples

For histopathological examination, the right brain half is fixed in a 4% neutrally buffered formaldehyde solution. Similar to the recommendations of the Working Group on Nervous System Sampling of the Society of Toxicologic Pathology for sampling and processing of the nervous system during nonclinical general toxicity studies (Bolon et al., 2013b), histopathological examination of the brain in Type-I studies should include the caudate nucleus/putamen, cerebellum, cerebral cortex, choroid plexus, hippocampus, hypothalamus, medulla oblongata, midbrain, olfactory bulb, pons, and thalamus. In the porcine brain, these structures can be examined in 5-7 frontal (cross) sections. The approximate positions of the section levels are determined by anatomical landmarks, recognizable on the medial surface of the halved brain, as shown in Fig. S134.

Alternatively, the fixed brain half can completely be sectioned into approximately 5-8 mm thick cross sections. To consistently acquire homologous sections and section levels, slicing/positioning devices may be used to cut the tissue as shown in Fig. S137. The resulting tissue slabs are visually inspected for gross pathological alterations, and the relevant slabs are selected for paraffin embedding (Fig. S132-S135). If necessary, the tissue slabs are cut into smaller pieces to fit into standard embedding cassettes (Bolon et al., 2013b, Pardo et al., 2012). Figure S135 shows the approximate location of important brain structures within the tissue slabs and the corresponding histological sections from different section levels of the brain. A more detailed description of the neuroanatomical areas included in comparable brain section levels of the cynomolgus monkey is provided by Pardo et al. (2012). The histopathological examination is not limited to the brain structures mentioned above and includes all other structures present in the sections.

Location, number and orientation of sections:	Brain cross sections from five defined levels: 1st level: Frontal lobe, 2nd level: Basal nuclei, 3rd level: Thalamus, 4th level: Pons, 5th level: Brainstem and cerebellum (Fig. S133, S134, and S135).
Fixation/Embedding:	FF-PE (Fig. S136).

Note: The necessary fixation time largely depends on the size (volume) of the brain tissue to be preserved. A tissue:fixation solution volume ratio of at least 1:10 is recommended. For better immersion in the fixation solution, gentle agitation should be applied to the sample during the first few days of the fixation process. The fixation solution should be exchanged in regular intervals to maintain its pH and avoid fixation artifacts. At the author's institute, positive results were obtained by replacing the fixation solution twice, once after one day and again after four days of fixation. For a "normal" pig brain (halved, not sliced), the minimal fixation time in a 4% neutrally buffered formaldehyde solution at 20°C is approximately 6-8 days.

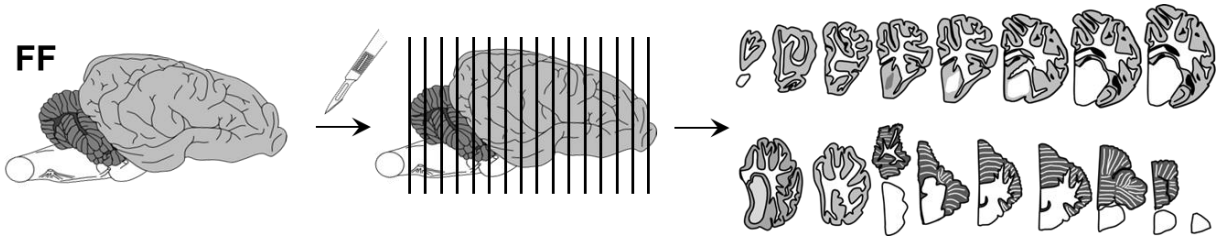


Fig. S132. *A schematic illustration of cross section preparation from fixed brain tissue for Type-I studies.*

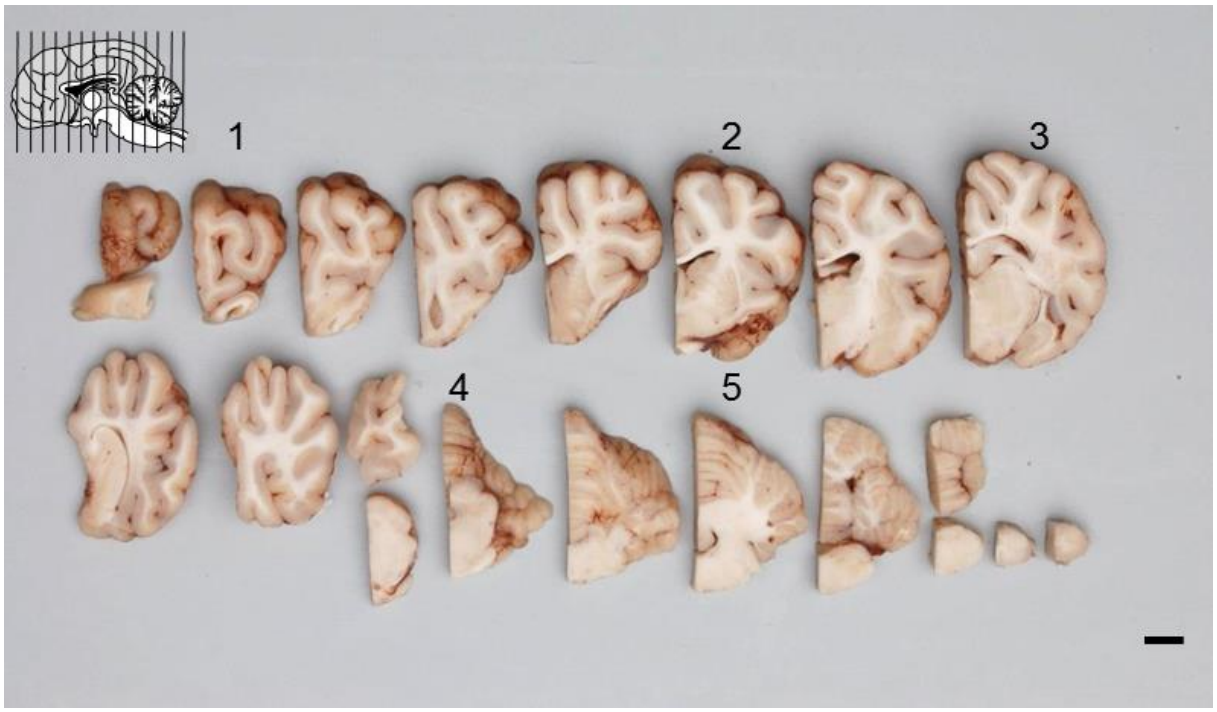


Fig. S133. *Cross sections of the brain cut from the fixed brain half. Tissue slices for paraffin embedding are marked by numbers that refer to the standard section levels shown in Fig. S134. Scale bar = 1 cm.*

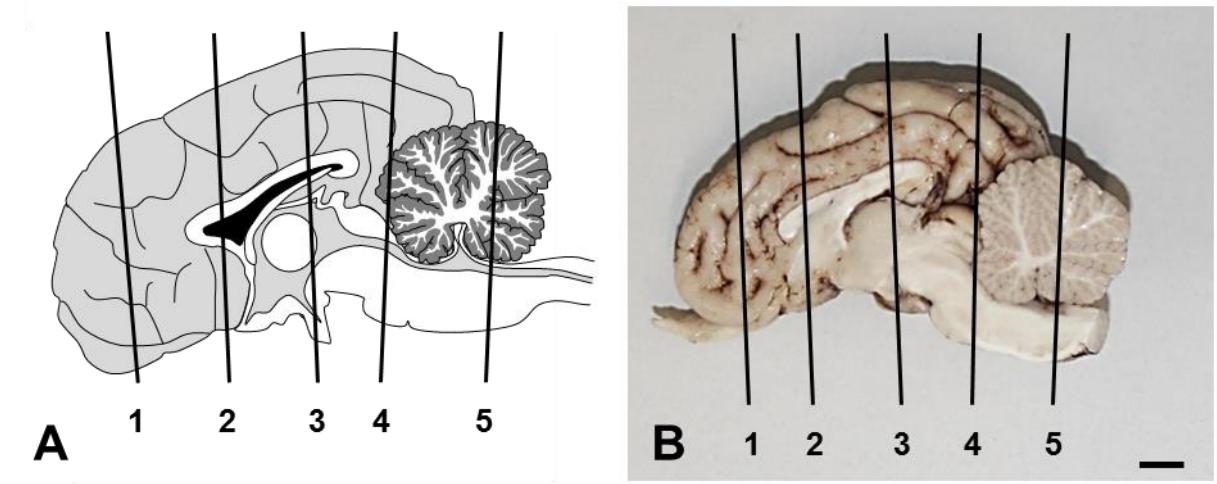


Fig. S134. *A schematic illustration (**A**) and image (**B**) of standard frontal (cross) sectional planes for the selection of brain tissue slices for subsequent preparation of FF-PE samples for paraffin histology, ISH and IHC analyses. **1**: Frontal lobe; **2**: Basal nuclei; **3**: Thalamus; **4**: Pons; **5**: Brainstem and cerebellum. Scale bar = 1 cm.*

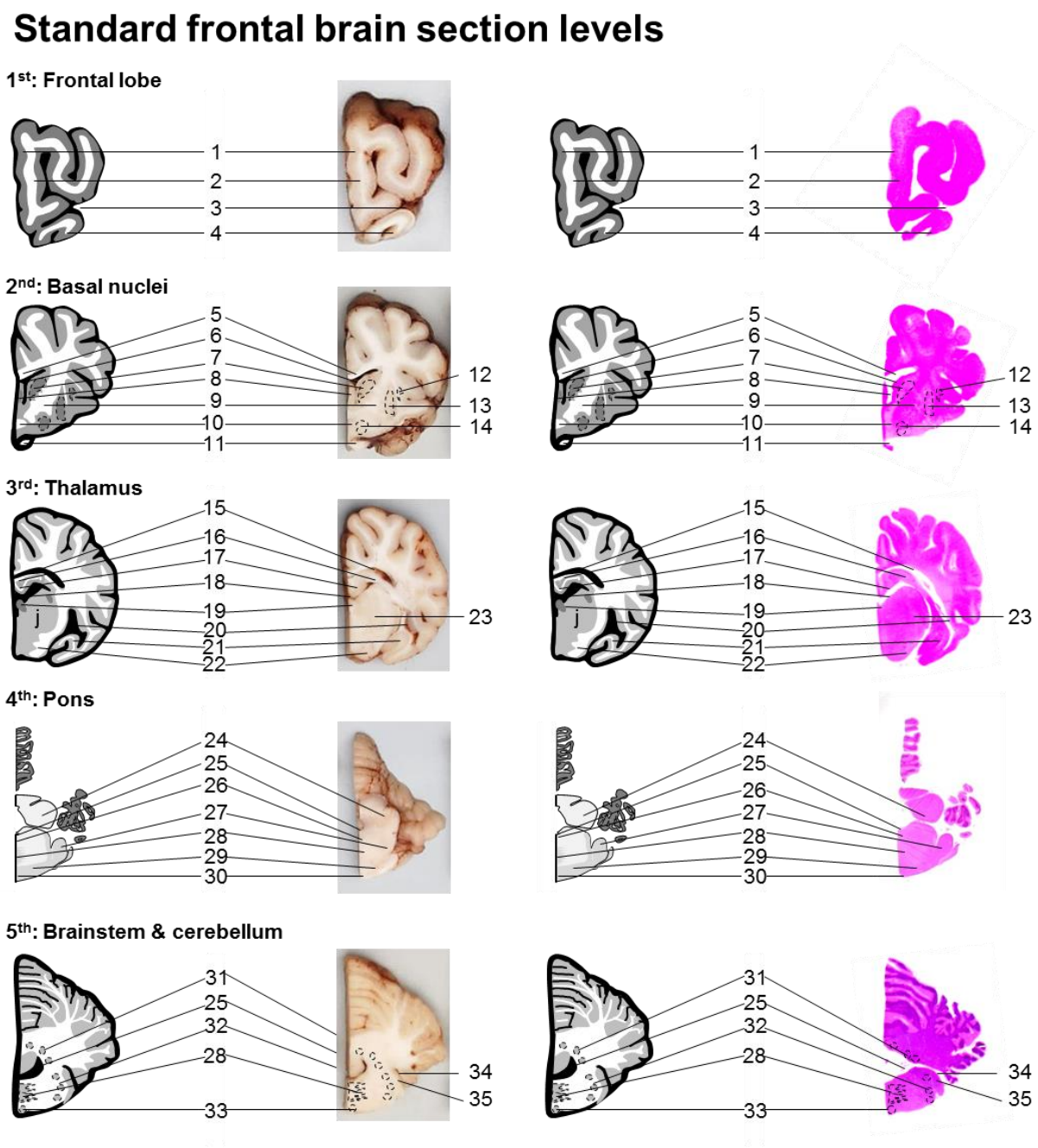


Fig. S135. *A schematic illustration, images, and histological slides of standard frontal (cross) sectional planes of the brain (formalin fixed tissue) with macroscopically discernable strictures (**1**-**35**): **1**: Cerebral cortex; **2**: Cerebral white matter; **3**: Rhinal sulcus; **4**: Olfactory peduncle; **5**: Corpus callosum; **6**: Lateral ventricle; **7**: Caudate nucleus; **8**: Septal nuclei; **9**: Internal capsule; **10**: Rostral commissure; **11**: Optic chiasm; **12**: Claustrum; **13**: Putamen; **14**: Globus pallidus; **15**: Lateral ventricle; **16**: Fornix; **17**: Hippocampus; **18**: Third ventricle; **19**: Habenula; **20**: Lateral ventricle; **21**: Hippocampus; **22**: Crus cerebri; **23**: Thalamus; **24**: Caudal colliculus; **25**: Fourth ventricle; **26**: Medial longitudinal fasciculus; **27**: Middle cerebellar peduncle; **28**: Reticular formation; **29**: Medial lemniscus; **30**: Pons; **31**: Cerebellar nuclei; **32**: Cranial nerve nuclei; **33**: Pyramid; **34**: Lateral recess; **35**: Trigeminal spinal tract. Scanned histology slides stained with HE. FF-PE tissue sections.*

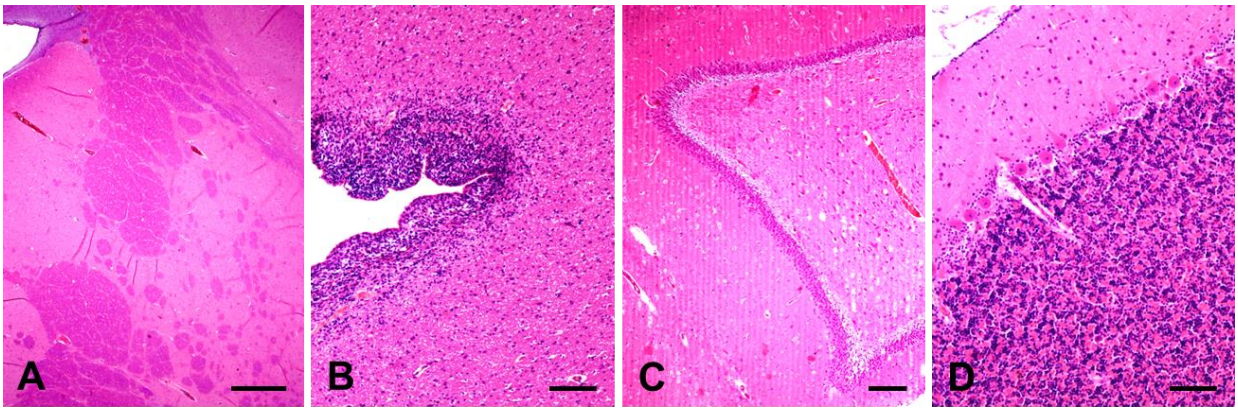


Fig. S136 (↑). Brain histology *(selected locations). Standard frontal (cross) sections of the brain.*
A*: Forebrain with basal nuclei.* ***B****: Periventricular (lateral ventricle) brain parenchyma.* ***C****: Dentate gyrus.*
D*: Cerebellar cortex. HE. FF-PE tissue sections. Scale bars = 1 mm (A), 100 µm (B-D).*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

For the histopathological examination of rodent and non-rodent brains in nonclinical general toxicity studies, the Working Group on Nervous System Sampling of the Society of Toxicologic Pathology recommends the sampling of 6-7 coronal bilateral sections (rodents) or 5-7 coronal hemisections in non-rodent species after formalin fixation of the brain. Brain sections should contain the caudate nucleus/putamen, cerebellum, cerebral cortex, choroid plexus, hippocampus, hypothalamus, medulla oblongata, midbrain, olfactory bulb (rodents only), pons, and thalamus (Bolon et al., 2013b).
Similarly, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of 3-5 transverse brain sections taken at defined levels for histopathological examination of the brain in regulatory toxicity studies (Morawietz et al., 2004).
The Technical Guide for Nervous System Sampling of the cynomolgus monkey for General Toxicity Studies recommends the generation of six frontal brain sections taken at the levels of the frontal pole, rostral commissure, rostral thalamus, caudal thalamus, middle cerebellum with brainstem, and occipital lobe (Pardo et al., 2012). After sectioning, the tissue is trimmed to fit into standard embedding cassettes. The Type-I-study sampling protocols provided for porcine models principally recommend the generation of samples with comparable section levels and section orientations to allow for an adequate histopathological examination of the same brain regions in routine studies.

2.8.1.1.3. Brain samples in Type-II and Type-III studies

General sampling strategy

The brain has a highly complicated architecture with numerous functionally and/or morphologically distinguishable structures. Applying systematic random sampling procedures using the entire brain as the reference compartment is not typically useful because of the high spatial morphological diversity of the different brain regions. Thus, a very large number of specimens would have to be sampled from the brain to be representative for the entire organ. However, most scientists are interested in distinct properties of particular brain regions. The sampling of brain regions of interest (BROI) requires that (1.) the borders of the respective BROI can unambiguously be identified in the section surface of the brain tissue slice, (2.) the total volume of the BROI can be adequately determined (Cavalieri volumetry), and (3.) the samples are processed (fixation, embedding media, orientation of tissue samples relative to the section plane, etc.) using a method compatible with the subsequent analyses. For example, various quantitative stereological analysis methods for the estimation of neuron cell number, mean cellular/nuclear volumes, brain capillary surface areas, or nerve fiber lengths in a particular brain region require different embedding materials and sample orientations. There are

excellent publications available that thoroughly describe different sampling procedures, sample processing techniques and analysis methods for a vast range of quantitative stereological brain parameters (Kristiansen and Nyengaard, 2012, Schmitz and Hof, 2005). A common feature of quantitative stereological analysis sampling strategies is the requirement that certain parameters are determined in advance; therefore, the sampling strategy can be optimized for the parameters of interest. In contrast, the generation of sample materials suitable for examination of any type of downstream quantitative stereological analysis not specified at the time point of sample generation is not feasible *per se*. Several studies have reported on the sufficient quality of human brain tissue for ultrastructural analyses and distinct immunohistochemical analyses after prolonged fixation times of up to 10 years (Liu and Schumann, 2014, Pikkarainen et al., 2010). However, the storage of brain tissue in a fixation solution for extended periods of time can markedly decrease the tissue quality for certain analyses. Therefore, the sampling procedures described for Type-II and -III studies in this guide must necessarily always represent compromises between a maximum of different possible downstream analysis types (PE-histology, IHC, ISH, PR-histology, EM, quantitative stereology) and the need for special samples and sample processing for special analysis parameters

In Type-II and -III studies, samples for molecular analyses and histopathological examination are collected in a similar method as Type-I studies. However, the left and the right brain halves are randomly allocated for molecular analyses or histopathological examination in Type-II and -III studies.

Principally, the **generation of brain samples for molecular analyses** (Table S20) does not differ between Type-I, II and III studies. However, samples from larger BROIs, such as the neocortex, cerebellar cortex, thalamus, and caudate nucleus, are generated using systematic random sampling (Fig. S139). In case additional locations are required, the frozen glass-plated brain-tissue slabs should be stored at -80°C after the standard sampling procedures.

The **generation of brain samples for qualitative and quantitative histopathological analyses** does not differ in principle between Type-I, II and III studies. However, thinner cross sections (2-4 mm) need to be cut from the fixed brain half to generate samples for subsequent analyses in Type-II and -III studies. This procedure can be completed using a self-constructed tissue-slicing device (Fig. S137). The fixed brain is embedded in agar with the guide rail of the tissue slicer and then exhaustively sectioned in equidistant parallel sections orthogonal to the longitudinal brain axis. The tissue slabs are counted and placed on the same side (Fig. S138). Next, the slabs are scanned in conjunction with a size scale (Fig. S138). This process allows for the planimetric measurement of distinct BROIs within every brain tissue section using scanned images. Thus, the total volume of the fixed half brain or BROI can be determined following the Cavalieri principle (volume = mean section profile area x mean section thickness x number of sections). For standard histology, ISH and IHC analyses using light microscopy, tissue slices from defined brain parts are selected and embedded in paraffin. From the remaining agar-embedded brain-tissue slabs and/or from the defined BROIs in these slabs, subsamples are taken for downstream analyses, such as plastic histology, EM, and quantitative stereological analyses (Table S21). This subsampling is completed using systematic random sampling. The tissue slabs are overlaid with a cross grid printed on a transparency, and the grid sizes depend on the organ size, BROI, and number and area of slabs (Fig. S140). The systematic random sampling of tissue locations is performed as described in section 1.4. The sampled locations can be marked with small pieces of paper (Fig. S141). An adequately sized tissue piece is excised from each of the sampled BROI locations and further processed as indicated in Fig. S140, Fig. S141, and Table S21.

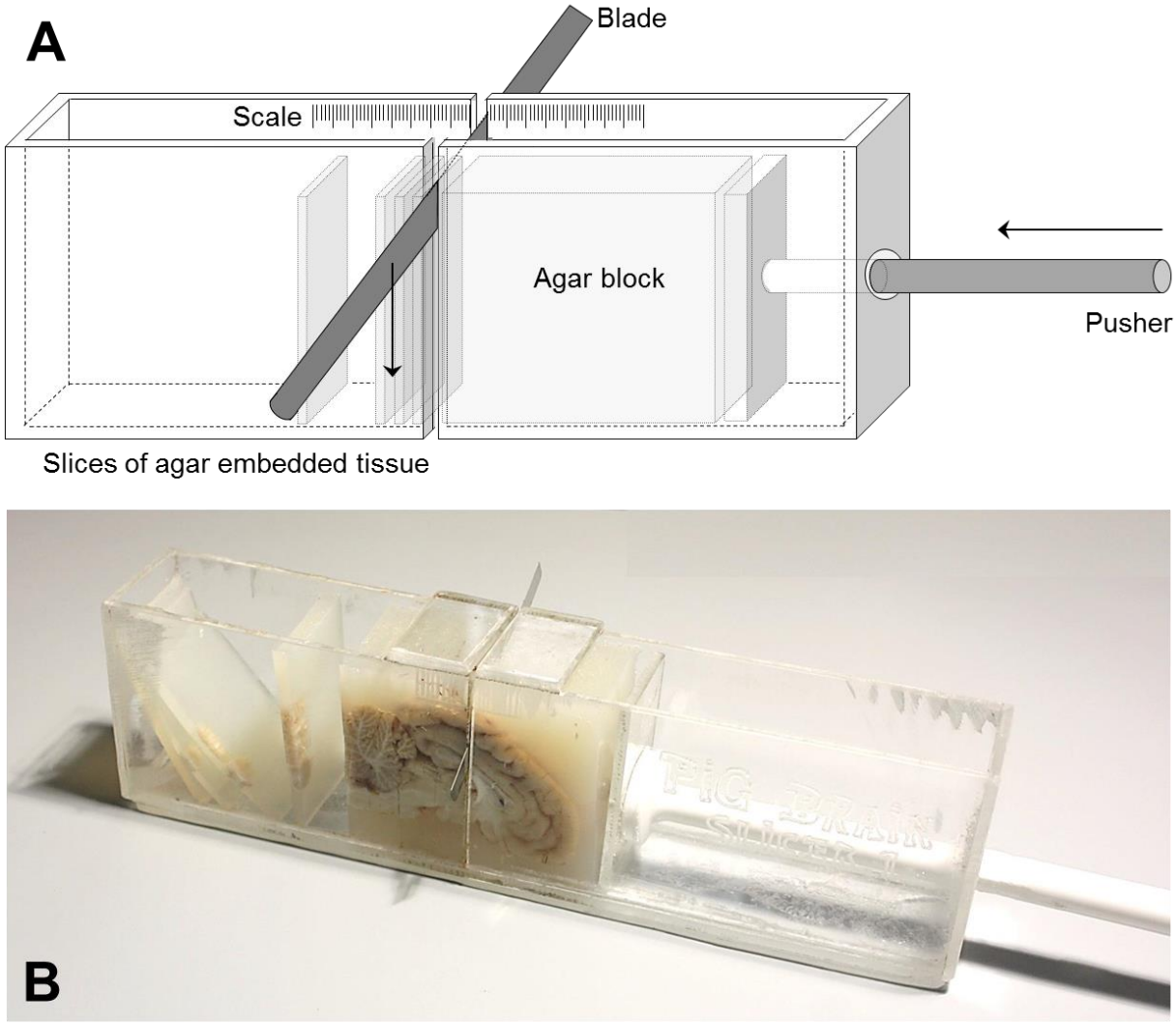


Fig. S137. *Serial sectioning of agar-embedded brain tissue in a self-constructed tissue slicer. A schematic illustration (**A**) and image (**B**) of brain sections.*

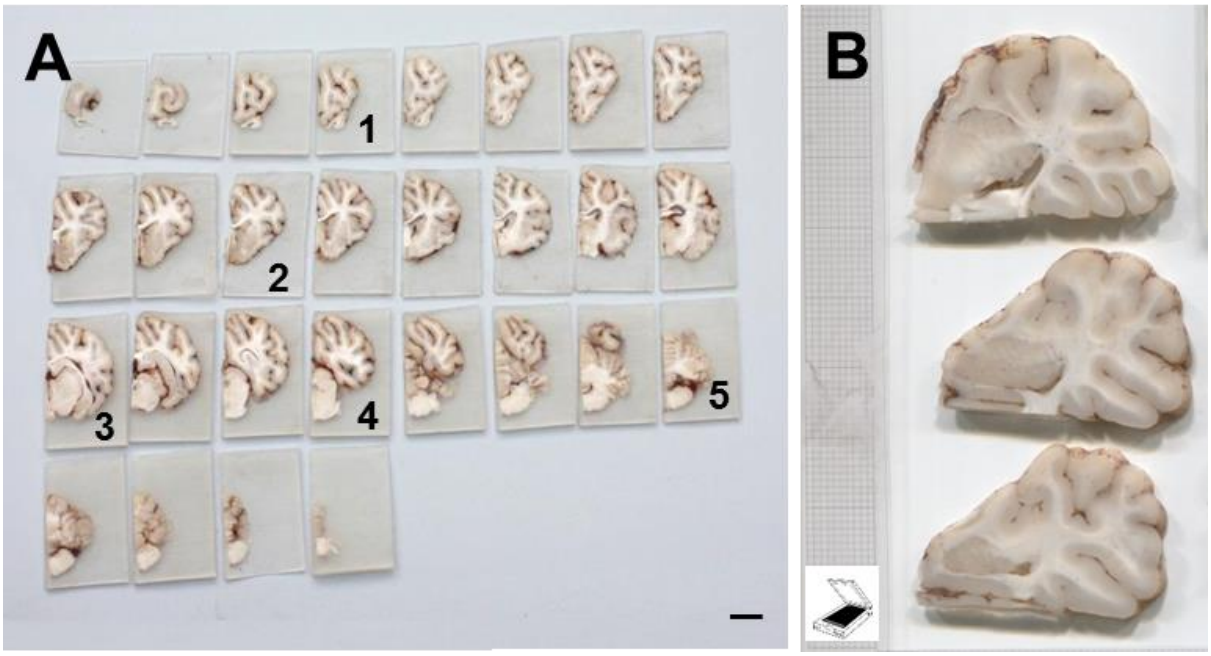


***Fig. S138 (↑). A**: Cross sections (~4 mm) of agar-embedded fixed brain tissue (left). The hardened agar is a semitransparent gray-fawn material that surrounds the brain tissue. The brain slices selected for paraffin embedding are marked by numbers that refer to the standard section levels shown in Fig. S134. Scale bar = 3 cm. **B**: Scanned image of three brain cross sections for Cavalieri volumetry of the brain and distinct BROIs.*

Table S20. Numbers of brain samples generated for molecular analyses in Type-I-III studies.

	Sampling method*/number of samples per BROI	
BOI	**Type-I studies**	**Type-II and Type-III studies**
Neocortex	DCL (location: 1 in Fig. S135)/**1**	SRS (10 mm)/**10**
Cerebellar cortex	DCL (location: 2 in Fig. S135)/**1**	SRS (4 mm)/**6**
Caudate nucleus	DCL (location: 3 in Fig. S135)/**1**	SRS (10 mm)/**6**
Thalamus	DCL (location: 4 in Fig. S135)/**1**	SRS (10 mm)/**6**
Hippocampus	DCL (location: 5 in Fig. S135)/**1**	DCL/**3**
Hypothalamus	DCL (location: 6 in Fig. S135)/**1**	DCL/**2-3**
Pons	DCL (location: 7 in Fig. S135)/**1**	DCL/**1-2**

**Sampling methods: SRS: Systematic random sampling (recommended cross grid size); DCL: Deliberately chosen location.*

Remarks

Generation of brain samples for molecular analyses does not differ in principle between Type-I, II and III studies. However, samples from larger BROIs, such as the neocortex, cerebellar cortex, thalamus, and caudate nucleus, are generated using systematic random sampling in Type-II and -III studies. This procedure is analogous to the sampling process illustrated in Fig. S140 and Fig. S141, except that sagittal sections of frozen brain tissue are sampled (Fig. S139). In comparably small BROIs, such as the hippocampus and hypothalamus, systematic random sampling is not applicable because these BROIs span only one or two brain tissue slices. Therefore, these BROIs are harvested completely. After sampling the standard locations, the frozen glass-plated brain-tissue slabs should be stored (-80°C) in case additional locations are subsequently required.

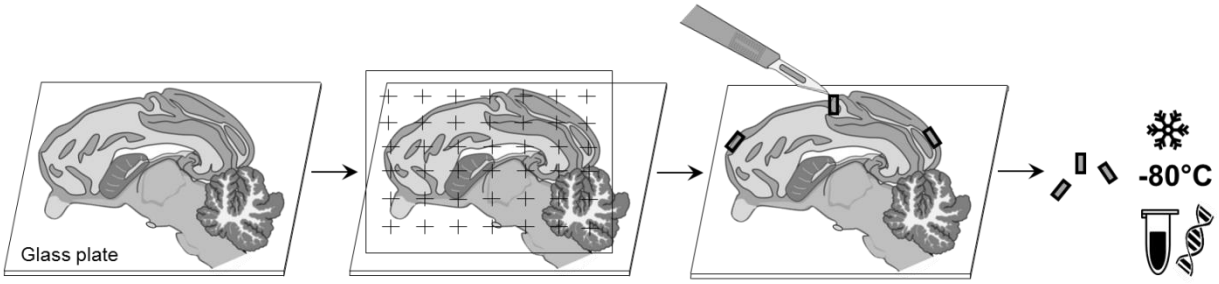


***Fig. S139.** A schematic illustration of the systematic random sampling of the neocortex in frozen brain tissue slices for molecular analyses. Note that only one brain tissue slice is shown. However, the sampling process is performed on all tissue slices of the brain half that contain the BROI.*

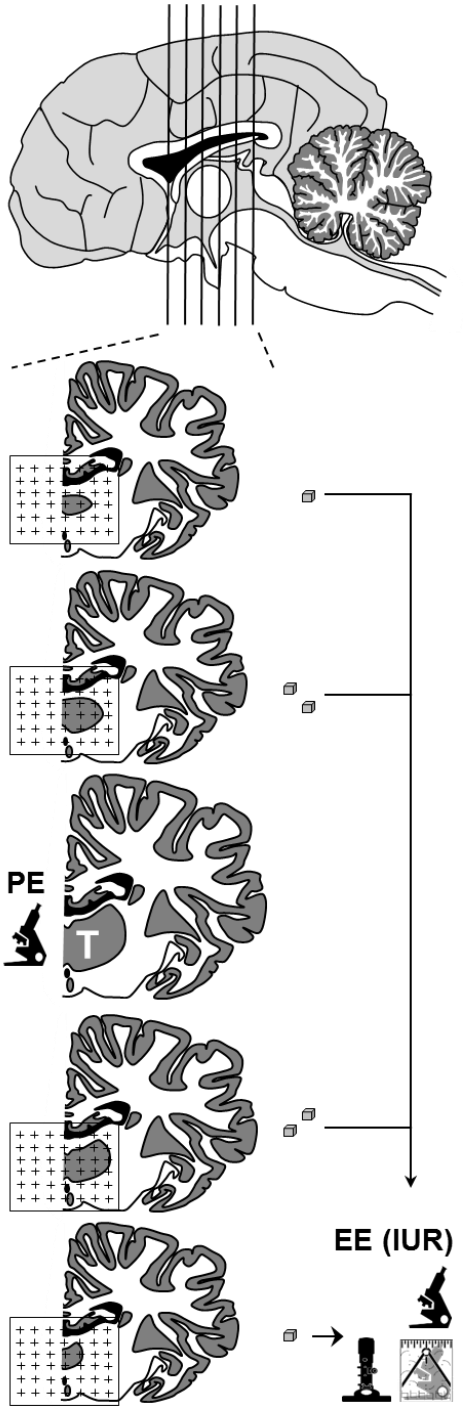
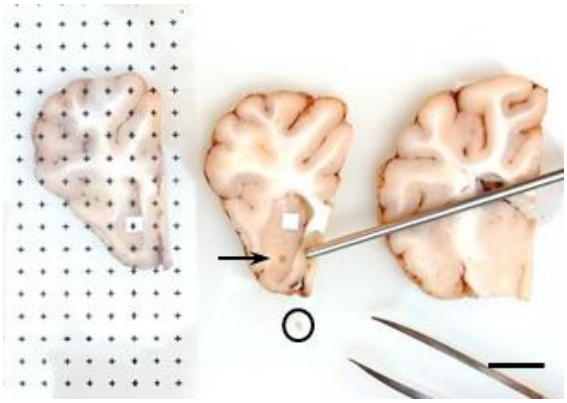


Fig. S140 (←). *A schematic illustration of the systematic random sampling of BROIs. In this example, samples from the thalamus (T) are generated for histology, electron-microscopy and quantitative stereological analyses. The section profiles of the thalamus are present in five of the ~4-mm-thick cross sections of the formalin-fixed agar embedded brain half. Here, only these five slices are shown. From these five slices, the middle slice is completely embedded in paraffin for routine histology. The thalamic section profiles present in the remaining four slices are overlaid with a cross grid printed on a transparency (grid size ~4 mm) for the systematic random sampling of six specimens (each ~4x4x4 mm). The samples are embedded in Epon for quantitative stereological analyses. Depending on the potential parameters of interest, the samples can be cut into semithin or ultrathin sections or be re-embedded in Epon for the generation of IUR sections (ORIENTATOR or ISECTOR). After the thalamic samples are obtained, the next BROI is subjected to systematic random sampling.*

Fig. S141 (↓). *Systematic random sampling of the caudate nucleus in cross sections of the formalin-fixed agar-embedded brain half. Pieces of paper are used to mark the sampled positions. Samples 1x1x3 mm in size (circled tissue) are punched out for Epon embedding (arrow) using a trocar (file-sharpened cannula). Scale bar = 1 cm.*



Brain samples for paraffin histology, ISH and IHC analyses in Type-II/III studies

In Type-II and in Type-III studies, 4 slices of the 4-mm-thick, formalin-fixed, agar-embedded brain tissue are selected for paraffin histology, ISH and IHC analyses using the standard frontal (cross) brain section planes (section 2.8.1.1.2., Fig. S132 – S135) for the frontal lobe, basal nuclei, thalamus, pons, cerebellum and brainstem.

Table S21. Number of specimens sampled from 4-mm-thick cross sections of the brain to be processed for Epon embedding for quantitative histopathological analyses in Type-II/III studies.

BROI	Sampling method*/number of samples per BROI	
	Type-II studies	**Type-III studies**
Neocortex	SRS (10 mm)/**10**	SRS (10 mm)/**10**
Cerebellar cortex	SRS (10 mm)/**10**	SRS (10 mm)/**10**
Caudate nucleus	-	SRS (10 mm)/**10**
Thalamus	SRS (10 mm)/**6**	SRS (10 mm)/**6**
Hippocampus	-	DCL/~**6**
Hypothalamus	-	DCL/~**6**
Pons	-	DCL/~**4**

**Sampling methods: SRS: Systematic random sampling (recommended cross grid size); DCL: Deliberately chosen location. Size of samples: Approximately 4x4x4 mm.*

Remarks

After systematic random sampling of BROIs, the remaining brain tissue slices are embedded in paraffin.

Time and personnel requirements

At least 35 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one or two assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Bolon et al., 2013a, Bolon et al., 2013b, Kaufmann et al., 2012, Kristiansen and Nyengaard, 2012, Morawietz et al., 2004, Pardo et al., 2012, Schmitz and Hof, 2005, Zachary, 2012)

2.8.1.2. Pituitary gland

Relevant anatomical features/preparation

The pituitary gland is harvested after removal of the brain. It is located directly behind the optic chiasm. To remove the gland, two incisions are made in the dura mater to the right and left of the gland. The pituitary gland can then be dissected from the cavernous sinus. During removal of the pituitary gland, the trigeminal nerves and their ganglia should be spared (Fig. S142 and Fig. S143).

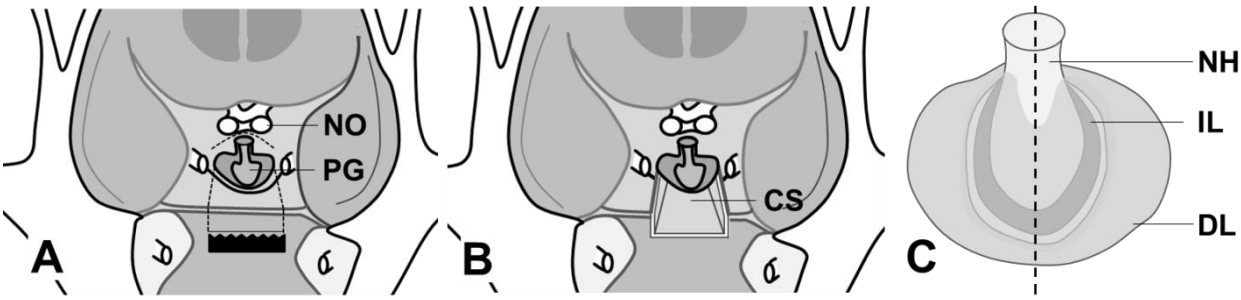
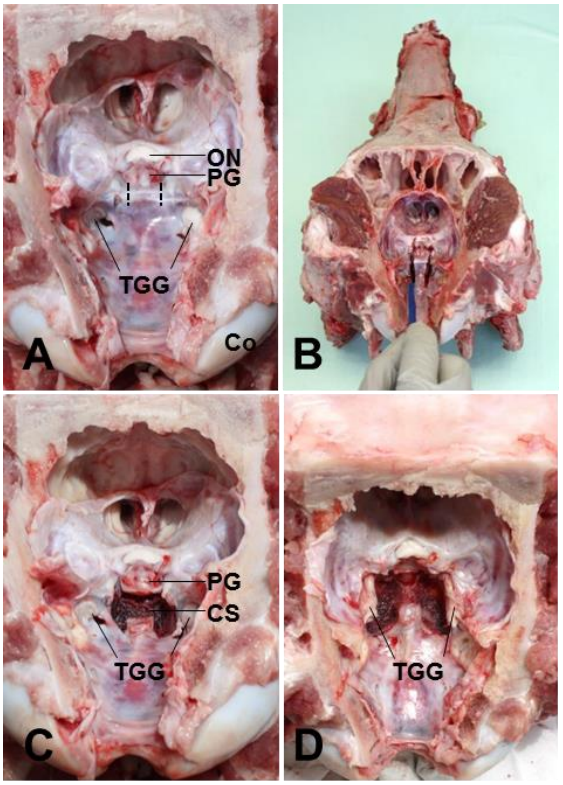


Fig. S142. *A schematic illustration of pituitary gland (PG) removal. A dorsal view of the skull after removal of the brain is shown. NO: Optic nerve (optic chiasm).* ***A****: Incision lines into the dura mater to the right and left of the pituitary gland are indicated.* ***B****: The pituitary gland is then removed from the cavernous sinus (CS).* ***C****: A schematic illustration of the excised pituitary gland. NH: Neurohypophysis; IL: Intermediate lobe; DL: Distal lobe.*

General examination parameters

After careful removal of adjacent dura mater, vessels, and connective tissue from the pituitary gland, the gland is weighed and examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.



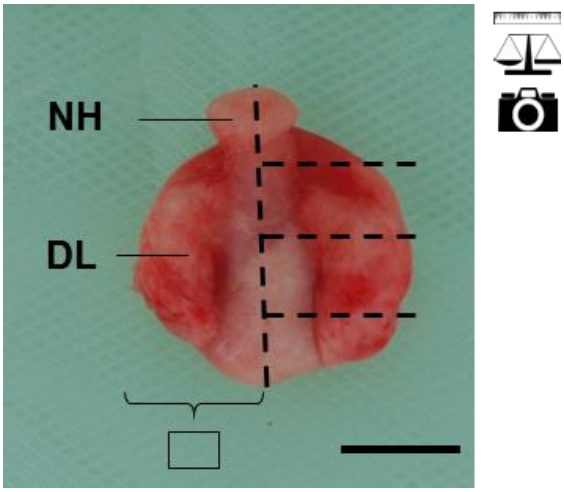


Fig. S144 (↑). *Freshly harvested pituitary gland with Type-I-study sampling locations indicated. The left half of the pituitary gland is frozen at -80°C for subsequent molecular analyses (black rectangle), and the right half is horizontally sectioned (black dashed lines) for histopathological examination. NH: Neurohypophysis; DL: Distal lobe (pars distalis). Scale bar = 5 mm.*

Fig. S143. Location and removal of the pituitary gland (PG). *Dorsal view of the skull after removal of the brain. ON: Optic nerve (optic chiasm).* ***A****: Incision lines into the dura mater to the right and left of the pituitary gland are indicated by dotted lines. TGG: Trunks of the trigeminal nerves/trigeminal ganglia. Co: Occipital condyle.* ***B****: Incision of the dura mater.* ***C****: The pituitary gland is removed from the cavernous sinus (CS).* ***D****: The site after removal of the pituitary gland. Note the location of the TGG.*

2.8.1.2.1. Pituitary gland samples in Type-I studies

The pituitary gland is sagittally cut into two halves along its rostro-caudal axis as indicated in Fig. S144 and Fig. S145. The left half is frozen on dry ice for molecular analyses and stored at -80°C until further investigation. The right half is fixed in 4% FA or PFA. After fixation, the tissue is cut into equidistant slices approximately 1-2 mm in thickness and embedded in paraffin for histology. Slices are cut in horizontal orientation orthogonal to the dorso-ventral axis of the organ and the sectional plane used to halve the organ (Fig. S144 and Fig. S145). This section orientation allows for the generation of more slices from the organ than sagittal cuts. The availability of a greater number of sections is advantageous, e.g., when the volume proportions of the different compartments of the pituitary gland (neurohypophysis, NH; intermediate lobe, IL; distal lobe, DL) are to be determined from their respective cross section profiles using the Delesse principle. This approach allows for several downstream analyses. Frozen samples can be subjected to DNA, RNA, and protein analyses (OMICS profiling). Qualitative histology, ISH and IHC analyses can be performed on FF-PE sections. Equidistant sections of the pituitary gland are generated; thus, distinct quantitative stereological parameters, such as optical disector analyses, can be applied, and dimensionless variables independent of tissue shrinkage, such as the volume density of single structural compartments or distinct cell types within the pituitary, can be determined in paraffin sections. The absolute volume can be calculated because the total pituitary gland volume is known/calculable by the weight and density of the pituitary gland.

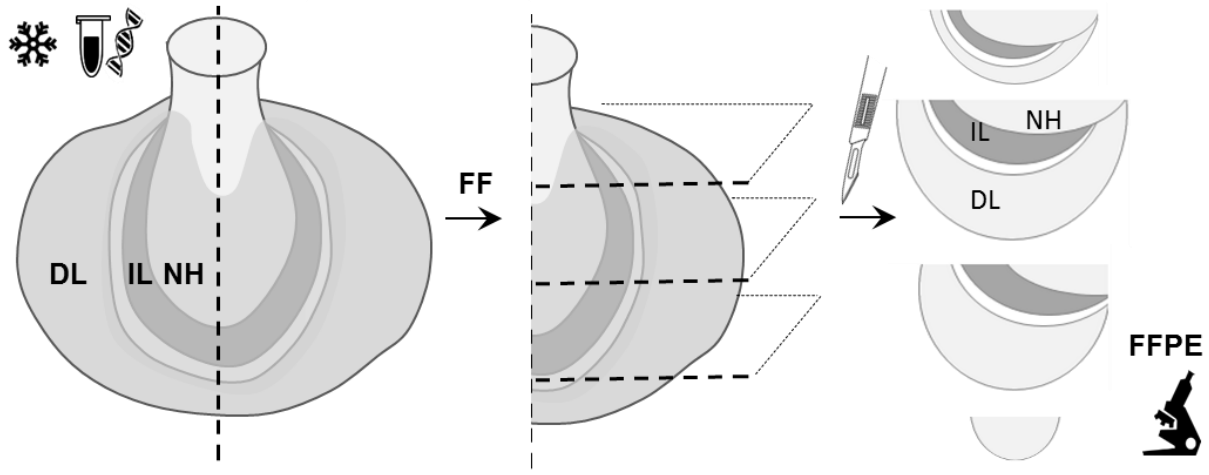


Fig. S145 (↑). *The preparation of pituitary gland samples in Type-I studies. The pituitary gland is sagittally cut into halves (with equal volume proportions of the neurohypophysis (NH), the intermediate lobe (IL) and the distal lobe (DL) in both halves). The left half of the pituitary gland is frozen at -80°C for subsequent molecular analyses, and the right half is horizontally sectioned for histopathological examination.*

Samples for molecular analyses

Location:	Left half of the pituitary gland. The section orientation is indicated in Fig.S144 and Fig. S145.
Number of samples:	One.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Right half of the pituitary gland. The section orientation is indicated in Fig. S145 and Fig. S144.
Fixation/Embedding:	FF-PE (Fig. S149).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

The pituitary gland is commonly included in the list of protocol organs for general toxicity studies (Bolon et al., 2013b). The revised guides for organ sampling and trimming in rats and mice recommend the preparation of one transverse section through the fixed pituitary gland parallel to the caudodorsal surface of the gland. The section includes all three portions of the pituitary (DL, IL, NH) (Kittel et al., 2004). The samples generated according to the Type-I-study sampling protocols allow for an adequate evaluation of the porcine pituitary gland in routine studies. However, considering the larger size of the porcine pituitary gland and the need to incorporate the collection of samples for molecular analyses into the sampling protocol, the Type-I-study sampling plan recommends the generation of additional samples from the porcine pituitary gland compared with routine toxicity studies in rodents.

2.8.1.2.2. Pituitary gland samples in Type-II and Type-III studies

Using the Type-II/III-study sampling guides described below, pituitary gland samples suitable for advanced quantitative stereological analyses can be generated. However, sample generation for molecular analyses is not recommended in this guide. If necessary, samples for molecular analyses can be generated by laser-microdissection from sections of FF-PE tissue. If samples for molecular analyses are important for a specific study, preference should be given to the sampling guide for Type-I studies.

Sampling

The freshly dissected pituitary gland is weighed to the nearest mg and cut into two halves as described above. One half is chosen randomly and fixed in a 2.5% glutaraldehyde solution (GA), and the other half is fixed in a 4% formaldehyde solution. Both, the GA- and formalin-fixed halves are embedded in agar and sliced in equidistant parallel thin (1 mm thick) slices. The formalin-fixed half is sliced in a horizontal direction as described above. The GA-fixed half is sliced in a sagittal orientation. In sagittal sections, the borders between the distal lobe and the pouch of Rathke and the intermediate lobe/neurohypophysis are easily recognizable. The slicing of the pituitary gland is facilitated with a tissue slicing device (Fig. S146). The slices are placed on the same side and scanned in conjunction with a size scale for the subsequent planimetry of section surfaces to determine the Cavalieri volume and embedding-related tissue shrinkage. The slices of the formalin-fixed gland are completely embedded in paraffin while maintaining the orientation of their sectional planes. These samples can be used for qualitative histology, ISH and IHC analyses, estimation of distinct quantitative stereological parameters, such as optical disector analyses, and determination of dimensionless variables independent of tissue shrinkage, such as the volume density of single structural compartments or distinct cell types within the pituitary. Molecular analyses using laser-microdissection from FF-PE tissue sections can be completed. The slices cut from the GA-fixed half of the gland are placed on the same side, and subsamples from the neurohypophysis (NH) and distal lobe (distal portion of the adenohypophysis, DL) are used for systematically random sampling. The selected samples are excised using a trocar with ~1 mm inner diameter. Systematic random sampling is necessary because the single cell types present in the different parts of the pituitary gland are not equally distributed within their reference compartments (Rosol et al., 2013). Meaningful sampling of the intermediate lobe (IL), a thin band of tissue attached to the NH, is not possible because the IL cannot be macroscopically identified with sufficient certainty. The excised tissue pieces are processed for generation of IUR sections (ORIENTATOR/ISECTOR) and embedded in Epon resin. These samples can be used for the estimation of virtually all quantitative stereological parameters, semithin section histology and ultrastructural analyses by TEM (Fig. S147 and Table S22).

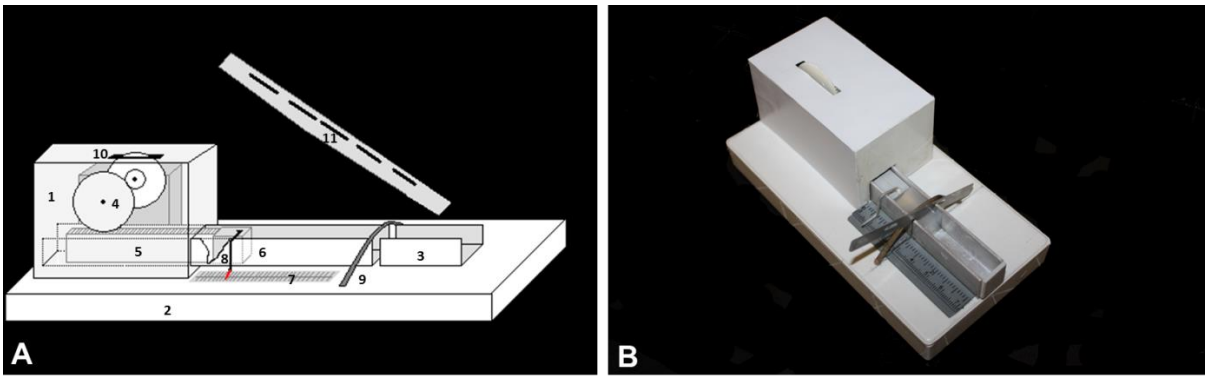


***Fig. S146. Tissue slicing device. A**: A schematic illustration. **B**: Image of the device. The device consists of a profile rail (3) that holds and guides the form-locking block of agar-embedded tissue. A propulsion mechanism, consisting of gear wheels (4), a gear rack (5), and a die (6) mounted in a housing (1), and a slot (10) for the main gear wheel, allows for the precise and smooth forward movement of the agar block inside the profile rail. The amount of propulsion distance can be determined from the scale (7) using an indicator (8) adapted to the die (6) at the front of the gear rack (5). The agar block (and the embedded tissue) is cut using a microtome blade (11) or similar instrument with an interruption of the profile rail using an installed blade guide (9). The complete device is mounted on a base (2). The device can be easily be built using common household tools and super glue. The device allows for the sectioning of tissue with adjustable and constant section thicknesses of at least ~1 mm.*

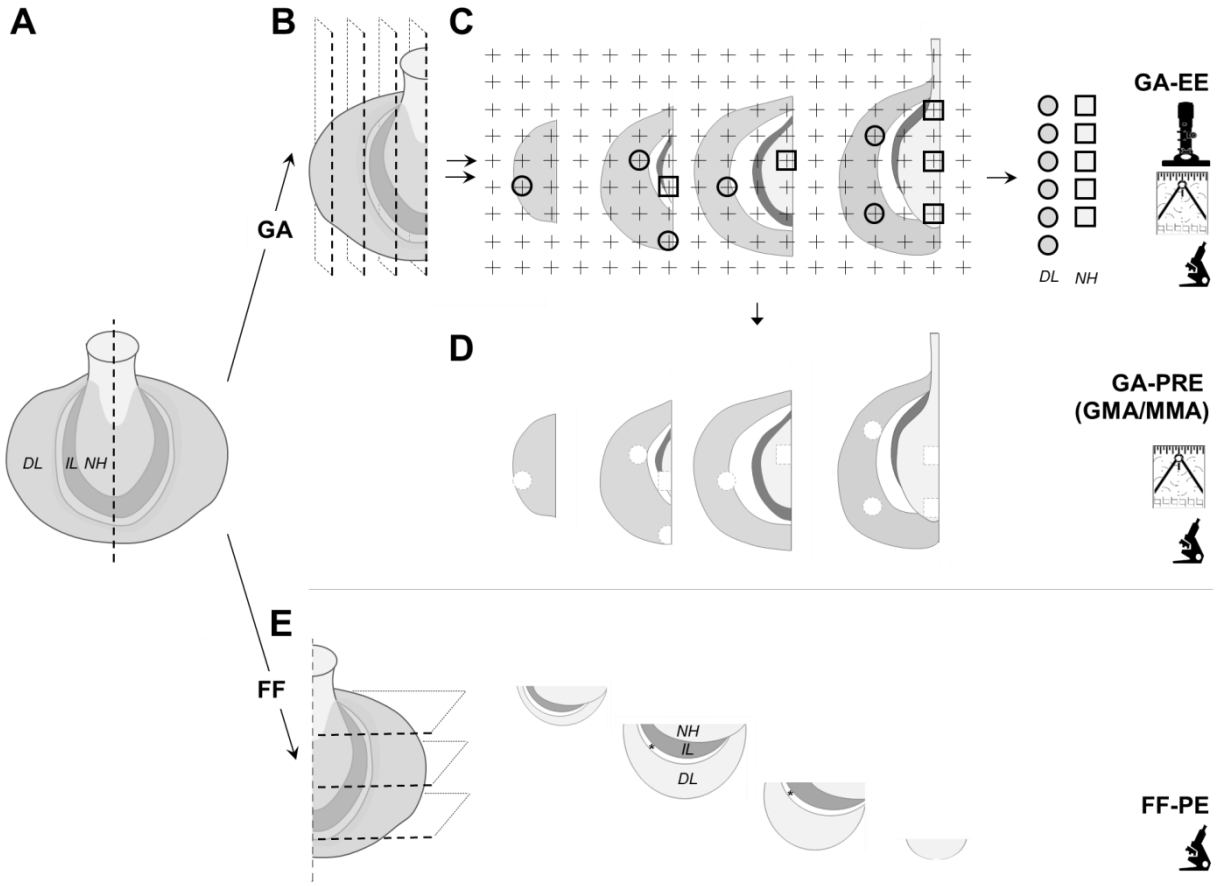


***Fig. S147. Processing of the pituitary gland for advanced histopathological analyses (in Type-II/III studies)**. **A**: The pituitary gland is sagittally cut into two halves. **B**: One half is randomly chosen, fixed in FA, embedded in agar and cut into sagittal sections. **C**: The slices are put on the same surface and overlaid with a cross grid printed on a plastic transparency for the systematic random sampling of six samples of the distal lobe (DL) and five samples of the neurohypophysis (NH). The sampled specimens are excised with a trocar and embedded in Epon for subsequent semithin section histology, quantitative stereology and ultrastructural analysis. If appropriate, IUR sections are prepared. **D**: The remaining tissue slabs are embedded in GMA/MMA for plastic histology and quantitative stereological analysis. **E**: The second half of the pituitary gland is fixed in formalin, sliced as described above and processed for FF-PE and subsequent analyses. Pouch of Rathke (*).*

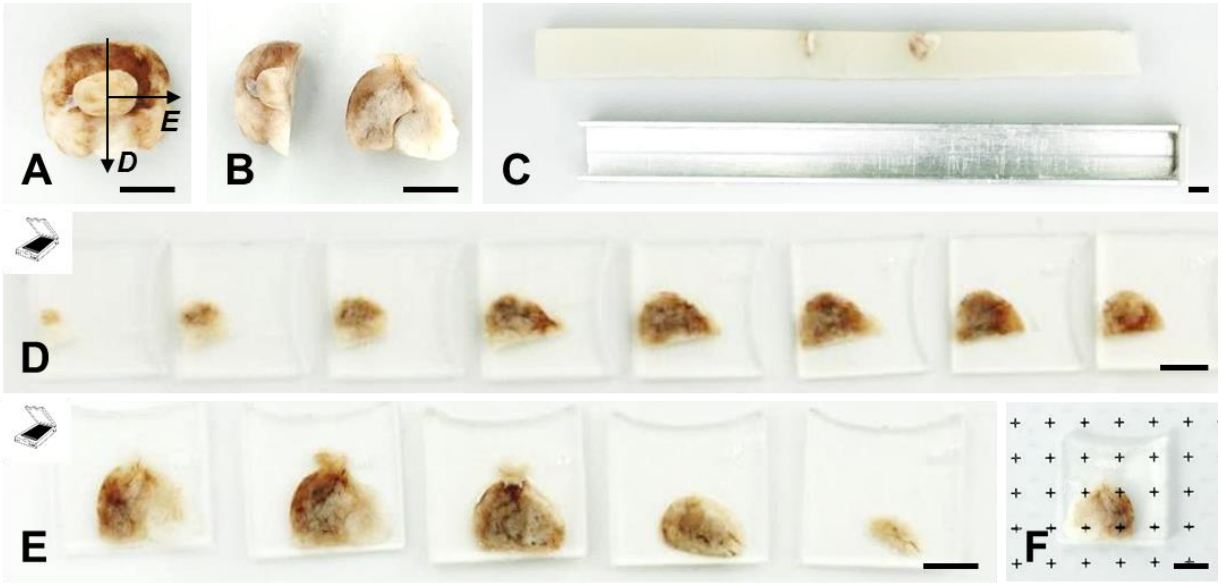


***Fig. S148. Processing of the pituitary gland for advanced histopathological analyses (in Type-II/III studies). A**: The pituitary gland is sagittally cut into two halves. Sagittal (↓D) and horizontal (→E) sectional directions are indicated. One half of the gland is fixed in GA, and the other half of the gland is fixed in FA. **B**: Bisected fixed pituitary gland. **C**: While maintaining their recommended orientations, the halves are embedded in agar using a casting rail corresponding to the size of the profile rail of the tissue-sectioning device (Fig. S146). **D**: Sagittal tissue slices of the FA-fixed half of the pituitary gland and horizontal slices of the GA-fixed half of the pituitary gland are cut and scanned for volumetry and determination of embedding-related tissue shrinkage. **F**: Detailed enlargement of pituitary gland section overlaid with a cross grid printed on a plastic transparency for systematic random sampling. Scale bars = 5 mm.*

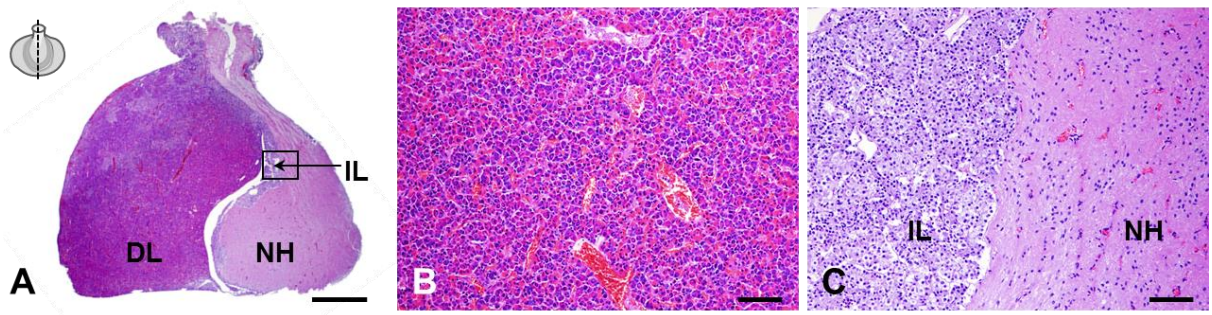


***Fig. S149. Pituitary gland histology. A**: Midsagittal section. DL: Distal lobe. IL: Intermediate lobe. NH: Neurohypophysis. The position of the image shown in **C** is indicated by a black box. **B**: DL. Note the acidophilic, basophilic and chromophobic cells in the DL. **C**: IL and NH. FF-PE. HE. Scale bars = 1 mm (A), 100 µm (B, C).*

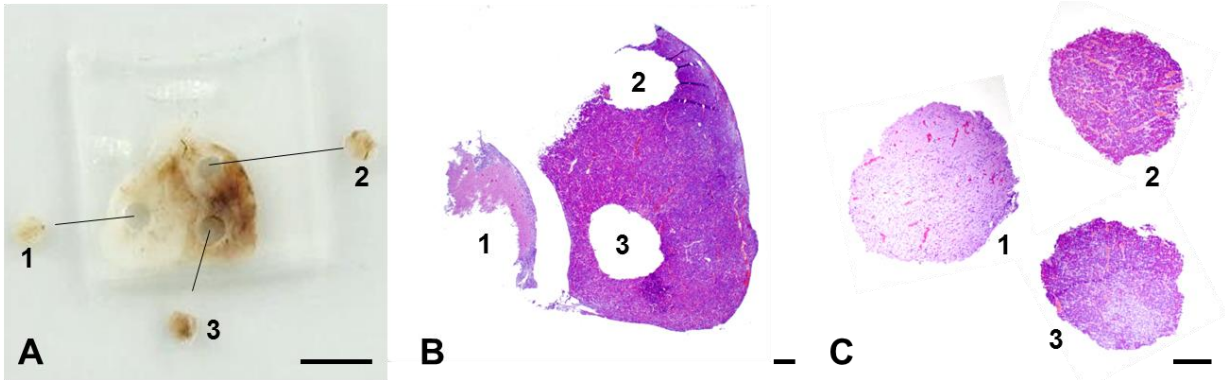


***Fig. S150. Demonstration of the accuracy of differential sampling of the distal lobe (DL) of the pituitary gland and the neurohypophysis (NH). A**: Agar-embedded midsagittal section of the pituitary gland. Samples of the NH (1) and DL (2, 3) are taken with a trocar at systematically randomly sampled locations. **B**: Histology of the tissue slice after excision of the samples (the corresponding sampling positions are indicated). **C**: Histology of the excised samples demonstrate the accuracy of the differential sampling approach. FF-PE. HE. Scale bars = 1 mm (A), 100 µm (B, C).*

Table S22. The type and number of samples generated from the pituitary gland in Type-II/III studies.

Fixation and embedding medium	Sectioning and number of locations/samples per location in Type-II and Type-III studies
FA(PFA) **FF-PE**	*Sagittal sections. All slices (7-10) are embedded in paraffin. No subsamples are taken.*
GA **GA-EE**	*Horizontal sections (4-6). Five specimens from the NH* and six samples from the DL** are sampled. Samples are embedded in Epon. IUR-sectionable samples (ISECTOR) are prepared if required.*
GA-GMA/MMA	*The remaining tissue slices are embedded in GMA/MMA.*

**NH: Neurohypophysis; **DL: Distal lobe*

Time and personnel requirements

At least 10 minutes must be scheduled for the execution of the complete Type-II/III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Blutke et al., 2014, Bolon et al., 2013b, Kittel et al., 2004, La Perle, 2012, Rosol et al., 2013)

2.8.1.3. Pineal gland

In this guide, pineal gland sampling is recommended for FF-PE histology and molecular analyses in Type-III studies, but this procedure is not typically performed during routine necropsies. Advanced sampling/tissue processing for quantitative stereological analyses is not recommended in this guide.

Relevant anatomical features/preparation
The pineal gland is located at the caudo-dorsal margin of the third ventricle (Fig. S151). The gland is assessed when the brain is dissected longitudinally (section 2.8.1.1.2.).

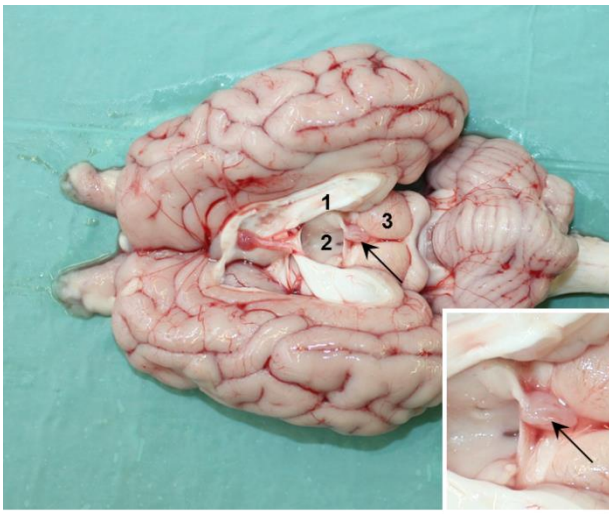


Fig. S151. Location of the pineal gland *(three-month-old pig). The pineal gland (arrow) is located at the caudo-dorsal margin of the third ventricle (2) and is most accessible after the dorsal longitudinal dissection of the corpus callosum (1). Rostral colliculus (3).*

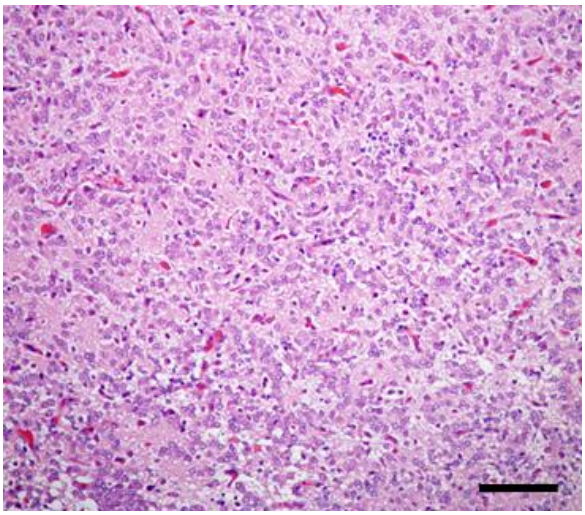


Fig. S152. Pineal gland histology. *FF-PE. HE. Scale bar = 100 µm.*

General examination parameters
The pineal gland is weighed, measured, and examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are collected if required.

Samples for histology and molecular analyses
The pineal gland is longitudinally cut into two halves. The left half is frozen at -80°C for molecular analyses. The right half is routinely fixed in a 4% neutrally buffered formalin solution and embedded in paraffin (Fig. S152).

Related literature
(Yamamoto et al., 2010)

2.8.1.4. Spinal cord, cranial nerves, and ganglia

In this section, sampling of the spinal cord, of the vagus nerve, of spinal ganglia (dorsal root ganglia, DRG), and of trigeminal ganglia is described. In routine (Type-I) studies, only the spinal cord is sampled, whereas additional samples of cranial nerves (except the optic nerve), cranial nerve ganglia, and DRG are only collected in Type-II and Type-III studies.

2.8.1.4.1. Spinal cord

Relevant anatomical features/preparation

For removal of the spinal cord from the vertebral column in young pigs, the dorsal vertebral arches are removed using Liston forceps. This method is not recommended for older pigs because the dorsal vertebral arches are too massive and hard to be cut by hand. Here, the musculature around the vertebral column and the dorsal spinous processes are removed using a knife and saw. Next, the dorsal vertebral arches are cut open using an oscillating saw (Fig. S153). Very experienced necropsy assistants (or pathologists) may be able to split the vertebral column lengthwise using a butcher axe or band saw. Using this method, the spinal cord may occasionally be severely damaged; thus, this technique should be performed only if absolutely necessary. If possible, the spinal cord is removed together with the roots of the spinal nerves and their ganglia. Alternatively, the dorsal root ganglia samples can be harvested before the spinal cord is removed from the vertebral column (Fig. S153). If only defined spinal cord segments (C1-C2, T6-T8, L4-L5) are to be harvested for examination in Type-I studies, the technique for spinal cord collection from non-rodent species in nonclinical general toxicity studies recommended by the Working Group on Nervous System Sampling of the Society of Toxicologic Pathology (Bolon et al., 2013b) can be used. Short sections (two to three vertebrae in length) of the cervical, thoracic, and lumbar vertebral column are isolated after the respective intervertebral joints have been cut using a thin knife (cranial to vertebrae C1 and C3 for the upper cervical C1-C2 spinal cord segments, cranial to T6 and T8 for the mid-thoracic T6-T8 spinal cord segments, and cranial to L3 and L5 for the lumbar intumescence region of L4-L5). Next, the spinal nerve roots are cut and the spinal cord segments are carefully removed. Especially in larger pigs, application of this technique reduces the time needed for sample collection compared with a full-length preparation of the entire spinal cord. However, it should be noted that due to the shortening of the spinal cord relative to the vertebral column during postembryonal growth, the L4-L5 spinal cord segments are located cranial to the corresponding vertebral bodies (Böhme, 1992). Therefore, the third and fourth lumbar vertebra must be excised to collect the L4-L5 spinal cord segments.

After removal of the spinal cord, the dura mater is opened longitudinally using a fine pair of scissors to facilitate the harvesting of tissue samples and tissue penetration of fixation liquid. If the spinal cord was removed intact, an identification of single spinal cord segments can be achieved by counting the roots of the spinal nerves. According to the applied sampling protocol, samples for molecular analyses can be obtained next, and the remaining tissue is transferred into fixation solution. The split vertebral column is then inspected macroscopically. For further examination, samples of the vertebral column, such as the vertebrae and vertebral discs, can be harvested; however, the sampling of these structures is not included in these sampling guidelines.

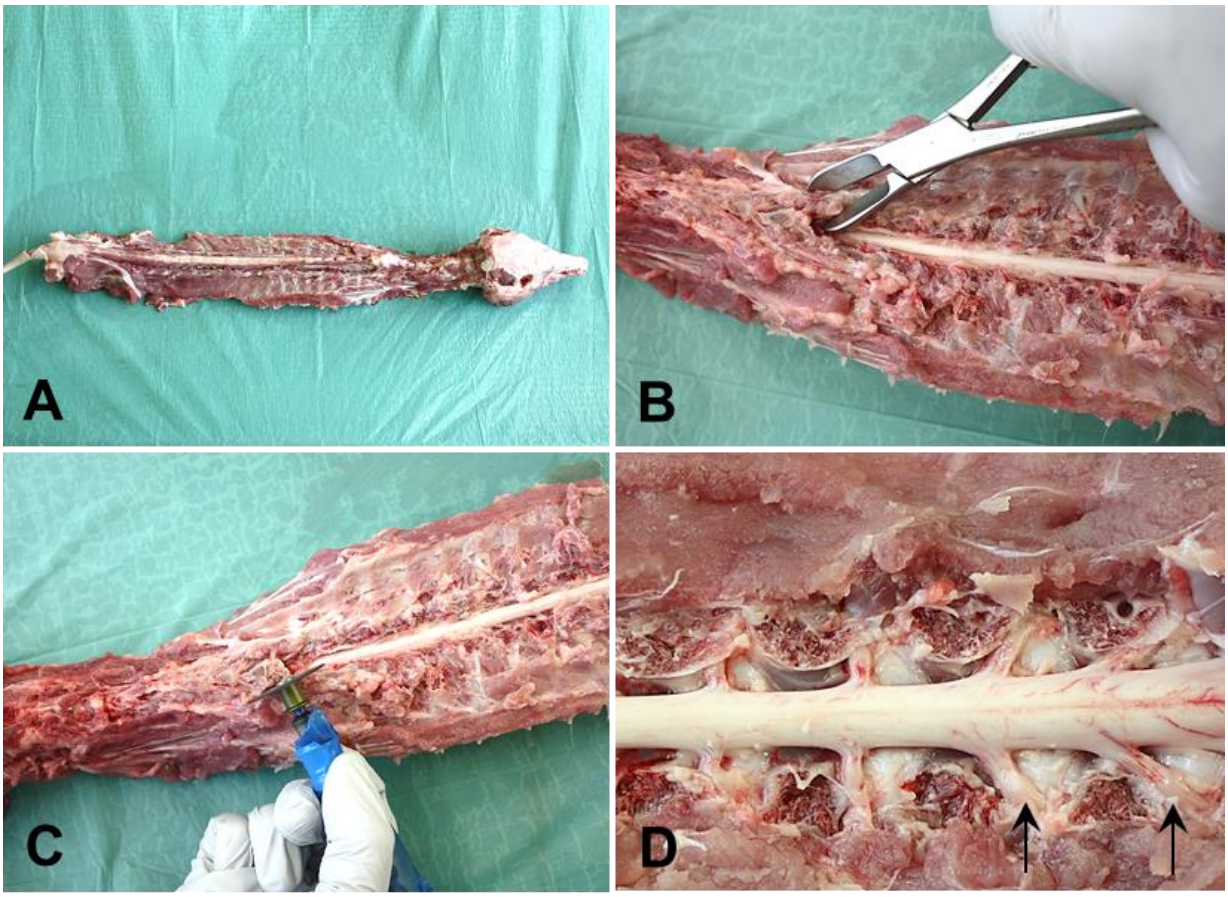


Fig. S153. Spinal cord dissection *(three-month-old pig).* ***A****: Vertebral column after removal of the musculature and the spinous processes.* ***B, C****: Removal of the dorsal vertebral arches using Liston forceps (B) and an electric saw (C).* ***D****: Dorsal aspect of the spinal cord after removal of the dorsal vertebral arches (lumbo-sacral segments). The dorsal root ganglia are marked by arrows.*

General examination parameters

If the spinal cord is intact, its length and weight are determined (Fig. S154 and Fig. S155). Next, the spinal cord is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

2.8.1.4.1.1. Spinal cord samples in Type-I studies

For molecular and histopathological analyses in Type-I studies, samples of spinal cord segments C1-C2, T6-T8, and L1-L3 are collected as described above and illustrated in Fig. S154.

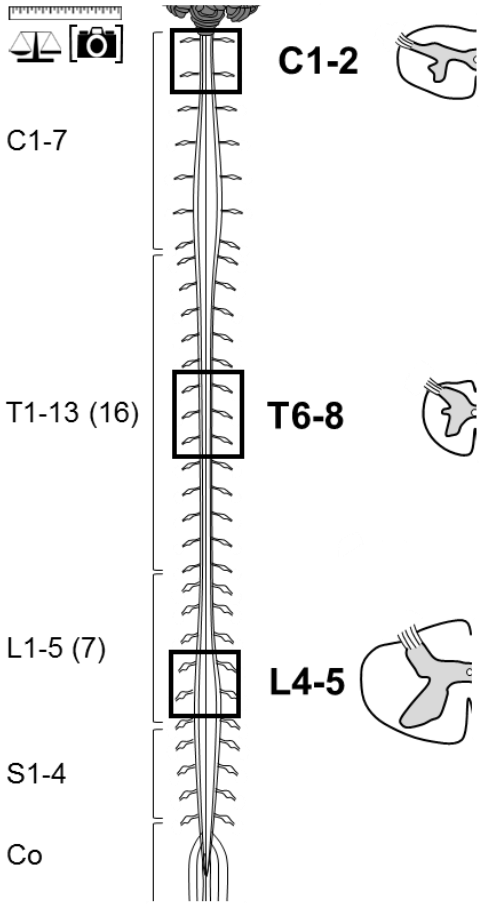


Fig. S154. *A schematic illustration of the spinal cord and section profiles of distinct cross sections at different segments demonstrating the distribution of gray and white matter. The spinal cord segments are indicated as C1-7 for cervical segments, T1-13(16) for thoracic segments, L1-5 for lumbar segments, and S1-4 for sacral segments. Co: Medullary cone. The Type-I-study sampling locations (spinal cord segments C1-2, T6-8, and L4-5) are indicated by rectangles.*

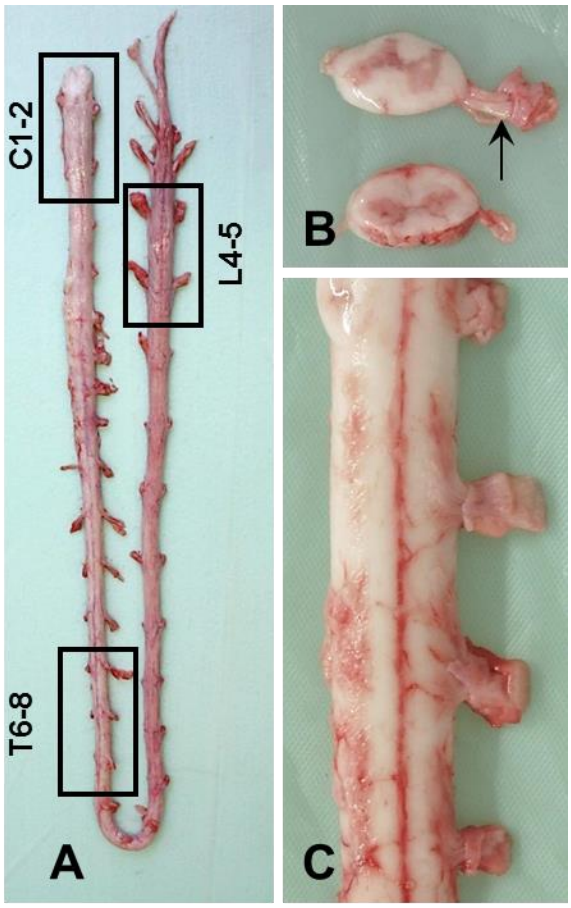


Fig. S155. A*: Spinal cord from a six-month-old boar. The samples for molecular analyses (black rectangles) and the corresponding spinal cord segments (C1-2, T6-8, L4-5) for Type-I studies are indicated.* ***B****: Cross section of the spinal cord with adjacent dorsal root ganglion.* ***C****: Magnification of the spinal cord with spinal nerve roots (dorsal view).*

Samples for molecular analyses

For molecular analyses, one cross section approximately 1-2 mm in thickness is taken from each spinal cord segment: C1-C2, T6-T8, and L4-L5. The excised sections are each divided into halves as shown in Fig. S156 and separately stored at -80°C until further investigation.

Location:	Spinal cord segments C1-C2, T6-T8, and L4-L5 (Fig. S154 and Fig. S156).
Processing:	The adjacent spinal meninges and adipose tissue are carefully removed from the spinal cord. A thin transversal slice is used to divide the spinal cord into two halves (Fig. S156). The samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

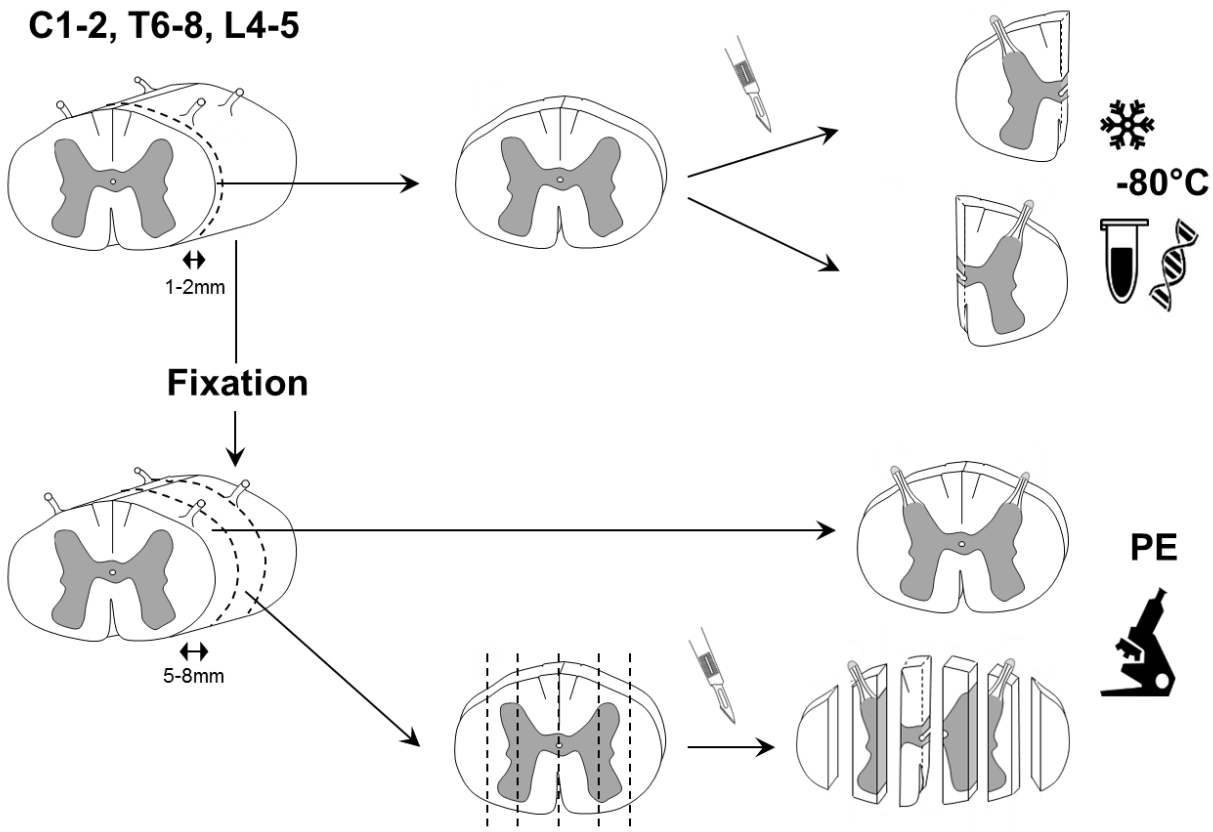


Fig. S156. *A schematic illustration of the generation of spinal cord samples for molecular analyses and histopathological examination in Type-I studies.*

Histology samples

After the samples for molecular analyses are obtained, the remaining spinal cord tissue is fixed in 4% neutrally buffered formalin. For histopathological analyses, both cross and longitudinal spinal cord sections are prepared.

Location, number and orientation of sections:	Two approximately 5-8 mm thick cross sections are cut from each of the cervical (C1-C2), mid-thoracic (T6-T8), and lumbar intumescence regions (L4-L5) near the location of the samples taken for molecular analyses. One of the two cross sections per spinal cord segment is cut into approximately six equidistant sagittal slices to obtain longitudinal spinal cord sections as indicated in Fig. S156. Thus, one complete cross section and approximately six longitudinal sections are prepared from each sampled spinal cord region.
Fixation/Embedding:	FF-PE (Fig. S157 and Fig. S158).

Fig. S157. *Histology slide of a cross-sectioned and longitudinally sectioned spinal cord segment. FF-PE. HE.*

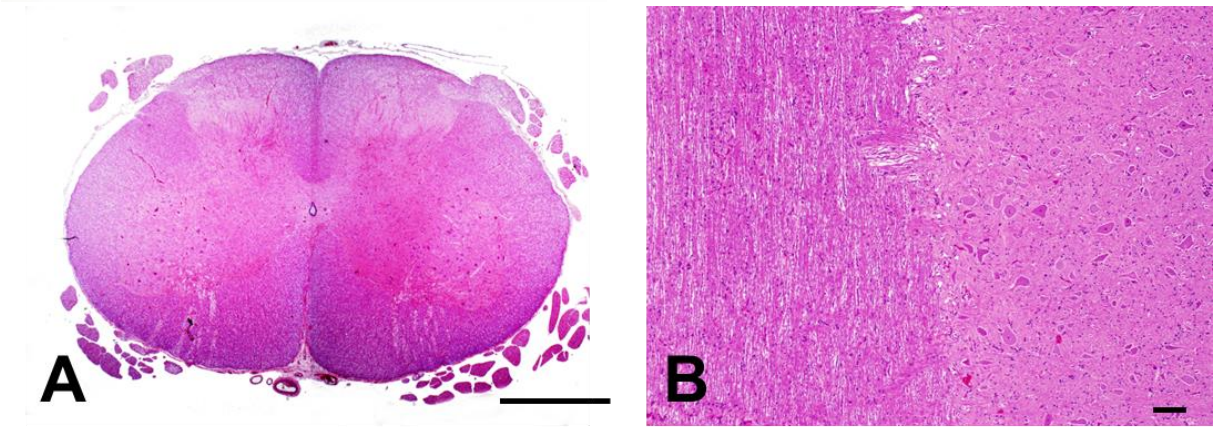


Fig. S158. Spinal cord histology *(lumbar intumescence).* ***A****: Cross section. Scale bar = 5 mm.* ***B****: Longitudinal section. Scale bar = 100 µm. FF-PE tissue. HE.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

The revised guides for organ sampling and trimming in rats and mice in regulatory toxicity studies, recommend the preparation of three cross sections of the upper cervical, mid-thoracic, and 4th lumbar spinal cord segment (Morawietz et al., 2004).

For histopathological examination of the rodent and non-rodent spinal cord in nonclinical general toxicity studies, the Working Group on Nervous System Sampling of the Society of Toxicologic Pathology recommends the sampling of cervical, mid-thoracic, and lumbar spinal cord segments with subsequent formalin fixation and cross and longitudinal/oblique sections for paraffin histology (Bolon et al., 2013b).

The Technical Guide for Nervous System Sampling of the cynomolgus monkey for General Toxicity Studies recommends the generation of cross and oblique sections of the C1–C4, T10–T12, and L1–L4 segments, including dorsal root ganglia and dorsal and ventral nerve roots (Pardo et al., 2012).

The sampling protocols for Type-I studies recommend the generation of samples for histopathological examination of comparable segments (C1-C2, T6-T8, L4-L5) with similar section orientations (cross and longitudinal sections). This method allows for an adequate evaluation of the porcine spinal cord in routine studies.

2.8.1.4.1.2. Spinal cord samples in Type-II and Type-III studies

In Type-II and -III studies, spinal cord segments undergo systematic random sampling. The samples are then processed for molecular analyses and cryo-histology of fresh spinal cord tissue or for qualitative and quantitative histopathological analyses of formalin-fixed tissue. The sampling strategy is described in the legend for Fig. S159. A list of spinal cord samples for Type-II and Type-III studies is provided in Table S23.

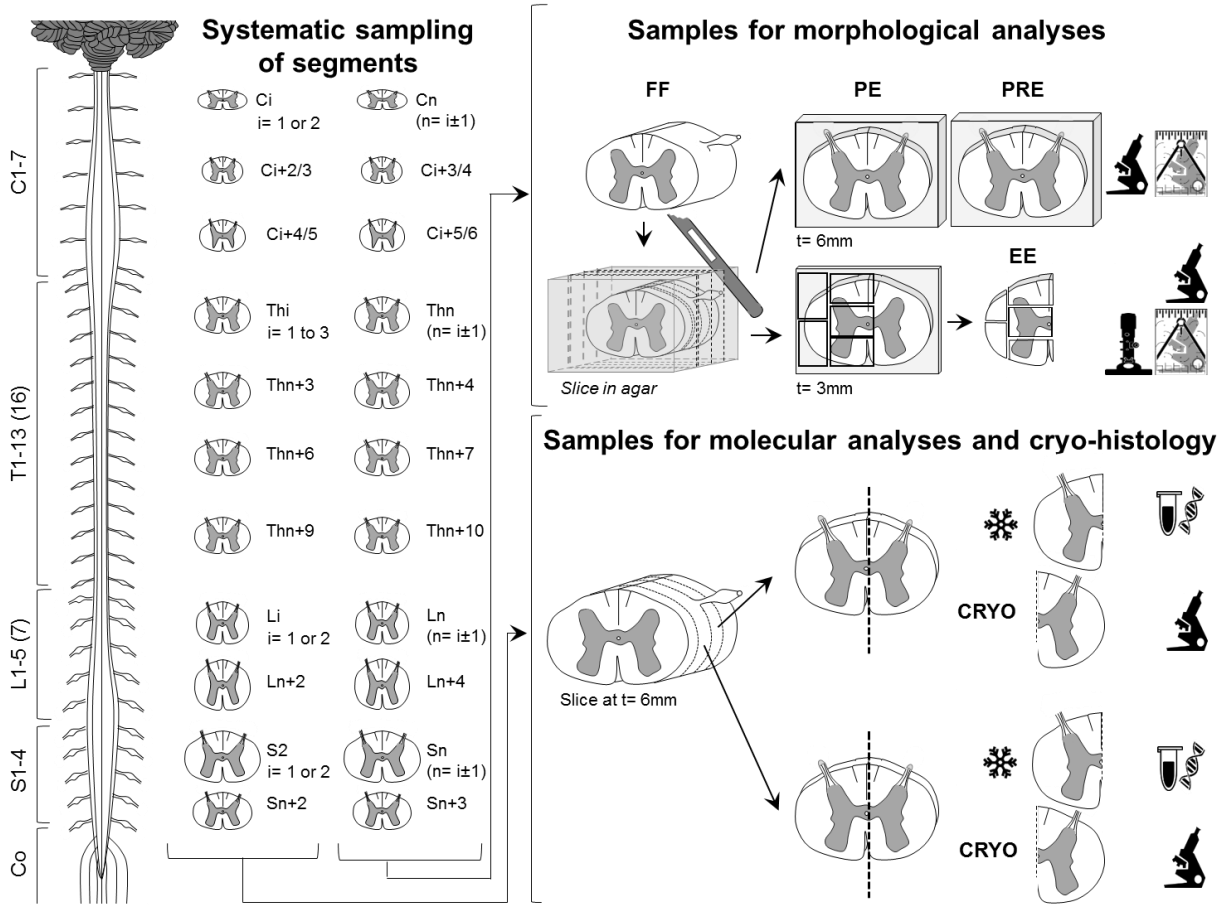


Fig. S159. *A schematic illustration of spinal cord sample preparation in Type-III studies. For each spinal cord region (C: Cervical; T: Thoracic; L: Lumbar; S: Sacral), a defined number (Table S23) of spinal cord segments is systematically randomly sampled. Half of the sampled segments per spinal cord region are fixed in a 4% formaldehyde solution. For molecular analyses and cryo-histology, two ~6 mm thick slices are cut from each unfixed (fresh) sampled spinal cord segment at the approximate level of the exit of the spinal nerve root from the spinal cord. The resulting tissue slabs are then cut into halves as indicated. One of the halves is frozen on dry ice and stored at -80°C until further processing for molecular analyses, and the other half is processed for cryo-histology by embedding in freezing-blocking medium and freezing in liquid nitrogen-cooled isopentane. The samples are stored at -80°C. For qualitative and quantitative histopathological analyses, the fixed spinal cord segments are embedded in agar for facilitated sectioning. For each segment, two cross sections approximately 6 mm thick (t) are cut at the level of the exit of the spinal nerve root from the spinal cord. One of these slices is embedded in paraffin for FF-PE-histology, ISH, IHC, and/or laser-microdissection of defined areas. The other slice is embedded in PRE (GMA/MMA) for histological examination and quantitative stereological analyses. From an additional ~3 mm thin slice, samples for Epon embedding are cut as indicated. Approximately six 4x4x3 mm samples will account for the tissue from one half of the spinal cord slice. The Epon-embedded samples may be sectioned for semithin section histology, TEM, or quantitative stereological analyses. If GA-EE samples for TEM examination are required (not shown), a thin slice (2-3 mm) can be cut from the agar-embedded fresh spinal cord tissue and then preserved with a fixatixe, such as glutaraldehyde solution.*

Table S23. The type and number of spinal cord samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

Study type	Spinal cord section	Number of segments sampled for molecular analyses and cryo-histology	Number of samples for molecular analyses per spinal cord segment	Number of samples for cryo-histology per spinal cord segment	Number of segments sampled for histopathological analyses	Number of FF-PE samples per segment	Number of FF-PRE samples per segment	Number of 3 mm thick spinal cord slices for FF-EE (six FF-EE samples/slice)
II	Cervical	3	1	1	3	1	1	-
III		3	1	1	3	1	1	1 of each sampled segment
II	Thoracic	4	1	1	4	1	1	-
III		4	1	1	4	1	1	1 of each sampled segment
II	Lumbar	2	1	1	2	1	1	-
III		2	1	1	2	1	1	1 of each sampled segment
II	Sacral	2	1	1	2	1	1	-
III		2	1	1	2	1	1	1 of each sampled segment

Time and personnel requirements

At least 30 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time needed for removal of the spinal cord and for processing of the samples until freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Beck et al., 2010, Bolon et al., 2013a, Bolon et al., 2013b, Morawietz et al., 2004, Navarro et al., 2012, Pardo et al., 2012, Zachary, 2012)

2.8.1.4.2. Dorsal root ganglia samples

Generation of spinal ganglia samples (dorsal root ganglia, DRG, Fig. S153, Fig. S155, and Fig. S160) for histopathological and molecular analyses is recommended for only Type-II and -III studies. Histopathological examination of the DRG should be performed in neurotoxicity studies. DRG are collected from the spinal cord intumescences (n = 4 DRG/intumescence for Type-II studies; n = 8 DRG/intumescence for Type-III studies) (Bolon et al., 2013b). If possible, the same number of DRG should be collected from the right and left side. Harvested DRG are randomly assigned for different downstream analyses as shown in Fig. S160. For each two DRG (4 in Type-III studies), the samples are processed for cryo-histology, FF-PE, GA-EE and molecular analyses. For histology, sagittal (longitudinal) DRG sections are prepared. The DRG allocated to molecular analyses must be "cleaned" by removal of adjacent dura mater, adipose tissue and spinal nerve fibers under a photomicroscope (Fig. S160) using sharp-tipped tweezers and scissors. The DRG samples should be labeled appropriately to identify the spinal cord segment from which they were derived.

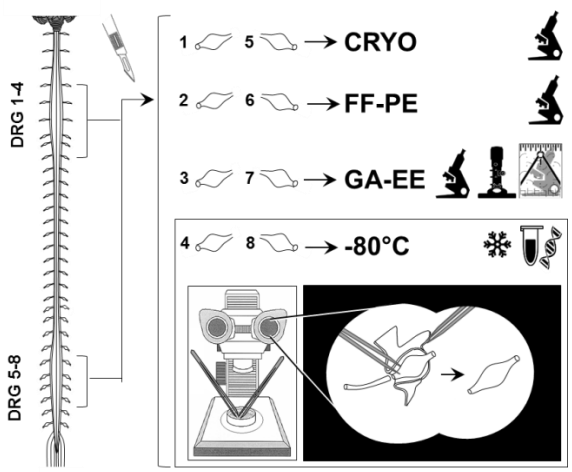


Fig. S160. *A schematic illustration of DRG sampling in Type-II studies.*

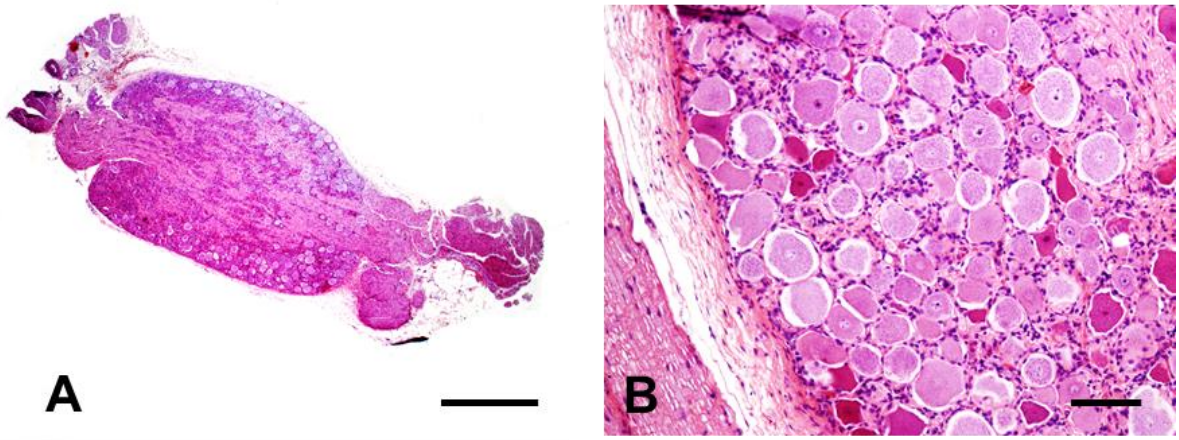


***Fig. S161. DRG histology. A**: Longitudinal section. Scale bar = 1 mm. **B**: Detailed enlargement. Scale bar = 100 µm. FF-PE tissue. HE.*

Related literature
(Bolon et al., 2013b, Pardo et al., 2012)

2.8.1.4.3. Vagus nerve samples

Vagus nerve samples are collected for only Type-II and Type-III studies. Samples of the thoracic portion of the left and of the right vagus nerve (Fig. S162) are collected and processed using the methods described for peripheral nerves (section 2.8.2.).

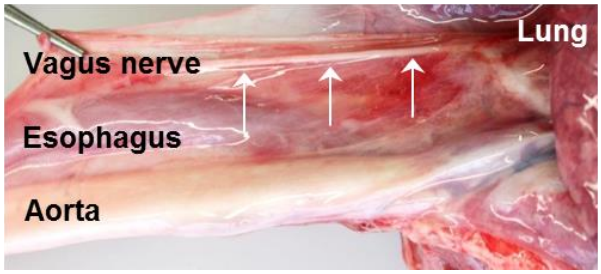


Fig. S162. *Location of the vagus nerve (arrows) in the thoracic cavity.*

2.8.1.4.4. Trigeminal ganglia samples

The generation of trigeminal ganglia samples for histopathological and molecular analyses is recommended for only Type-II and -III studies. After removal of the brain and pituitary gland, the trigeminal ganglia can be harvested. The ganglia are located to the right and left slightly caudal to the pituitary and enwrapped in the dura mater (section 2.8.1.2., Pituitary gland, and Fig. S163). One ganglion is randomly chosen, fixed in a formaldehyde solution, and embedded in paraffin for preparation of longitudinal sections (Fig. S164). The other ganglion is processed as described for dorsal root ganglia (Fig. S160), frozen on dry ice, and stored at -80°C for subsequent molecular analysis.

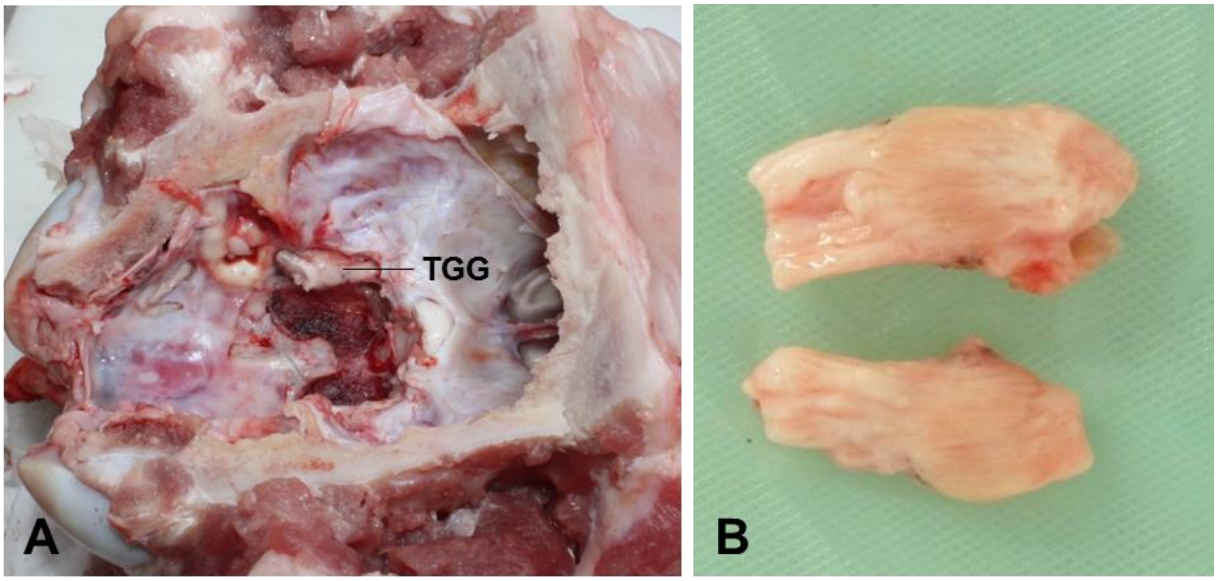


Fig. S163. A*: Dorsal aspect of the skull after removal of the brain and the pituitary gland. The left trigeminal ganglion (TGG) is indicated.* ***B****: Isolated trigeminal ganglia.*

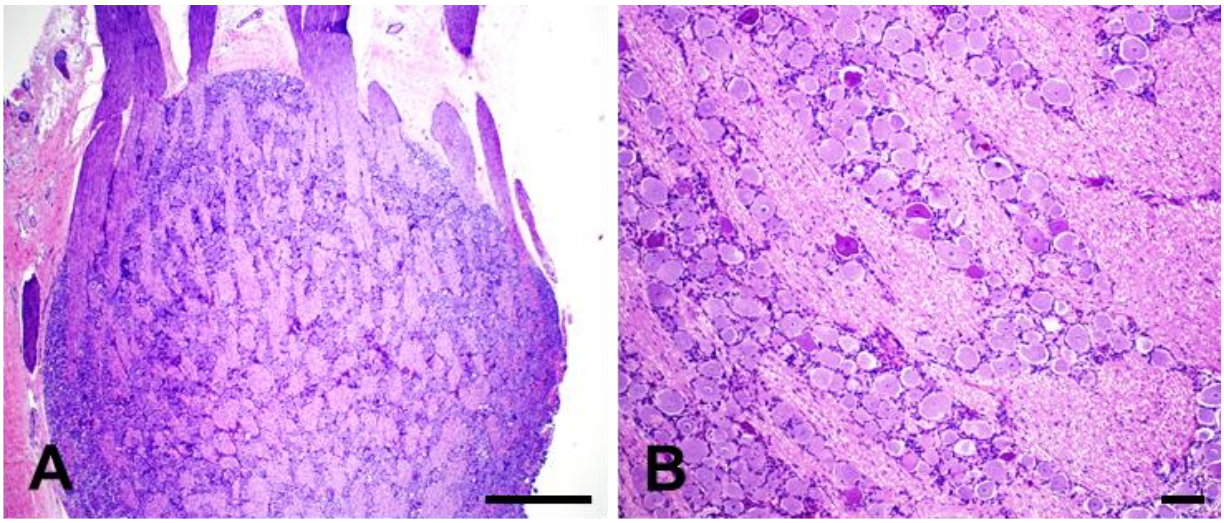


***Fig. S164. Histology of the trigeminal ganglion. A**: Longitudinal section. Scale bar = 1 mm. **B**: Detailed enlargement. Scale bar = 100 µm. FF-PE tissue. HE.*

Related literature
(Botti et al., 2011, Hart and Terenghi, 2004)

2.8.2. Peripheral nervous system

Relevant anatomical features/preparation
After removal of the abdominal and thoracic organs, samples of peripheral nerves are taken. This guide provides protocols for sampling the nerves of the brachial plexus (radial nerve, ulnar nerve), lumbosacral plexus (sciatic nerve, common fibular nerve and tibial nerve), and sympathetic trunk. For the nerve preparation, the overlying skeletal muscles are removed (Fig. S165). Nerve samples destined for histopathological examination or nerve fiber teasing are carefully excised with adjacent perineural tissue to avoid mechanical damage. The samples are then placed in embedding cassettes or attached to appropriately labeled index cards indicating the nerve, body side, and proximal and/or distal end of the sample. Next, the samples are transferred to a fixation solution. Nerve samples for molecular analyses need to be "cleaned" from the adjacent connective tissue before further processing, which can be accomplished using a photomicroscope. However, this procedure can be challenging, especially when samples of tiny nerve structures, such as the sympathetic trunk, are examined.
The **radial nerve and the ulnar nerves** are best accessed from the medial side of the forelimb after its removal from the trunk. Fig. S165 shows the relevant anatomical landmarks.
The **sciatic nerve, the tibial nerve, and the common fibular nerve** can be accessed from the lateral or medial side of the hindlimb before it is removed from the trunk. The sciatic nerve and its main branches are located under the hip muscles proximal to the tuber coxae and directly laterally adjacent to the sacrotuberous ligament between the sacral and the iliac bone. The relevant anatomical landmarks are shown in Fig. S165.
The **sympathetic trunk** can be easily accessed after evisceration of the thoracic cavity. The sympathetic trunk is located in the dorsal portion of the thoracic cavity and runs parallel to the thoracic aorta. The relevant anatomical landmarks are shown in Fig. S166.

General examination parameters

The peripheral nerves are examined for individual anatomical course variations and pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology and molecular analyses are taken from the altered site(s) if required.

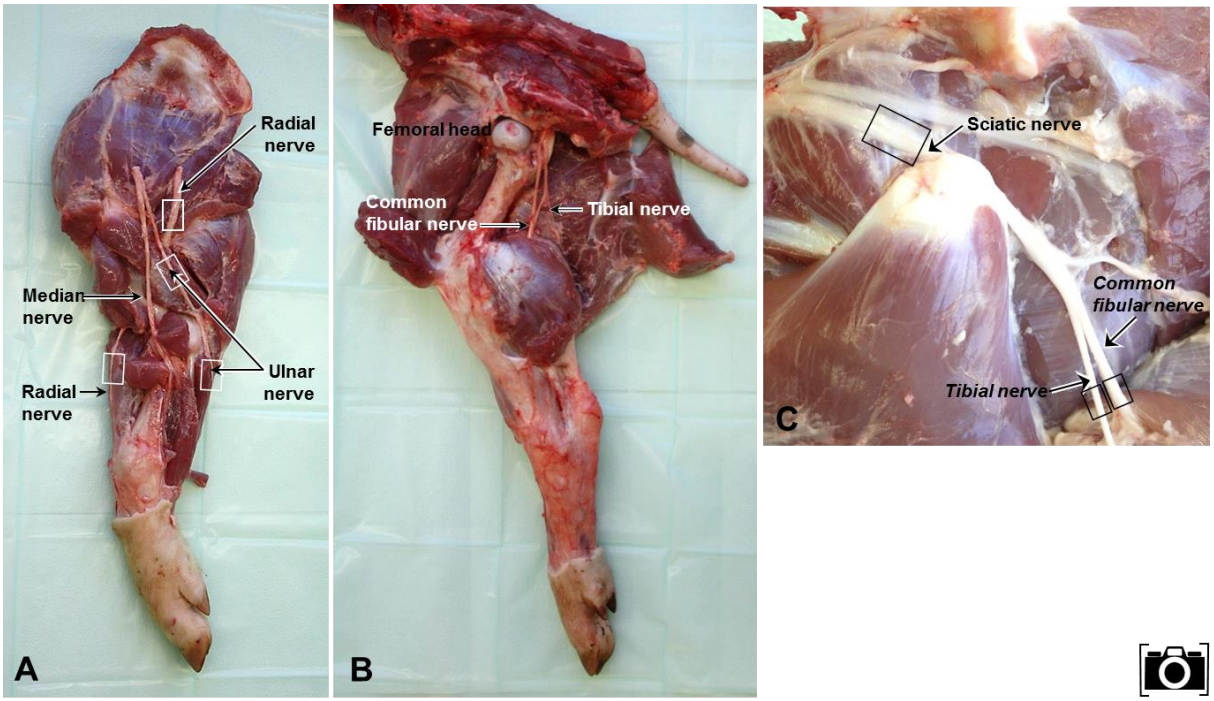


***Fig. S165. A**: Right forelimb, medial aspect. Brachial plexus with the radial nerve and ulnar nerve. **B**: Right hindlimb, medial aspect. Lumbosacral plexus with the sciatic nerve, common fibular nerve, and tibial nerve. **C**: Left hip, lateral aspect. The sampling locations are indicated by rectangles. In Type-I studies, only samples of the sciatic nerves are collected.*

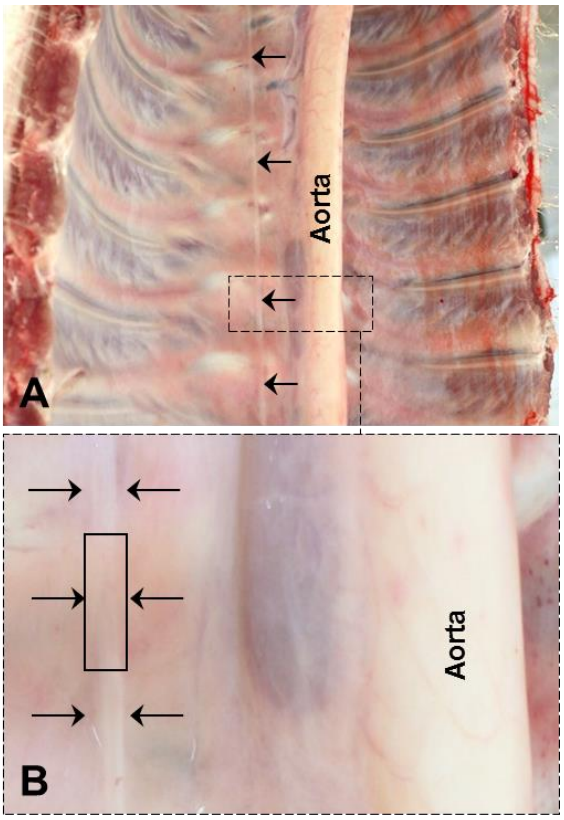


***Fig. S166.** Anatomical location of the sympathetic trunk in the thoracic cavity. **A**: Overview. The sympathetic trunk is marked by arrows. **B**: Detailed enlargement. The sympathetic trunk is marked by arrows. The sampling position for Type-II and Type-III studies is marked by a black rectangle.*

2.8.2.1. Peripheral nerve samples in Type-I studies

In Type-I studies, samples of both sciatic nerves (right and left side) are collected for molecular analyses and histopathological examination. The sampling of additional peripheral nerves and the sympathetic trunk and its ganglia is recommended for only Type-II and Type-III studies.

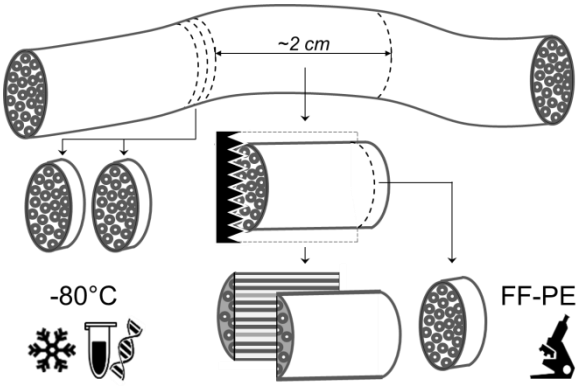


Fig. S167. *A schematic illustration of peripheral nerve sampling in Type-I studies.*

Samples for molecular analyses

Location:	Proximal end of both (right and left) sciatic nerves (Fig. S165 and Fig. S167).
Number of samples:	Two per side.
Remarks:	Prior to freezing, the nerve samples are “cleaned” from adjacent connective tissue using a photomicroscope.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Proximal end of both (right and left) sciatic nerves near the location of the samples for molecular analyses. One cross section and one longitudinal section are taken from each side (Fig. S165 and Fig. S167).
Fixation/Embedding:	FF-PE (Fig. S168).

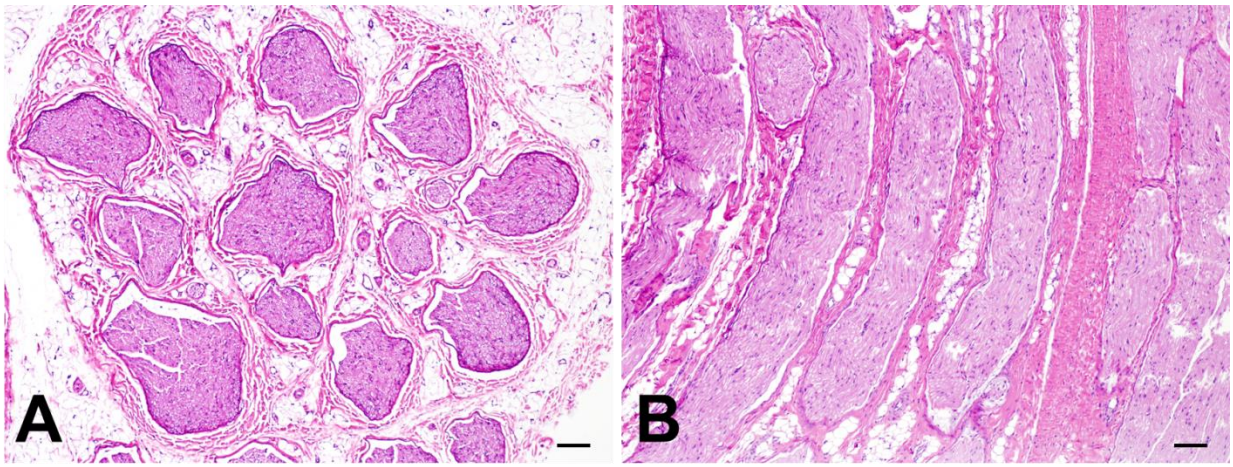


Fig. S168. Histology of the sciatic nerve. ***A****: Cross section.* ***B****: Longitudinal section. FF-PE. HE. Scale bars = 100 µm.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

The Working Group on Nervous System Sampling of the Society of Toxicologic Pathology (Bolon et al., 2013b) and the revised guides for organ sampling and trimming in rats and mice (Morawietz et al., 2004) recommend the bilateral sampling of the sciatic nerve for histopathological examination of the peripheral nerve system in rodent and non-rodent species in nonclinical general toxicity studies. For paraffin histology, cross and longitudinal sections are prepared from formalin fixed tissue samples. The Technical Guide for Nervous System Sampling of the cynomolgus monkey for General Toxicity Studies recommends the generation of cross and longitudinal sections of the sciatic nerve of one leg, or both legs if a local injection site trauma to the nerve is suspected. The nerve samples are taken at the mid-thigh level (Pardo et al., 2012).

The Type-I-study sampling protocols provided for porcine models recommend the generation of comparable sciatic nerve samples to allow for an adequate evaluation of porcine peripheral nerves in routine studies.

2.8.2.2. Peripheral nerve samples in Type-II and Type-III studies

General sampling strategy

In Type-II and Type-III studies, the sciatic nerves, additional peripheral nerves, and the sympathetic trunk are sampled at defined positions. A systematic random sampling procedure is not recommended. The sampling positions of the sciatic nerve, the radial nerve, ulnar nerve, common fibular nerve, and tibial nerve are shown in Fig. S165. The nerves from both sides (right and left) are sampled. Fig. S169 illustrates the subdivision of an excised peripheral nerve sample for downstream analyses (Table S24). The sympathetic trunk, including the sympathetic ganglia, is sampled at the approximate level of the 8th to the 10th rib as shown in Fig. S166. Only the left side is sampled. An approximately 2 cm long piece of the sympathetic trunk is fixed in a 4% formaldehyde solution and embedded in paraffin for histopathological analysis (Fig. S170). If samples for molecular analyses are required, 3-6 sympathetic ganglia are “cleaned” from the adjacent connective tissue under a photomicroscope before freezing at -80°C.

Table S24. The type and number of peripheral nerve samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

	Number of sampled locations/ number of samples per location	
Sample type	**Type-II studies**	**Type-III studies**
Number of sampled locations	*3 from right **and** from left side*	
	Number of specimens	
FF-PE*	1 per sampled location	1 per sampled location
CRYO*	1 per sampled location	1 per sampled location
GA-EE* or GA-NFT**	2 per sampled location	2 per sampled location
Molecular analyses (-80°C)	2 per sampled location	4 per sampled location

**Cross and longitudinal sections; **NFT: Nerve fiber teasing (if necessary).*

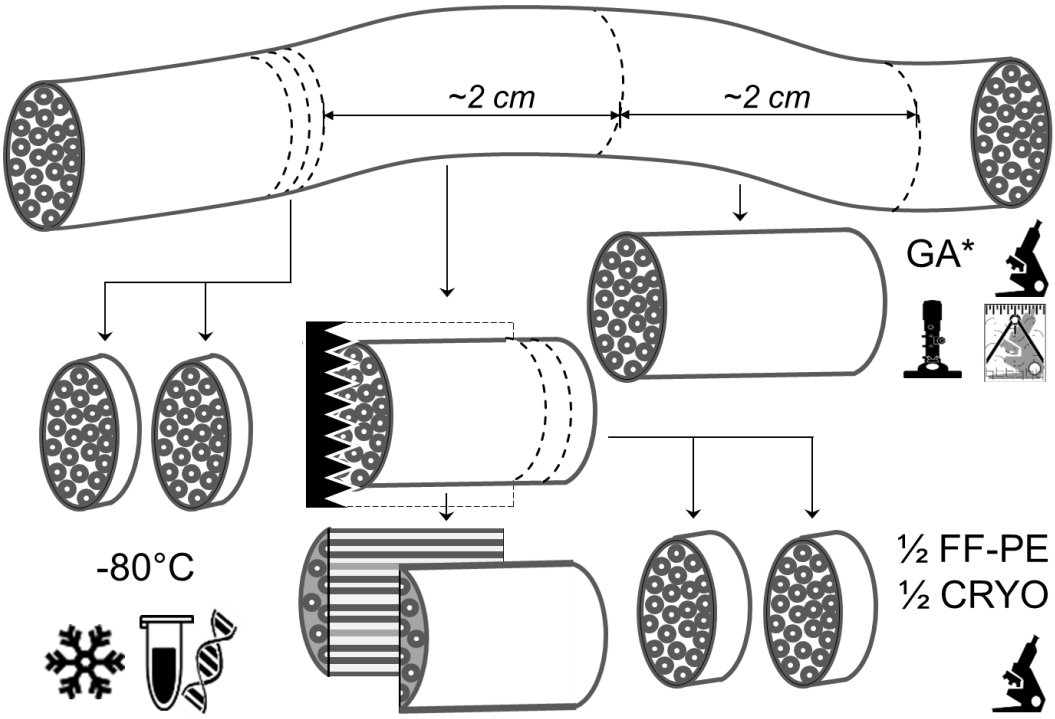


Fig. S169. *A schematic illustration of subsample preparation from an excised piece of nerve for downstream analyses in Type-II/III studies. Glutaraldehyde-fixed samples are used for the preparation of nerve fiber teasings or embedded in Epon for the preparation of IUR-sections (ORIENTATOR sections, or ORTRIPS) (Drogemuller et al., 2010).*

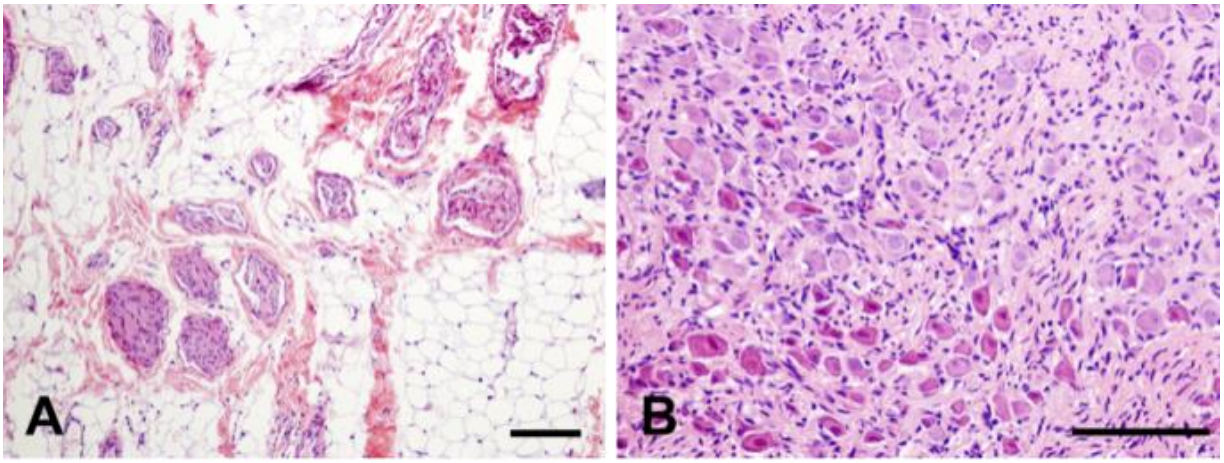


Fig. S170. Histology of the sympathetic trunk *(**A**, cross section) and a symphathetic trunk ganglion (**B**). FF-PE. HE. Scale bars = 1 mm (A), 100 µm (B, C).*

Time and personnel requirements

At least 40 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time needed for sample collection and sample processing until freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Bolon et al., 2013a, Bolon et al., 2013b, Botti et al., 2011, Drogemuller et al., 2010, Kristiansen and Nyengaard, 2012, Morawietz et al., 2004, Pardo et al., 2012, Zachary, 2012)

2.9. Thoracic cavity

2.9.1. Lungs and trachea

Relevant anatomical features/preparation

The organs of the oral cavity, neck and thoracic cavity are removed from the body together. To access the tongue, pharynx, and larynx, two incisions are made ventrally, following the medial sides of the right and the left ramus of the mandible from the throat to the chin. The thoracic cavity is opened by two paramedian incisions to the left and right of the sternum. After visual inspection of the thoracic cavity in situ, the pluck (heart, lungs, thymus, mediastinum), trachea, esophagus, larynx and tongue are removed from the body using a knife and scissors. After examination of the heart (section 2.9.2. and removal of the thoracic portion of the vagus nerve (in Type-II/III studies, section 2.8.1.4.3.), the lungs and trachea are isolated from the remaining organs as shown in Fig. S171 and Fig. S172.

General examination parameters

The size (longitudinal length) of both lungs is determined. Optionally, both lungs are weighed. The lungs are inspected for pathological alterations, particularly inflammatory lesions of the pleura, apical lobes, and ventral aspects of the middle and main lobes. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, mycology, and molecular analyses are taken from the altered site(s) if required.

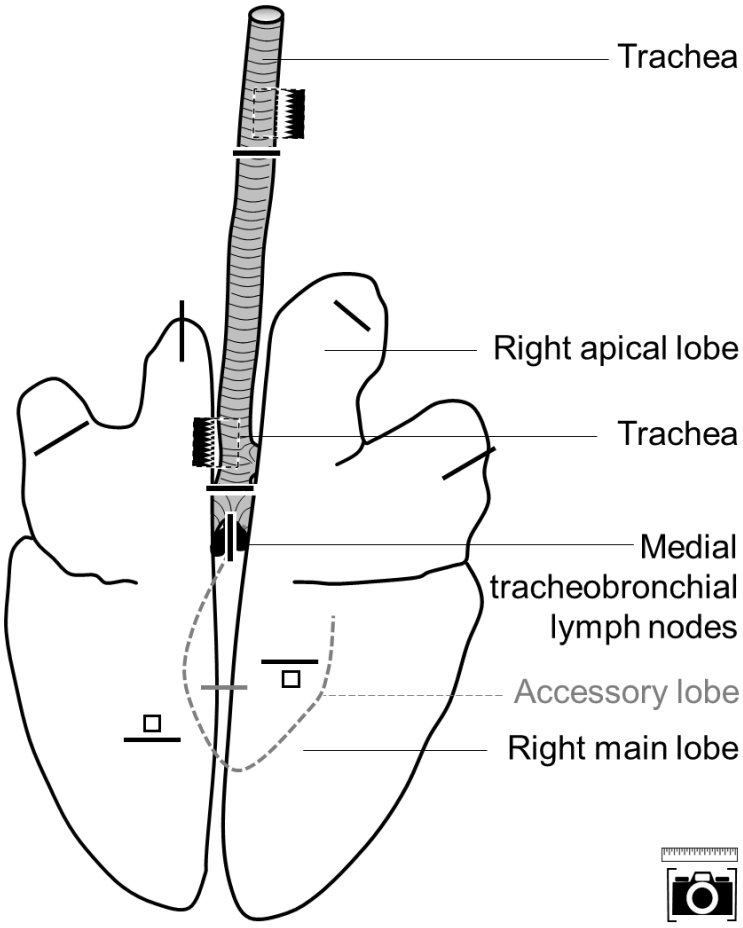


Fig. S171. *A schematic illustration of the lungs and trachea. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*

2.9.1.1. Lung and trachea samples in Type-I studies

Samples for molecular analyses

Location:	Two samples of tracheal mucosa are taken from the proximal cervical portion of the trachea and from the thoracic portion of the trachea as indicated in Fig. S171. Lung tissue samples are taken from the right and left main lobe as indicated in Fig. S171 and Fig. S172. If the left lung is fixed by airway-instillation of a fixation solution, a sample for molecular analyses is not obtained.
Number of samples:	Two lung samples and two samples of tracheal mucosa.
Remarks:	Homogeneous samples are cut from the lung parenchyma while avoiding the sampling of a nonhomogenous composition of different lung tissue compartments, such as bronchi, vessels, and parenchyma.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

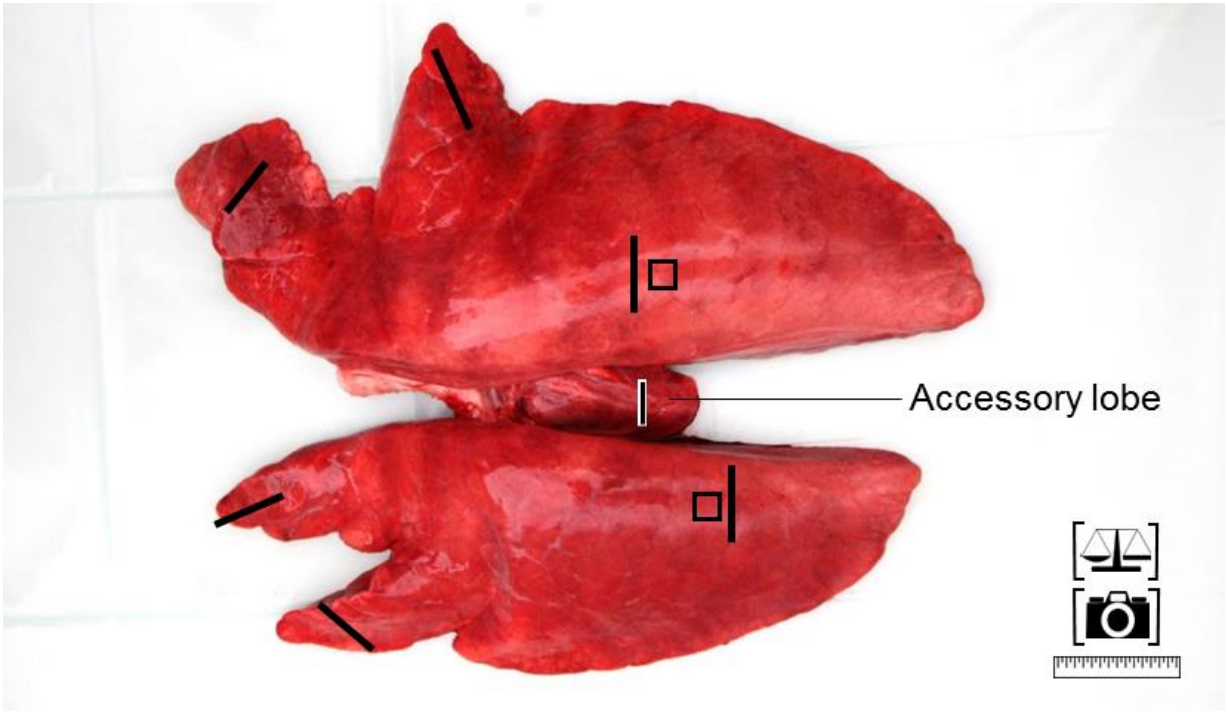


Fig. S172. *Dorsal view of the (fresh) lungs. The trachea has been cut off proximal to the bifurcation. If appropriate, both lungs (right and left) are weighed separately. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*

Histology samples

For a detailed assessment of lung morphology, which is required for inhalation toxicity studies, samples of lung tissue fixed by instillation of a fixation solution via the main bronchi under controlled pressure can be prepared (Fig. S174). If needed for Type-I studies, airway-instillation of a fixation solution is performed on the left lung as described in detail for Type-II and -III studies below (section 2.9.1.2).

Location, number and orientation of sections:

One cross section and one longitudinal horizontal section is taken from the proximal cervical and distal portion of the trachea as indicated in Fig. S171. Lung samples for histopathology are taken from the right and left main lobe (cross sections), right accessory lobe (cross section), and apical lobes (cross and longitudinal sections). The sections should contain pleura, lung parenchyma, vessels and bronchi (Fig. S173). One longitudinal section through the medial tracheobronchial lymph nodes (Fig. S173) is prepared. All sampling locations and section orientations are indicated in Fig. S171 and Fig. S172.

Fixation/Embedding: FF-PE (Fig. S173).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of different lung sections based on the drug administration route. In oral studies, instillation is strongly recommended. One longitudinal horizontal section of the left lobe is recommended. Additionally, transverse sections from the right cranial and caudal lobes may be taken. In inhalation studies, instillation is obligatory, and histology samples are taken from all lung lobes in longitudinal and transverse sectional orientations. Additionally, the preparation of one longitudinal and horizontal section of the trachea, including the bifurcation, is recommended (Kittel et al., 2004).

Compared to the total size of the porcine lung, the Type-I-study sampling plan recommends the generation of fewer samples than the recommendations for routine toxicity studies in rodents.

Nevertheless, the Type-I-study sampling protocols recommend the sampling of all lung lobes in different sectional orientations to allow for an adequate evaluation of the porcine lung and trachea in routine studies. In inhalation toxicity studies, the fixation of one lung by airway-instillation of a fixation solution is necessary, and additional samples may be collected from the fixed lung half.

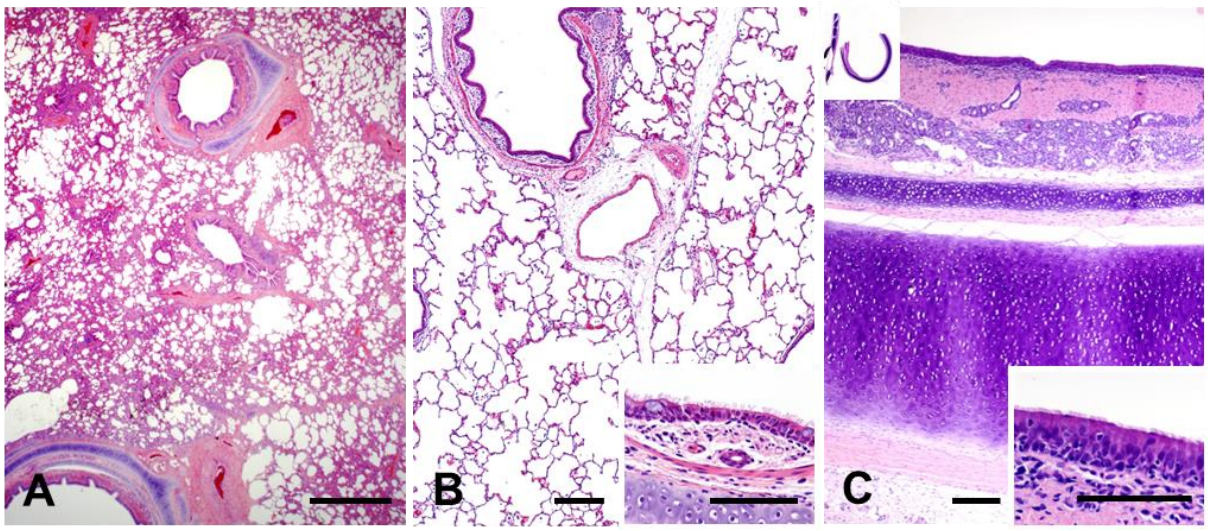


***Fig. S173. A**, **B**: **Lung histology.** The lung tissue sample in **A** was fixed by immersion after removal from the organ. Six-month-old pig, lung tissue sample from the apical lobe. FF-PE. HE. Scale bar = 1 mm. The lung tissue sample in **B** was fixed by airway-instillation of a fixation fluid (4% neutrally buffered formaldehyde solution). Three-month-old pig, lung tissue sample from the apical lobe. Inset: Bronchiolar epithelium. FF-PE. HE. Scale bars = 100 µm. **C**: **Trachea histology.** Top left: Photo of the histological slide. Bottom inset: Detailed enlargement of the tracheal mucosa with ciliated epithelium. FF-PE. HE. Scale bars = 1 mm, 100 µm (inset).*

2.9.1.2. Lung samples in Type-II and Type-III studies

In Type-II and -III studies, the right or left lung is randomly selected and fixed by instillation of a fixation solution via the main bronchi under controlled pressure, which will prevent the collapse of the tissue during the subsequent processing steps, provide high quality sections for the optimal assessment of lung morphology and allow for quantitative stereological analyses (Braber et al., 2010, Hyde et al., 2007, Weibel et al., 2007). In rodents, buffered glutaraldehyde is recommended for the fixation of the lungs for quantitative stereological analyses (Weibel et al., 2007). In this guide, a 4% neutrally buffered formaldehyde solution is used to allow for immunohistochemical analyses of the fixed lung tissue specimen. Advanced sampling protocols for the trachea are not recommended in these sampling guidelines.

Airway-instillation of a fixation solution

For instillation of a fixation solution, the same equipment for the vascular perfusion of the kidney (section 2.10.6., Kidney) is used (Fig. S174 and Fig. S175). The container/bottle with the fixation solution (4% neutrally buffered formaldehyde solution) is hung approximately 40-50 cm above the level of the lung. The randomly selected lung (right or left) is removed from the trachea, weighed and measured (length). The fixation solution is then instilled into the lung by inserting the end of the tube or a blunted straight irrigation cannula connected to the container with the fixation solution into the main bronchus(i). If the right lung is fixed, the fixation solution has to be instilled into the main bronchus and accessory cranial bronchus. The tube/irrigation cannula is carefully pushed forward into all accessible branches of the bronchus to instill the fixation solution into the entire lung parenchyma. A ligature is tied around the bronchial stump and the tube while the fixation solution is flowing, and the lung is placed into a bucket with fixation solution while maintaining the instillation pressure for at least four hours (Fig. S175). The tube and the ligature are then removed and the tissue is post-fixed by immersion for another 12-24 hours at room temperature.

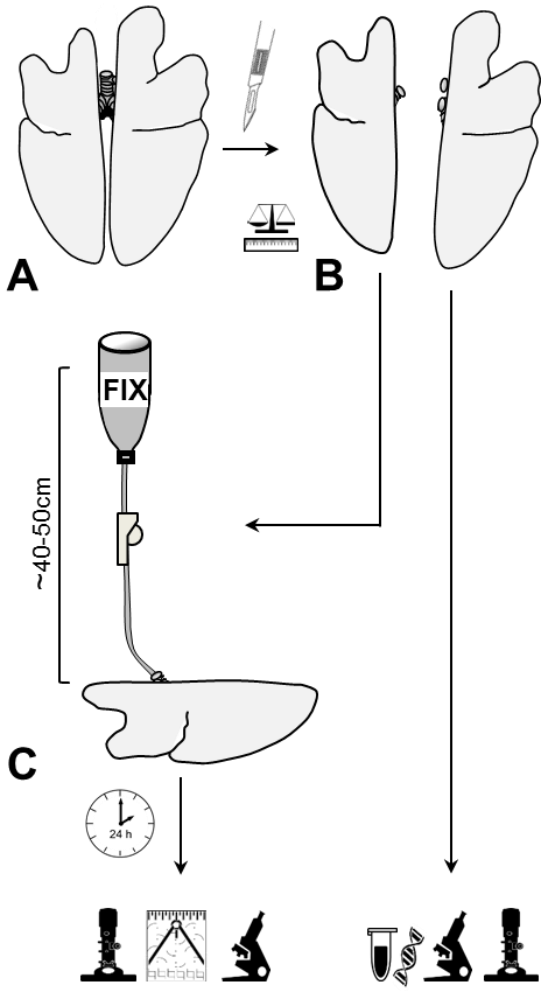


***Fig. S174. A schematic illustration of lung processing in Type-II and Type-III studies. A**, **B**: The right and left lung are separated, weighed and measured. **C**: One lung is fixed by the airway-instillation of a fixation solution and post-fixed by immersion. Next, the samples for qualitative and quantitative histopathological analyses are generated. Samples for molecular analyses are generated from the other (fresh) lung.*

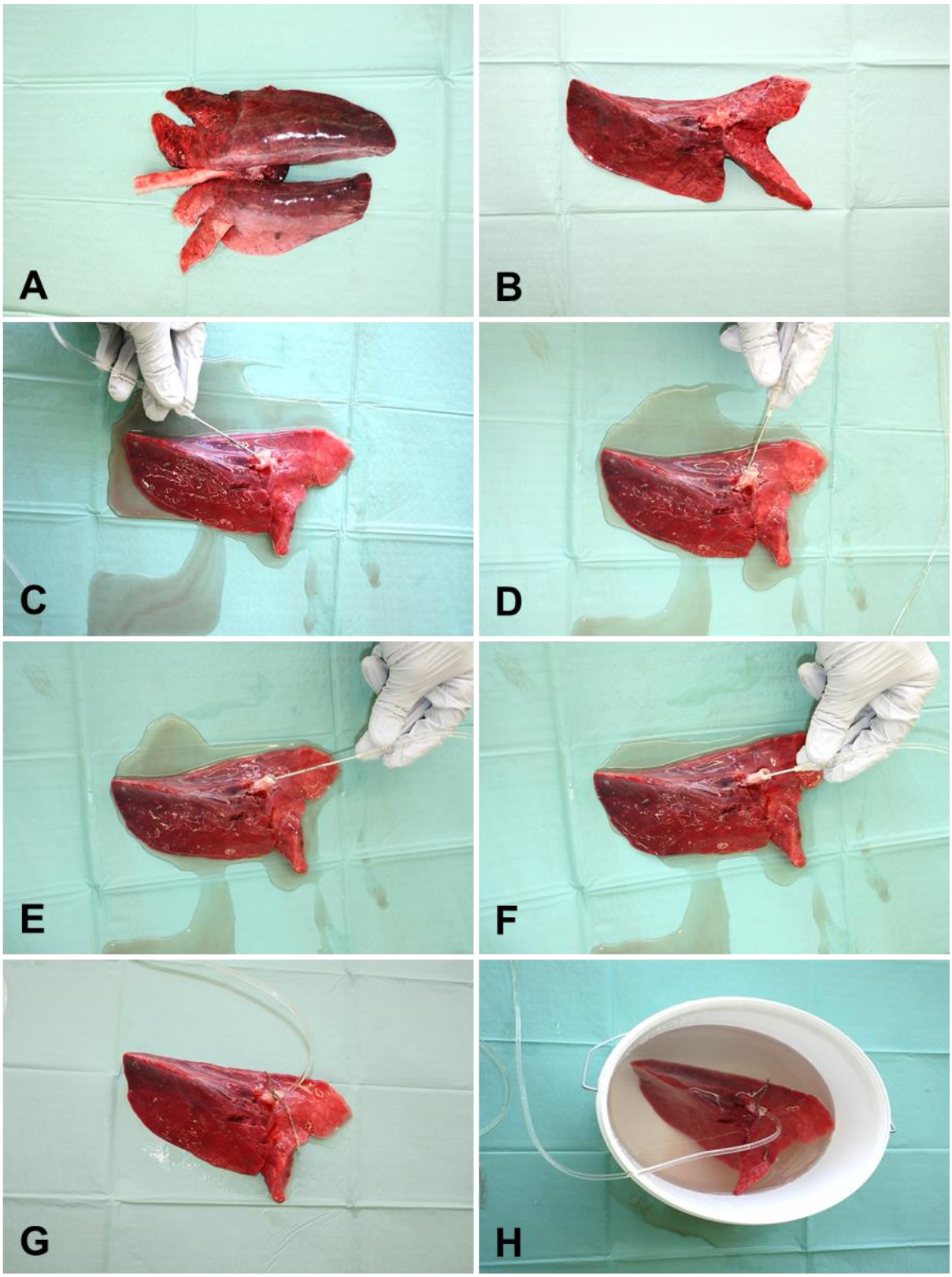


Fig. S175. Airway-instillation of a fixation solution. ***A****: Freshly dissected lungs from a three-month-old pig with euthanasia-related lung edema.* ***B****: Isolated left lung.* ***C****-****F****: A neutrally buffered 4% formaldehyde solution is instilled into the main bronchial branches of the apical, middle and caudal lobe via a blunted irrigation cannula.* ***G****: A tight ligature is tied around the bronchial stump and the tube while the fixation solution is flowing.* ***H****: The lung is placed into a bucket with fixation solution while maintaining the instillation pressure for at least four hours. Next, the tissue is post-fixed by immersion for another 12-24 hours at room temperature.*

General systematic random sampling strategy

The sampling positions are determined by systematic random sampling in the fresh lung and the lung fixed by airway-instillation of a fixation solution. The weights and the lengths of both lungs are determined. Each lung is cut into parallel equidistant slabs into 10-20 mm thick sections orthogonal to the longitudinal axis of the organ. The first section is randomly positioned between 0 and 20 mm from the margin of the organ (Fig. S176 and Fig. S177). The tissue slabs are counted and placed on the same side on plastic transparencies. For Cavalieri volumetry of the fixed lung (section 1.5.), the outline of every slab is traced with a waterproof pen on the transparency (Fig. S176 and Fig. S177). Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-4 cm, depending on the organ size and number and area of slabs). The systematic random sampling of eight (in Type-II studies) or twelve (in Type-III studies) tissue locations is performed as described in section 1.4. and indicated in Fig. S178. The sampled locations can be marked by placing pieces of confetti paper on the respective positions. An approximately 1.5x1.5x2-cm tissue piece is excised from each of the sampled locations regardless of the tissue structures present. If the sampled location contains vascular structures or bronchi, these structures must be excised together with the rest of the specimen to maintain representativity. The excised tissue specimens are subdivided into slabs for downstream analyses (Fig. S179 and Fig. S180, Table S25 and Table S26).

Fig. S176. A*: Serially sectioned fixed lung for systematic random sampling.* ***B****,* ***C****: For Cavalieri volumetry, the outline of every slab is traced with a waterproof pen on a plastic transparency.*

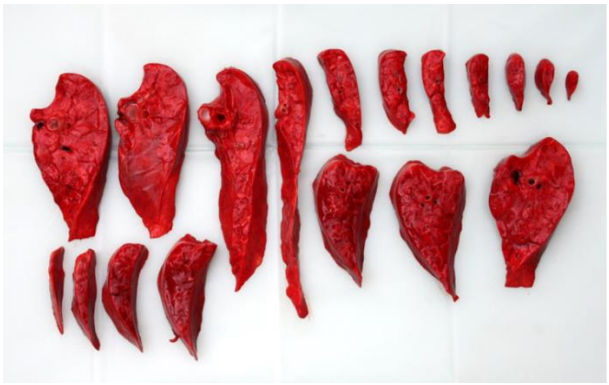


Fig. S177. *Serially sectioned fresh (unfixed) lung for systematic random sampling.*

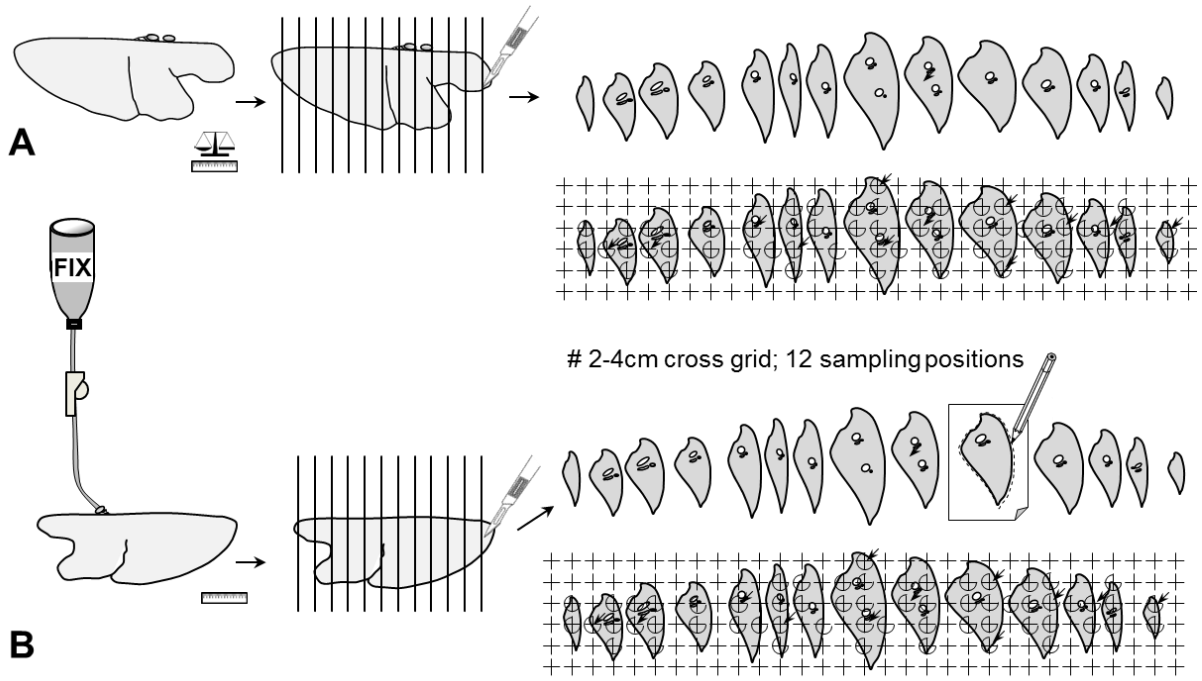


Fig. S178. *A schematic illustration of volumetry and systematic random sampling of the lungs in a Type-III study.* ***A****: The fresh (unfixed) and fixed lungs (****B****) are cut into parallel equidistant slabs with an approximate 10-20 mm thickness. For Cavalieri volumetry of the fixed lung, the outline of every slab is traced with a waterproof pen on a transparency. In each lung, 12 sampling locations are determined by systematic random sampling using a 2-4 cm cross grid.*

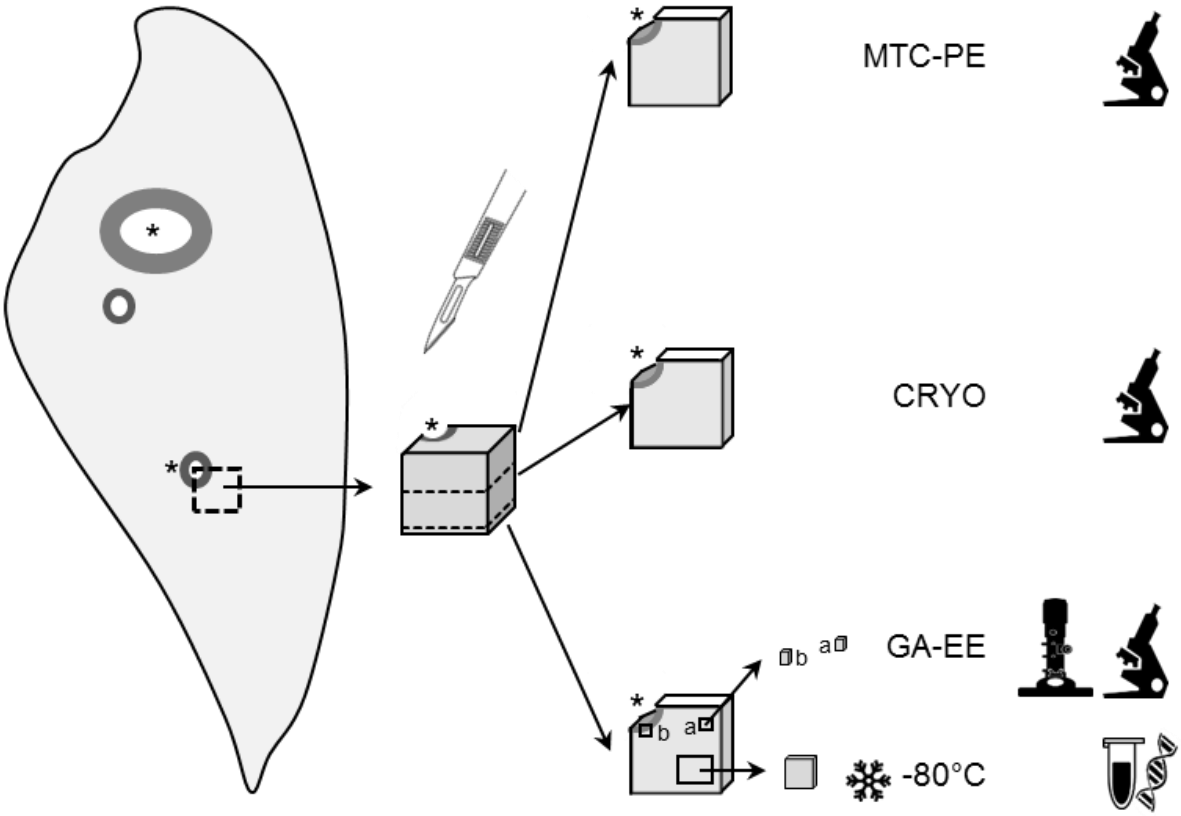


Fig. S179. *A schematic illustration of subsample preparation from one systematically randomly sampled location of* ***fresh lung tissue*** *for downstream analyses (Type-II/III studies). *Bronchus section. [a]GA-EE sample of lung parenchyma; [b]GA-EE sample of bronchus.*

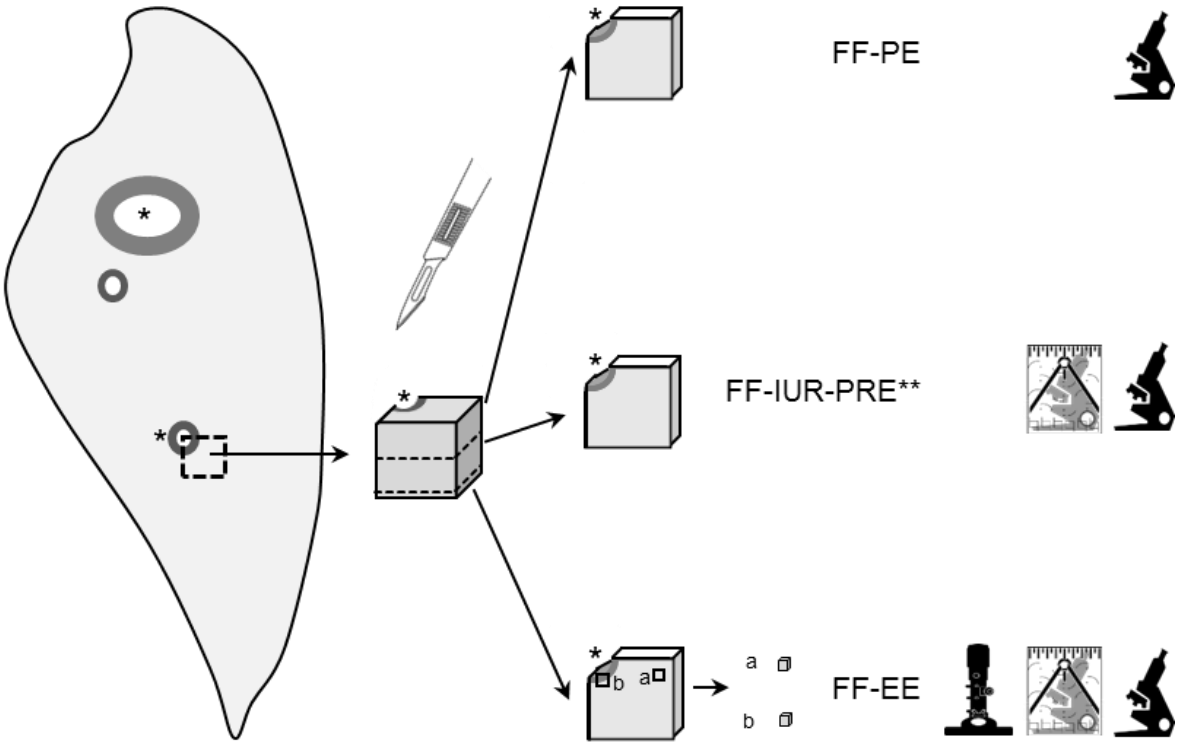


Fig. S180. *A schematic illustration of subsample preparation from one systematically randomly sampled location of* ***lung tissue fixed by airway-instillation of fixation fluid*** *for different downstream analyses (Type-II/III studies). *Bronchus section. [a]FF-EE sample of lung parenchyma; [b]FF-EE bronchial epithelium sample.**PR = GMA/MMA.*

Table S25. The type and number of lung samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

		Number of locations/samples	
Tissue	**Sample type**	**Type-II studies**	**Type-III studies**
Fresh lung tissue	**Sampled locations**	*8*	*12*
		Number of samples	
	MTC-PE	-	1 out of every 2nd location
	CRYO	1 per sampled location	1 per sampled location
	GA-EE	1 per sampled location	1 per sampled location
	Molecular analyses (-80°C)	1 per sampled location	1 per sampled location
Airway-instillation fixed lung tissue	**Sampled locations**	*8*	*12*
		Number of samples	
	FF-PE	1 per sampled location	1 per sampled location
	FF-EE	1 per sampled location	1 per sampled location
	FF-PRE (IUR)	1 per sampled location	1 per sampled location

Remarks

The subsamples for paraffin histology and PRE consist of ~5-mm-thick slabs taken from each of the systematically randomly sampled lung tissue samples (Fig. S179). An additional thinner ~2-mm-thick slab is taken from each of the systematically randomly sampled lung tissue samples (Fig. S179). From this slab, the subsamples for molecular analyses and/or Epon embedding (FA or GA fixation) are excised from areas of homogeneous lung parenchyma while avoiding sampling of specimens with heterogenous composition of different lung tissue compartments, such as bronchi, large vessels, and parenchyma.

For qualitative histological and ultrastructural examinations and molecular analyses of specific lung tissue structures, additional samples of bronchial tissue are taken from the systematically randomly sampled locations of fresh lung tissue (Fig. S179 and Table S26). If bronchus section profiles are present in more locations than needed for the acquisition of the recommended number of samples for GA-EE and molecular analyses, the tissue slabs are chosen by systematic random sampling. For example, six GA-EE bronchus samples are generated in a Type-III study. However, bronchial section profiles were present in 18 of the 20 systematically randomly sampled lung tissue locations. Thus, one bronchial GA-EE sample is taken from every third sampling location (18/6 = 3), and the first location is randomly determined between locations 1 and 3.

Table S26. Additional samples for molecular analyses and GA fixation and Epon embedding.

		Total number of samples	
Location	**Sample type**	**Type-II studies**	**Type-III studies**
Bronchial epithelium	**GA-EE**	*6*	*6*
	Molecular analyses (-80°C)	*6*	*6*

Time and personnel requirements

At least 35 minutes must be scheduled for the execution of the complete Type-II/III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Harkema et al., 2013, Hyde et al., 2007, Kittel et al., 2004, Knust et al., 2009, Nyengaard and Gundersen, 2006a, Ochs and Muhlfeld, 2013, Renne et al., 2009)

2.9.2. Heart

Relevant anatomical features/preparation

The organs of the oral cavity, neck and thoracic cavity are removed from the body together (section 2.9.1.). The pericardium is incised, opened, and inspected for pathological alterations. Next, the shape, surface, and color of the heart and the positions of the large vessels are visually evaluated. Using scissors, the heart is cut open as shown in Fig. S181-S183 to allow for examination and sampling of the atria, ventricles, heart valves and vessels (Bishop, 1999).

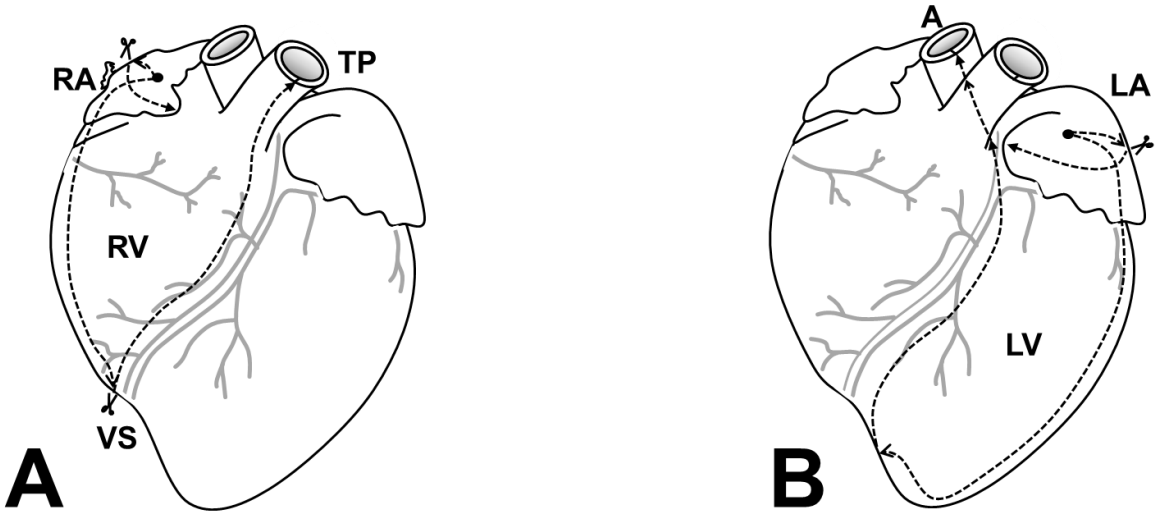


Fig. S181. *A schematic illustration of the cardiac incision lines.* ***A****: Right heart. The right atrium (RA) is cut open from the entry of the caval vein (•) to the apex of the auricle of the right atrium. Next, the incision is continued along the interatrial septum. The right atrioventricular orifice is opened, and the right ventricular (RV) myocardium is cut following the interventricular septum (VS) into the pulmonary trunk (TP). The right atrium, right ventricle, tricuspid valve and pulmonary valve are now accessible for further examination and sampling.* ***B****: Left heart. The left atrium (LA) is cut open from the entry of the pulmonary veins (•) to the apex of the auricle of the left atrium. Next, the incision is continued along the interatrial septum. The left atrioventricular orifice is opened, and the left ventricular (LV) myocardium is cut following the interventricular septum into the flow path of the aorta (A). The left atrium, left ventricle, bicuspid valve and aortic valve are now accessible for further examination and sampling.*

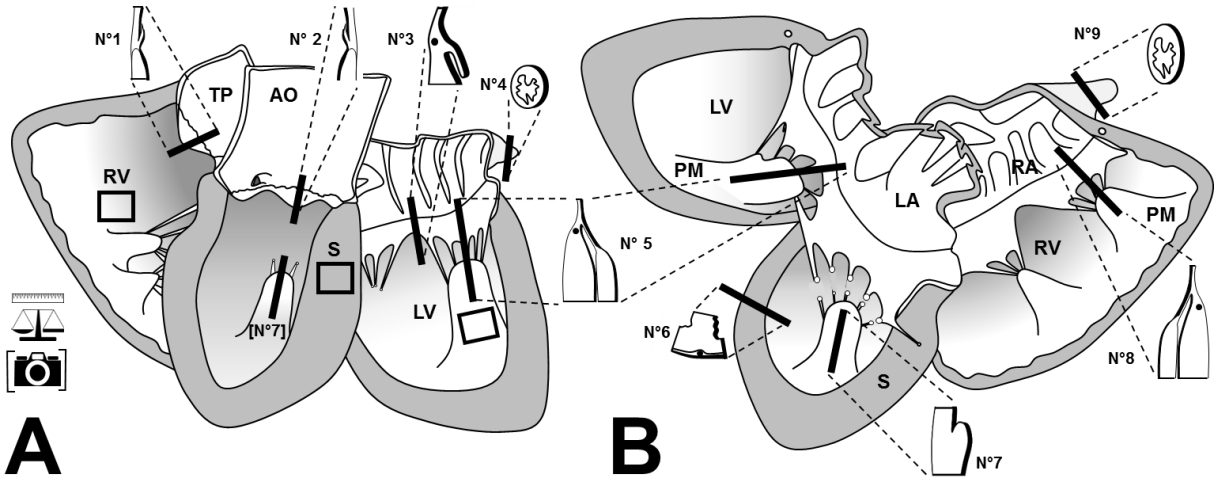
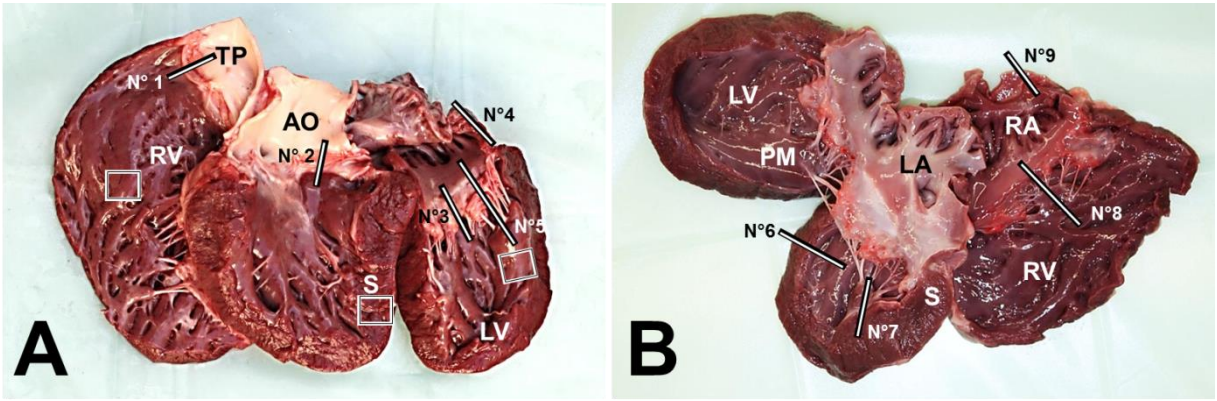


Fig. S182. (↑) and Fig. S183 (↓). A schematic illustration and images of the locations and orientations of sections for routine (Type-I study) histology and molecular analyses of the heart. A*: Opened heart with the left septal wall facing the observer. The sample locations for molecular analyses are indicated (□)* ***B****: Opened heart with the right septal wall facing the observer. Anatomical locations: Right atrium (RA), right ventricle (RV), interventricular septum (S), pulmonary trunk (TP), left atrium (LA), left ventricle (LV), aorta (A), and papillary muscle (PM). The section orientations are indicated by black/white lines and marked by numbers 1-10. In the schematic illustration, the profiles of the corresponding sections are indicated. Samples of an approximate 5 mm thickness are cut from the tissue.* ***N° 1****: Right ventricular myocardium, tricuspid valve and atrium;* ***N° 2****: Pulmonary valve, right ventricular outflow tract and pulmonary trunk (TP);* ***N° 3****: Left ventricular myocardium, bicuspid valve and atrium;* ***N° 4****: Auricle of the left atrium;* ***N° 5****: Left papillary muscle, left atrioventricular valve and atrium;* ***N° 6****: Interventricular septum;* ***N° 7****: Left ventricular septal wall and papillary muscle;* ***N° 8****: Right papillary muscle, tricuspid valve and atrium;* ***N° 9****: Auricle of the right atrium.*



General examination parameters

The weight of the (empty) heart is determined. The heart is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, mycology, and molecular analyses are taken from the altered site(s) if required.

2.9.2.1. Heart samples in Type-I studies

In addition to the heart samples described above and below, a section of the pericardium from the left ventricular region is prepared for histology (not shown). A pericardial sample for molecular analyses is not recommended in Type-I-III studies.

Samples for molecular analyses

Location:	Septal, right and left ventricular myocardium. Locations are indicated in Fig. S182 and Fig. S183.
Number of samples:	Three.
Remarks:	Homogeneous samples of the myocardium are collected while avoiding samples with a nonhomogenous composition of myo-, epi-, and endocardium and cardiac vessels.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Sampling locations (1-10) and section orientations are indicated in Fig. S182 and Fig. S183.
Fixation/Embedding:	FF-PE (Fig. S184). The heart is fixed after obtaining the samples for molecular analyses. The histology samples are taken from the fixed tissue.

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one longitudinal section through the ventricles and atria (Morawietz et al., 2004). Considering the size of the porcine heart and the necessity to incorporate the collection of samples for molecular analyses into the Type-I-study sampling protocol, a comparable sampling technique is not applicable in pigs. Thus, more samples need to be examined for a thorough investigation of all relevant heart structures. The sections recommended in Type-I-study sampling protocols were designed to allow for an adequate evaluation of all relevant structures of the porcine heart in routine studies.

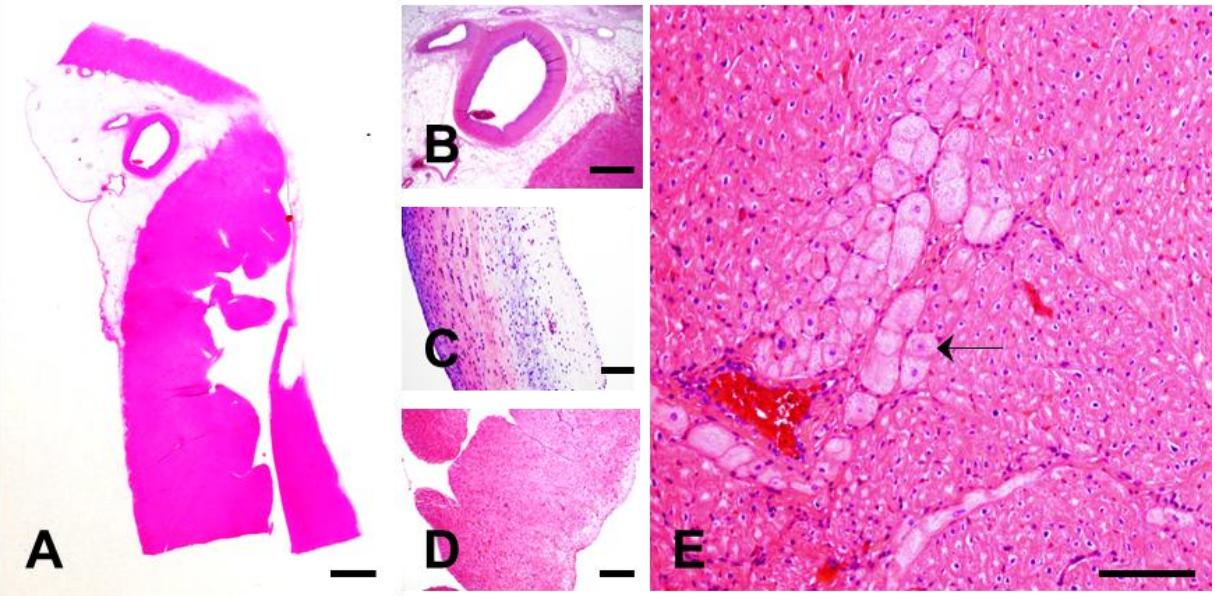


***Fig. S184 (↑). Heart histology**. **A**: Histological slide from sample location N° 5. The sample includes the left coronary artery, left papillary muscle, tricuspid valve and left atrium. **B**: Left coronary artery (location N° 5). **C**: Bicuspid valve (location N° 5). **D**: Auricle of the right atrium (location N° 9). **E**: Myocardium of the interventricular septum (location N° 6). The arrow indicates the Purkinje fibers (internal excitation conduction system of the heart). FF-PE. HE. Scale bars = 1 mm (A, B), 100 µm (C-E).*

2.9.2.2. Heart samples in Type-II and Type-III studies

General systematic random sampling strategy

The heart is cut open as described above and macroscopically examined. Next, blood clots are removed from the ventricles and atria, and the weight of the complete heart is determined. The thicknesses of the interventricular septum and of the right and left ventricular wall may be determined, although these determinations may vary greatly depending on the state of contraction of the heart (Berridge et al., 2013). The right ventricular and atrial free wall are removed from the remaining heart tissue by dissection along the interatrial and interventricular septum. Next, the standard histology samples (N° 1-9, in Fig. S185) are obtained because the standard locations are required for a meaningful qualitative histopathological evaluation. The samples are fixed in FA for routine paraffin histology (FF-PE). The atria are dissected from the ventricles, and the interventricular septum is removed from the left ventricle. The interatrial septum is left attached to the left atrium (Fig. S186). The atria, ventricles and septum are weighed separately. The inaccuracy related to the previous sampling of the standard histology samples is accepted as uncorrectable. Systematic random sampling for determination of sampling positions and organ size-dependent sample numbers for additional specimen collection is performed separately for the atria, ventricles, and interventricular septum (Fig. S188). For systematic random sampling, the tissue is **not** sliced into parallel slabs as described for many other organs in this guide. The thickness of the myocardium does not largely differ in the same

ventricle or atrium, and the samples are excised as transmural full thickness specimens that extend from the endocardial surface through the myocardium to the epicardium.
Therefore, a volume weighted sampling can be achieved by overlaying the different parts of the heart with a cross grid printed on a transparency (grid size: 1-2 cm, depending on the organ size). The systematic random sampling of six tissue locations from the right and left atrium, interventricular septum, and the right and left ventricle is shown in Fig. S188. The sampled positions can be marked by placing pieces of confetti paper on the respective positions. An approximately 2x1x2-cm tissue piece is excised from each of the sampled locations (full thickness). Maintaining their transmural orientation, the excised tissue specimens are subdivided into slabs for downstream analyses (Fig. S188). Samples from the heart valves are not sampled systematically (Table S27 and Fig. S187). Next, the coronary vessel samples are obtained (Fig. S34 in section 2.4.).

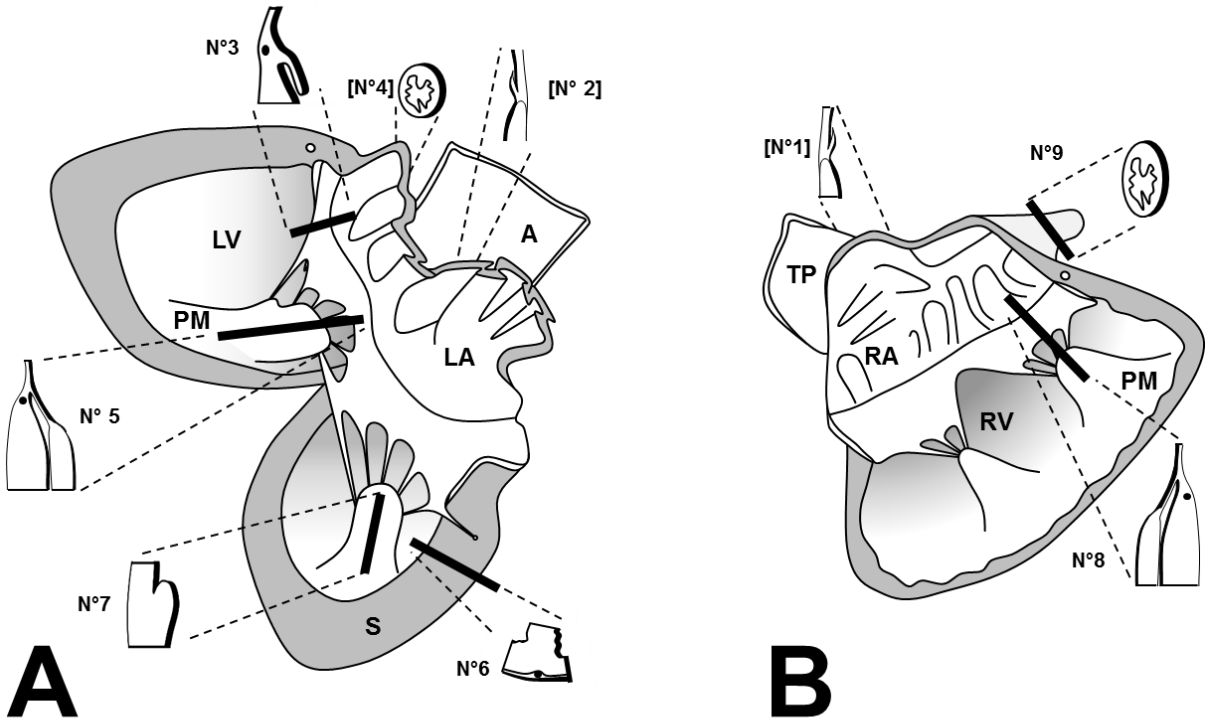


Fig. S185. Heart sampling in Type-II/III studies. First step*: After weighing the entire heart, the left ventricle, atrium and septum (**A**) are separated from the right ventricle and atrium by dissection along the interatrial and interventricular septum. Next, standard histology samples (N° 1-9) are taken and routinely processed for FF-PE histology.*

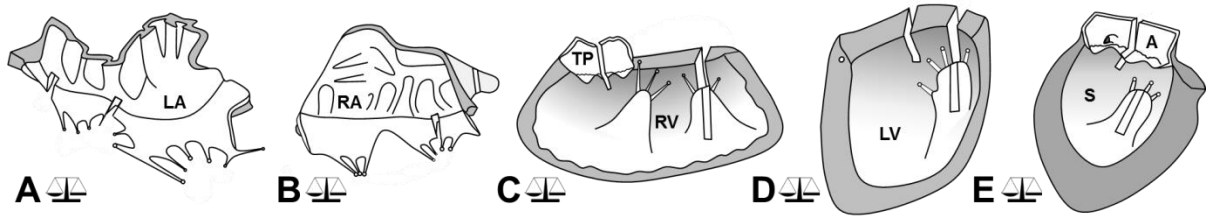


Fig. S186. Heart sampling in Type-II/III studies. Second step: *The atria (LA: Left atrium (**A**); RA: Right atrium (**B**)) are dissected from the ventricles (RV: Right ventricle (**C**), LV: Left ventricle (**D**)), and the interventricular septum (S in **E**) is removed from the left ventricle. The interatrial septum is left attached to the left atrium. Next, the weight of the atria, ventricles and septum is determined. TP: Pulmonary trunk. A: Aorta.*

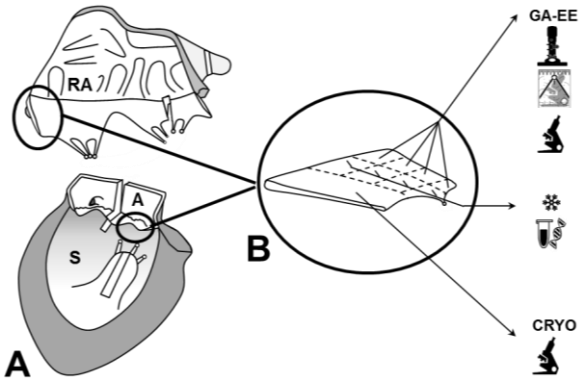


Fig. S187. Heart sampling in Type-II/III studies. Third step: Sampling of heart valves.
A*: A schematic illustration of subsample preparation from the tricuspid and aortic valves. RA: Right atrium; A: Aorta; S: Interventricular septum. From the middle of one cusp of each valve, 1-2 approximately 1 cm broad pieces are excised.* ***B****: Each excised sample is used for GA-EE, cryo-histology or molecular analyses.*

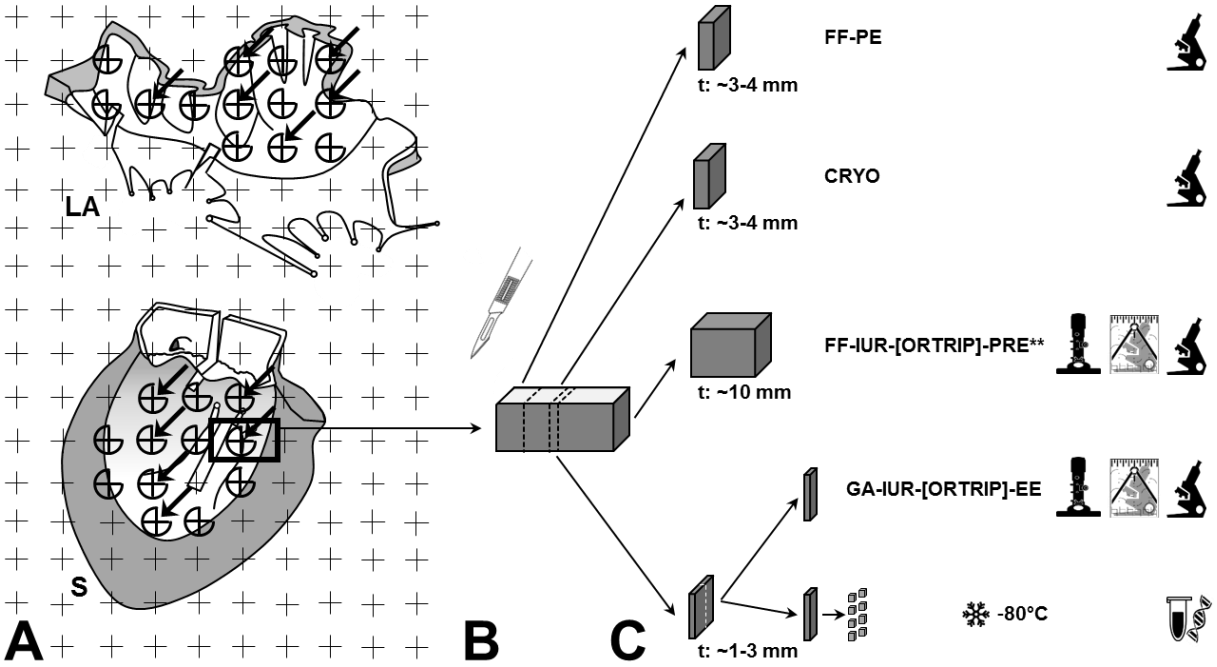


Fig. S188. Heart sampling in Type-II/III studies. Fourth step: *A schematic illustration of subsample preparation from systematically randomly sampled myocardium specimens for downstream analyses (Type-II/III studies).* ***A****: A schematic illustration of the systematic random sampling of six locations from the left atrium (LA, top) and interventricular septum (S, below).* ***B****: Transmural full-thickness specimens (~2x1x1 cm) are taken from each systematically randomly sampled location of myocardium.* ***C****: Each sample is divided into five subsamples of heart tissue while maintaining the transmural orientation of the samples. Thus, each of the subsamples contain an endocardial surface, a myocardial middle piece and an epicardial outer surface. Approximately 3-mm-thick slabs are processed for FF-, MTC-PE, and cryo-histology. A 1-3 mm thick slice is divided for molecular analyses and GA fixation. Another ~10 mm thick piece of heart tissue is fixed in FA. Next, six ORTRIP sections are produced (18 ORTRIPS per heart portion/compartment). For quantitative morphometric/ultrastructural analyses, three ORTRIP sections are embedded in GMA/MMA (*) and three ORTRIP sections are embedded in Epon.*

Time and personnel requirements

At least 50 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Table S27. The type and number of heart samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

Dissection and sampling	Compartment	DSP/SRS locations per compartment	Sample numbers per sampled location						Fig.
			FF-PE	MTC-PE	CRYO	MOL (-80°C)	FF-PRE ORTRIP	GA-EE	
Removal and opening of heart	Entire fresh heart (opened)	-	-	-	-	-	-	-	S181
Dissection of right and left heart, sampling of standard FF-PE histology specimen	LA + LV + S RA + RV	DSP	Loc. N° 1-9	-	-	-	-	-	S185
Dissection of atria, ventricles and septum, SRS of myocardial specimen	RA	6x (SRS)	1	0/1*	1	1	1	3	S186 and S188
	LA		1	0/1	1	1	1	3	
	RV		1	0/1	1	1	1	3	
	LV		1	0/1	1	1	1	3	
	S		1	0/1	1	1	1	3	
	Myocardial sample sizes and orientations: Transmural full-thickness samples. Sample sizes: FF-PE, MCT-PE, CRYO: ~3 mm thickness; MOL: ~2x2 mm base area; FF-PRE: ~1x1 cm base area; GA-EE: Three ~2x2x2 mm samples (top, middle, bottom) are excised from a transmural full-thickness specimen.								S188
Sampling of heart valves	Aortic valve	DSP 1 cusp	1	-	1	1	-	3	S187
	Pulmonary valve	DSP 1 cusp	1	-	1	1	-	3	
	Tricuspid valve	DSP 1 cusp	1	-	1	1	-	3	
	Bicuspid valve	DSP 1 cusp	1	-	1	1	-	3	

*Abbreviations: LA: Left atrium; RA: Right atrium; RV: Right ventricle; LV: Left ventricle; S: Interventricular septum; AV: Atrioventricular; DSP: Deliberate sampling position(s): SRS: Systematic random sampling. ORTRIP: Orthogonal triplet probe.*Sample numbers in Type-II/III studies.*

Related literature

(Berridge et al., 2013, Bishop, 1999, Mattfeldt et al., 1985, Miller et al., 2012, Morawietz et al., 2004, Muhlfeld et al., 2010)

2.10. Abdominal cavity

2.10.1. Stomach

Relevant anatomical features/preparation

After the pancreas is removed from the eviscerated intestines (section 2.10.3.) and the anatomical boundaries of the different intestinal segments have been identified (section 2.10.2.), the stomach is removed. The greater omentum is separated from the stomach. The stomach wall is opened along the greater curvature of the stomach as indicated in Fig. S189 using scissors. As with any hollow organ, the mucosa is not touched by hands or necropsy instruments to avoid damaging the epithelium.

General examination parameters

The organ size (dimensions) and weight of the stomach with and without contents are determined. The stomach is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

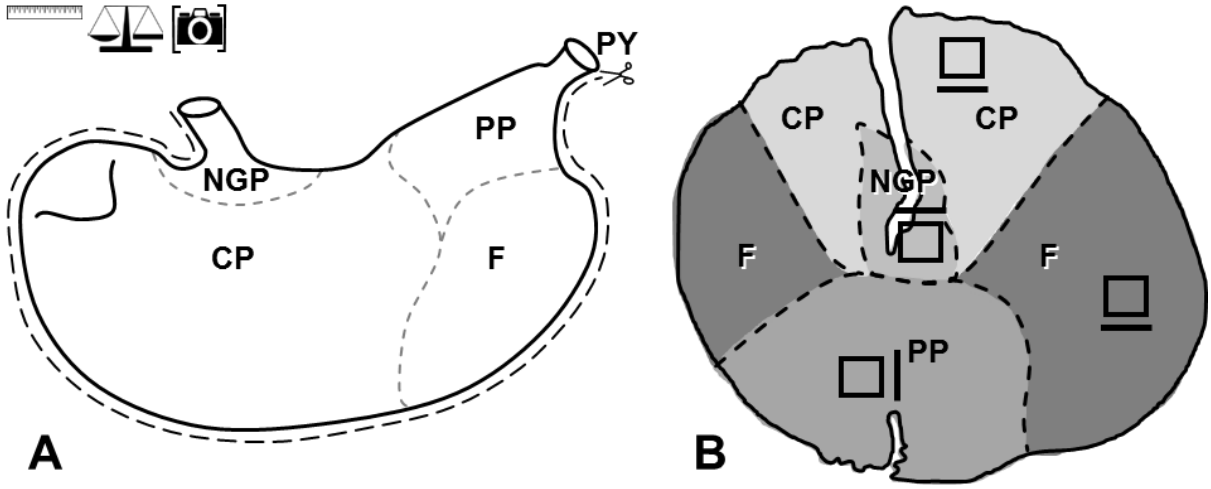


Fig. S189. *A schematic illustration of the closed (**A**) and opened stomach (**B**). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. NGP: Non-glandular portion, CP: Cardiac portion, F: Fundus of the stomach, PP: Pyloric portion, PY: Pylorus.*

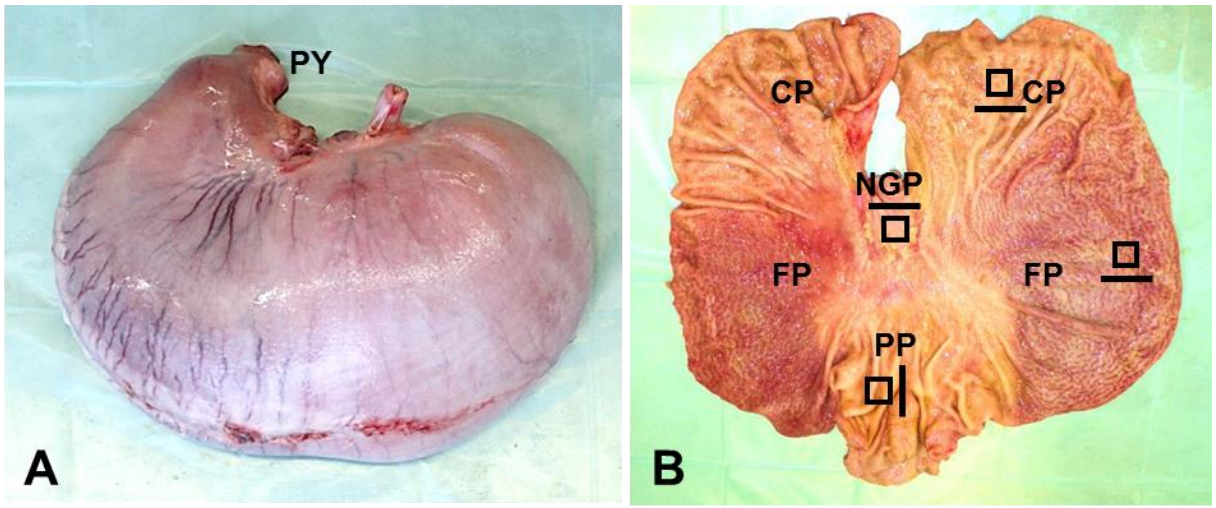


Fig. S190. *Closed (**A**) and opened stomach (**B**). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. NGP: Non-glandular portion, CP: Cardiac portion, F: Fundus of the stomach, PP: Pyloric portion, PY: Pylorus.*

2.10.1.1. Stomach samples in Type-I studies

Samples for molecular analyses

Location:	One sample is taken per gastric portion (NGP, CP, F, PP). The sampling locations and directions are indicated in Fig. S189 and Fig. S190.
Number of samples:	Four (one per gastric portion).
Remarks:	Samples are cut from the mucosa.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	One sample is obtained per gastric portion (NGP, CP, F, PP), next to the location of the samples for molecular analyses. The sampling locations and directions are indicated in Fig. S189 and Fig. S190.
Fixation/Embedding:	FF-PE (Fig. S191).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of 2-3 longitudinal vertical sections that represent all different gastric zones: (1.) the cardiac portion through the pyloric sphincter to the duodenum, (2.) across the limiting ridge with forestomach and the fundic portion of the glandular stomach, and (3.) through the fundus (Morawietz et al., 2004). The sampling protocols for Type-I studies recommend the generation of samples for histopathological examination from the same gastric regions, which will allow for an adequate evaluation of the porcine stomach in routine studies.

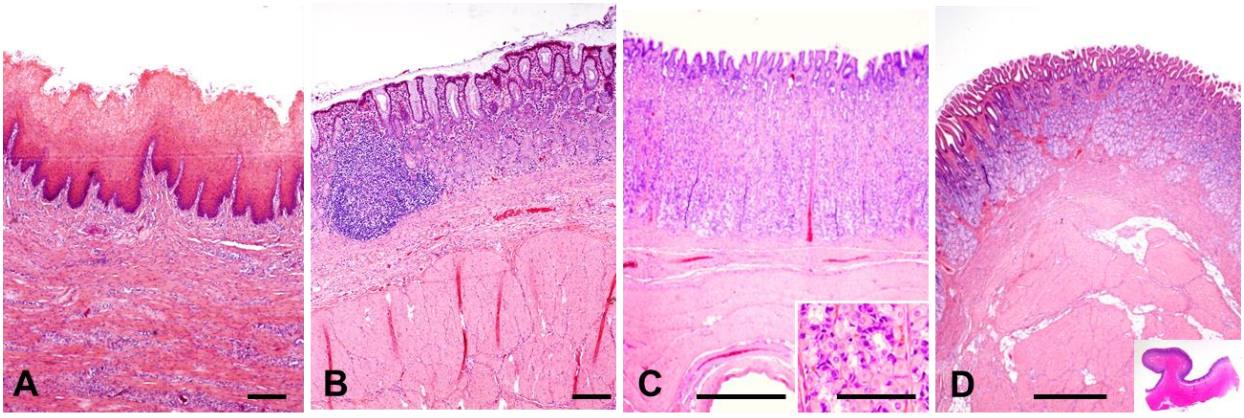


***Fig. S191. Stomach histology. A**: Non-glandular portion (keratinizing epithelium). **B**: Cardiac portion. **C**: Fundus of the stomach. Inset: Detailed enlargement. **D**: Pyloric portion. Inset: Histological slide with longitudinal section of the torus pyloricus. FF-PE. HE. Scale bars = 1 mm (C, D), 100 µm (A, B, inset in C).*

2.10.1.2. Stomach samples in Type-II and Type-III studies

General systematic random sampling strategy

The sampling positions and the organ size-dependent sample numbers are determined by systematic random sampling. The different glandular zones of the stomach are separated and weighed. Next, the tissue parts are overlaid with a cross grid printed on a transparency (grid size: 2-4 cm, depending on the size and area of the separated gastric wall parts). The systematic random sampling of tissue locations is performed as described in section 1.4. and shown in Fig. S192. The sampled positions can be marked by placing pieces of confetti paper on the respective positions (Fig. S192). An approximately 1.5x1.5 cm sized area of the gastric wall (full-thickness) is excised from each of the sampled locations. The excised tissue specimens are subdivided into slabs for downstream analyses (Fig. S193). Additionally, round mucosa samples are taken with the mucosal surface upside for VUR preparation for quantitative stereological analyses (Fig. S192 and Fig. S193) using a hole punch with an inner diameter of 1 cm and a hammer (Fig. S211 in section 2.10.2.2.). The type and number of stomach samples for histopathological examination and molecular analyses in Type-II and Type-III studies are listed in Table S28.

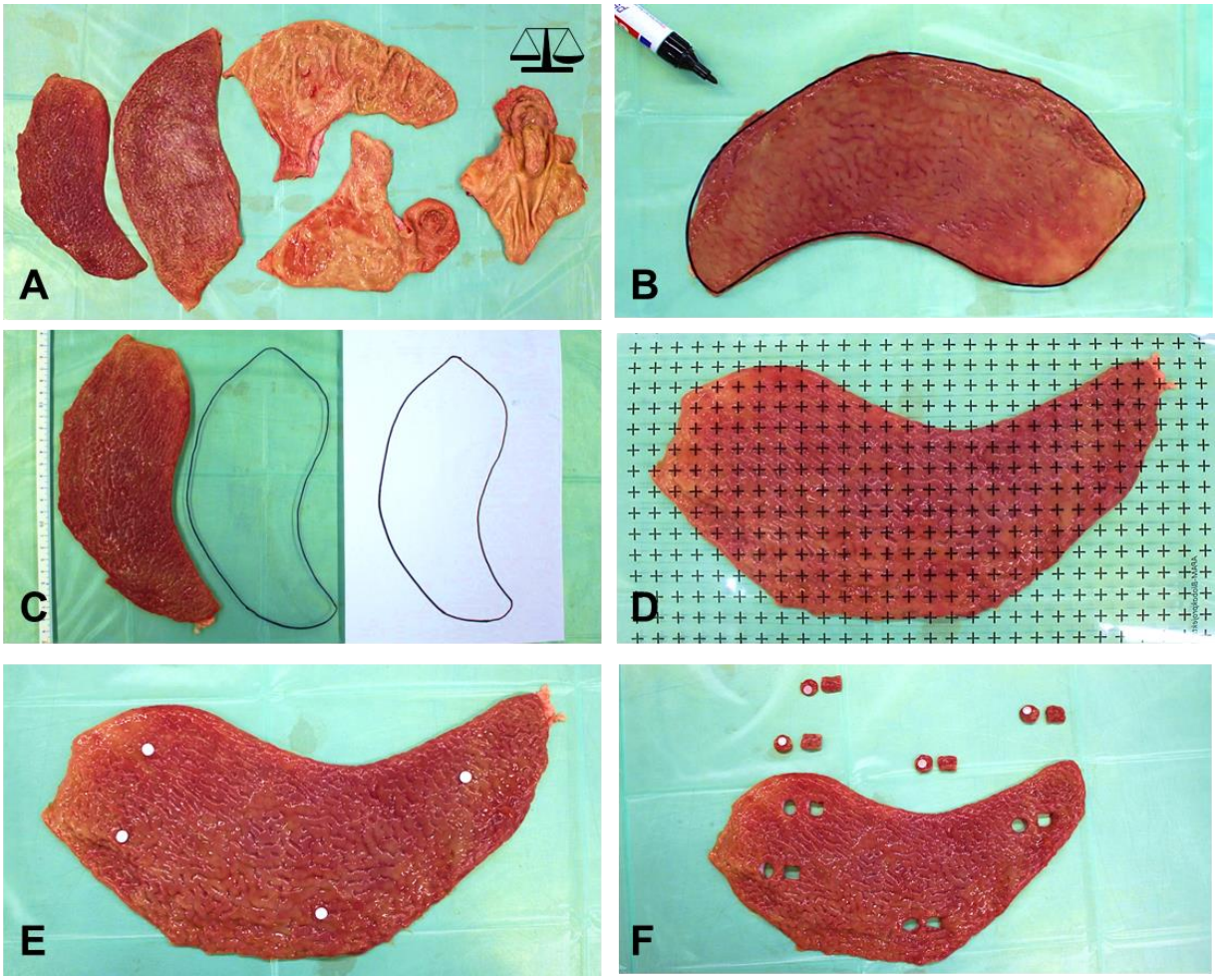


Fig. S192. *An illustration of the systematic random sampling of the fundus of the stomach in Type-II/III studies.* ***A****: The different gastric glandular zones are separated and weighed. The subsequent procedure is shown with one piece of the the fundus of the stomach.* ***B, C****: To determine the areas and volumes of the different gastric glandular zones, the outline of the sample is traced on a sheet of plastic transparency or a glass plate and then transferred to a sheet of paper.* ***D****: For systematic random sampling, the tissue is overlaid with a cross grid (1-2 cm).* ***E****: The systematically randomly sampled positions are marked by pieces of confetti.* ***F****: The samples for subsequent analyses are taken from the sampled positions (e.g., rectangular pieces of mucosa for histological, ultrastructural, and molecular analyses and round specimens for quantitative stereological analyses of VUR-sectionable samples).*

Table S28. The type and number of stomach samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

Sample type	Number of locations/samples Type-II studies	Type-III studies
Sampled locations (per gastric portion)	*6*	*8*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 out of every 2nd location
CRYO	1 out of every 3rd location	1 out of every 2nd location
GA-EE	1 per sampled location	1 per sampled location
FF-PRE (IUR)	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

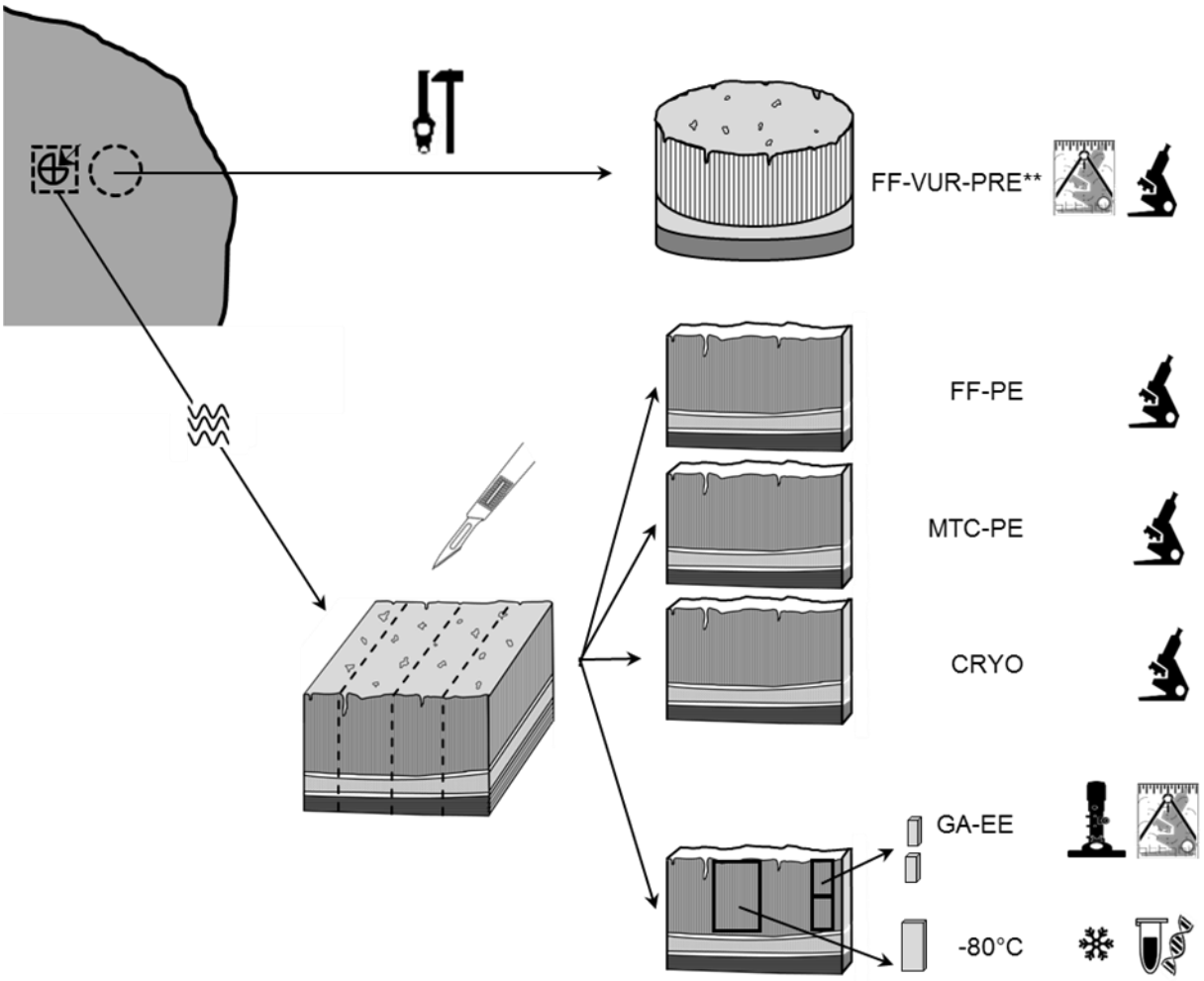


Fig. S193. *A schematic illustration of subsample preparation from one systematically randomly sampled location of stomach wall in a distinct gastric zone for downstream analyses (Type-II/III studies). *PR = GMA/MMA.*

Remarks

The subsamples for paraffin histology and PRE consist of ~5-mm-thick slabs taken from each of the systematically randomly sampled gastric mucosa samples (Fig. S192 and Fig. S193). An additional thinner ~2-mm-thick slab is taken from each of the systematically randomly sampled gastric mucosa samples (Fig. S193). From this slab, the subsamples for molecular analyses and GA fixation and Epon embedding are excised.

Time and personnel requirements

At least 30 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Bertram et al., 2013, Gelberg, 2012, Nyengaard and Alwasel, 2014, Ruehl-Fehlert et al., 2003)

2.10.2. Intestine

Relevant anatomical features/preparation

For removal of the gastrointestinal tract (GIT) with the typical colonic spiral from the abdominal cavity, the pig body is hung from the side and the abdominal wall is opened by a longitudinal incision in the ventral median line with additional relief incisions, if necessary. Next, the abdominal portion of the esophagus caudal to the diaphragm and the rectum proximal to the pelvis are ligated and cut. Next, the cranial and caudal root of the mesentery, hepatic portal vein and bile duct are cut. The GIT with the pancreas is then eviscerated. After the pancreas has been removed (section 2.10.3.), the intestinal bundle with mesentery, lymph nodes and vessels is examined for pathological alterations. Fig. S194 provides a schematic illustration of the porcine GIT with the anatomical boundaries of the different intestinal segments (Fig. S195), including the duodenum (pylorus to the distal end of the duodenocolic fold) and ileum (proximal end of the ileocecal fold to the ileal papilla). The most problematic part of the preparation of the porcine GIT is the separation of the colon from its mesentery. This procedure is best performed as shown in Fig. S195. First, the centripetal turns of the ascending colon are removed from the mesocolon by hand starting from the tip of the colonic spiral. Next, the centrifugal turns can be detached from the mesocolon, followed by the descending colon and the small intestine. If necessary, scissors are used to remove the mesentery from the intestine. The different intestinal segments are then laid in loops on the dissection table (Fig. S198).

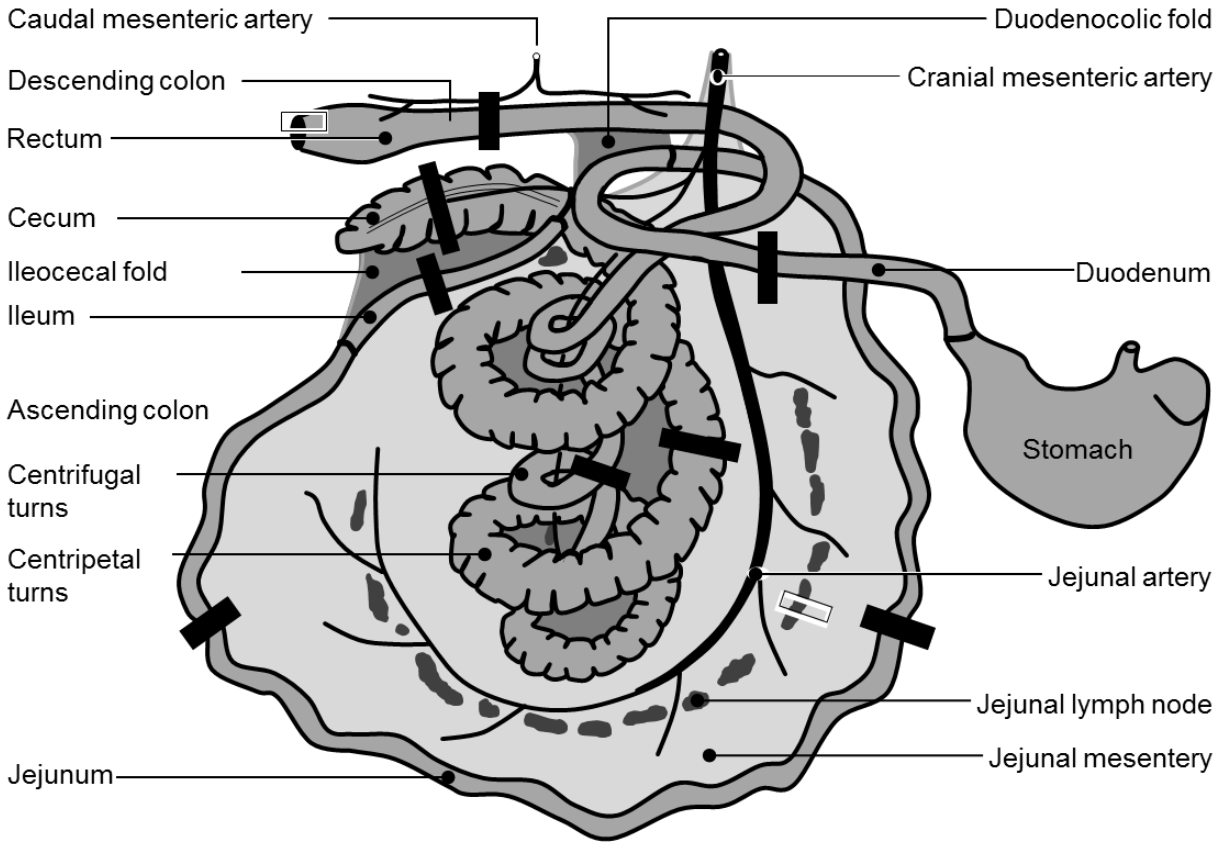


Fig. S194. *A schematic illustration of the porcine gastrointestinal tract (adapted from Ghetie and Pastea, (1958)). The intestinal segments, their anatomical boundaries, mesenteries, lymph nodes, and Type-I-study sampling locations (filled and empty rectangles) are indicated.*

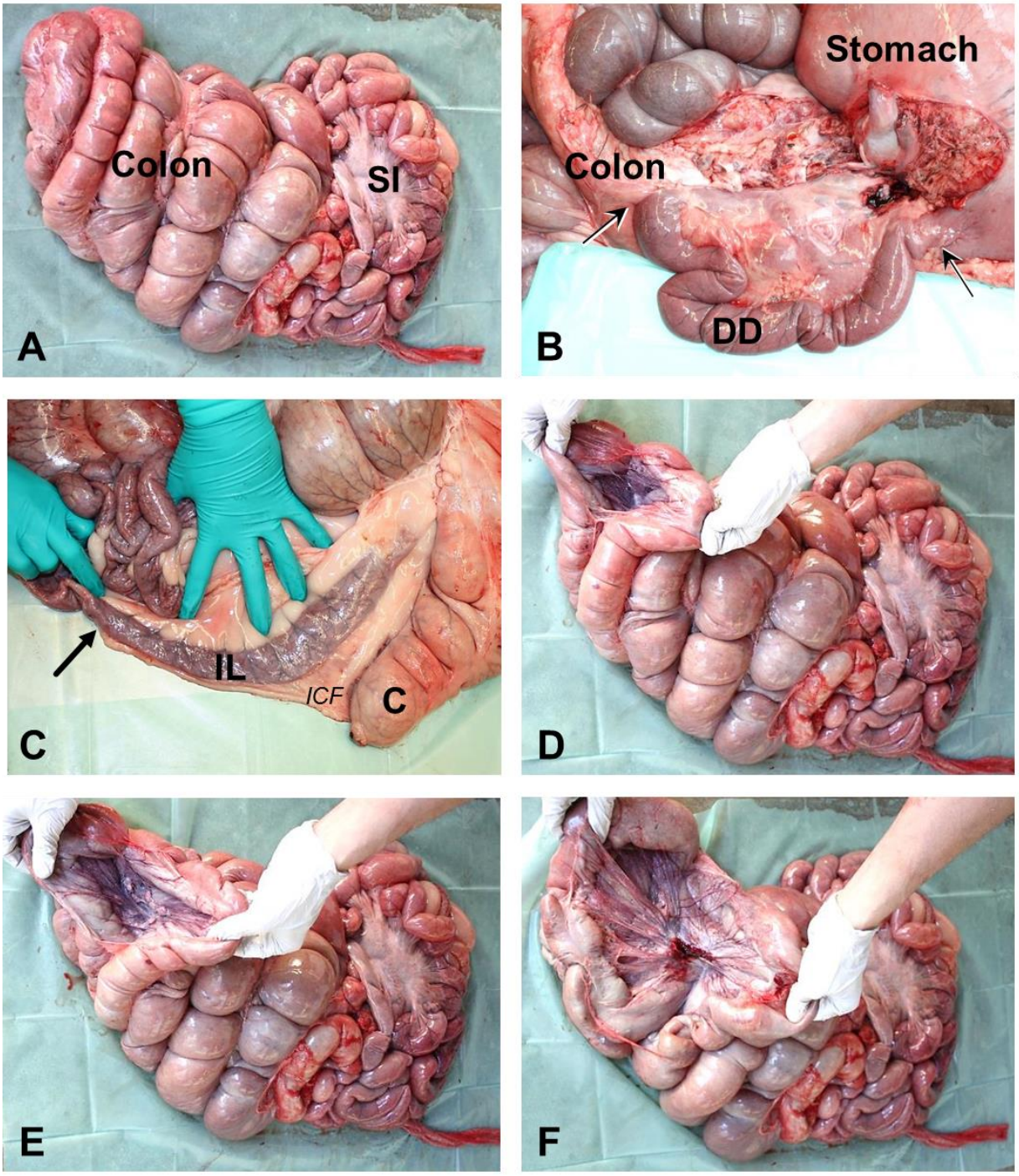


Fig. S195. *The anatomical boundaries of different intestinal segments and an illustration of the detachment of the mesentery from the colon.* ***A****: Eviscerated intestines. SI: Small intestine.* ***B****: Anatomic boundaries of the duodenum (DD). Arrows mark the beginning (pylorus) and end (duodenocolic fold) of the duodenum.* ***C****: Anatomical boundaries of the ileum (IL). The arrow indicates the proximal end of the ileocecal fold (ICF). C: Cecum.* ***D****-****F****: Separation of the colon from the mesocolon: The outer centripetal turns are stripped off the mesocolon by hand starting from the tip to the base of the colonic spiral.*

General examination parameters

The length of the intestinal segments is measured using a folding rule. If needed, the intestinal segments are weighed with ingesta/feces. After the respective samples (unopened intestinal pieces) have been harvested, the entire intestine is carefully opened longitudinally using scissors with rounded tips. Next, the ingesta can carefully be removed from the remaining gut to examine the mucosal surface. If necessary, the emptied gut segments are weighed. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

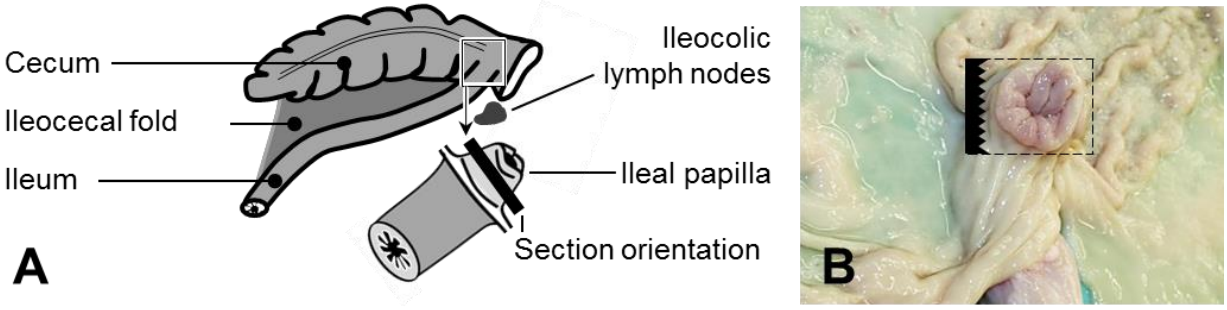


Fig. S196*: Topography and preparation of the ileal papilla. The orientation of the section through the ileal papilla (for Type-I studies) is indicated.*

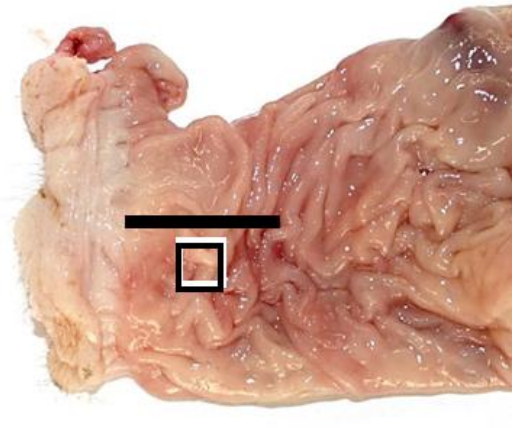


Fig. S197*: Longitudinally opened rectum. The location and orientation of the histology (black line) and molecular analyses (rectangle) samples for Type-I studies are indicated.*

2.10.2.1. Intestine samples in Type-I studies

The samples are taken from defined locations of the intestinal segments (Fig. S198). From each of the sampled locations, specimens are prepared for histopathological and molecular analyses (Fig. S199).

Samples for molecular analyses

Location, number of samples:	One mid-segmental sample is taken from the duodenum, one section is taken from the ileum, and one section is taken from the cecum. Two samples are prepared from the jejunum, three samples are taken from the colon (one from a centripetal turn, one from a centrifugal turn, and one from the descending colon), and one sample is taken from the rectal mucosa (Fig. S194 and Fig. S197).
Remarks:	The mucosa is carefully washed in PBS buffer before taking samples for molecular analyses. Separate specimens are taken from the antimesenteric side and lateral intestinal wall as indicated in Fig. S199.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA-, RNA-, and protein analyses, OMICS profiling.

Histology samples

Location, number of samples:	Unopened intestinal pieces approx. 1-3 cm in length are taken from the middle of the duodenum, ileum, and cecum. Two samples are taken from the jejunum, three samples are taken from the colon (one from a centripetal turn, one from a centrifugal turn, and one from the descending colon), and one sample is taken from the rectal mucosa (Fig. S194 and Fig. S197). The ileal papilla is always sampled (Fig. S196). Samples for histology are processed as indicated in Fig. S199. Cross sections of large caliber segments exceeding the size of embedding cassettes are processed by preparation of "Swiss rolls" (Fig. S200 and Fig. S201). Routinely, one sample of a jejunal lymph node and one sample of the ileocecal lymph node is taken.
Section orientation:	Cross sections (Fig. S196 and Fig. S199). The sample of the rectal mucosa is taken in a longitudinal orientation (Fig. S197).
Fixation/Embedding:	FF-PE (Fig. S202).

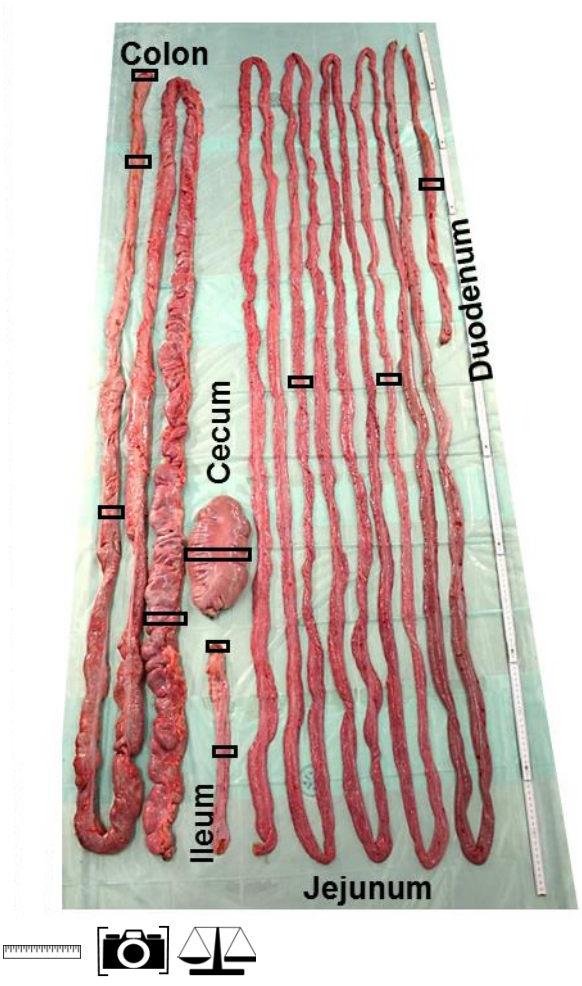


Fig. S198. *The intestinal segments and Type-I-study sampling positions (rectangles).*

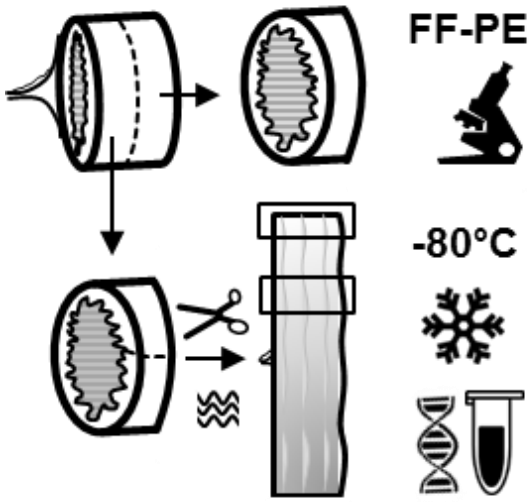


Fig. S199. *A schematic illustration of sample processing for routine screenings (Type-I studies).*

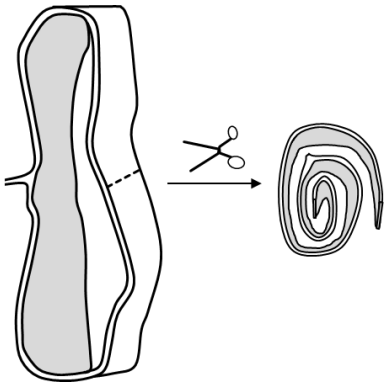


Fig. S200 (←). *Schematic illustration of "Swiss roll" preparation from large caliber intestinal cross sections for FF-PE.*

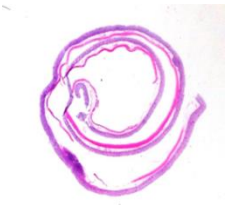


Fig. S201 (↑). *An image of a histological slide with a "swiss-rolled" piece of colon mucosa. HE.*

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of each one transverse (cross) section from the duodenum, jejunum, ileum, cecum, colon, and rectum (Ruehl-Fehlert et al., 2003). Considering the length of the porcine intestine, the collection of additional samples is recommended in the sampling protocols for Type-I studies to allow for an adequate evaluation of the porcine intestine in routine studies.

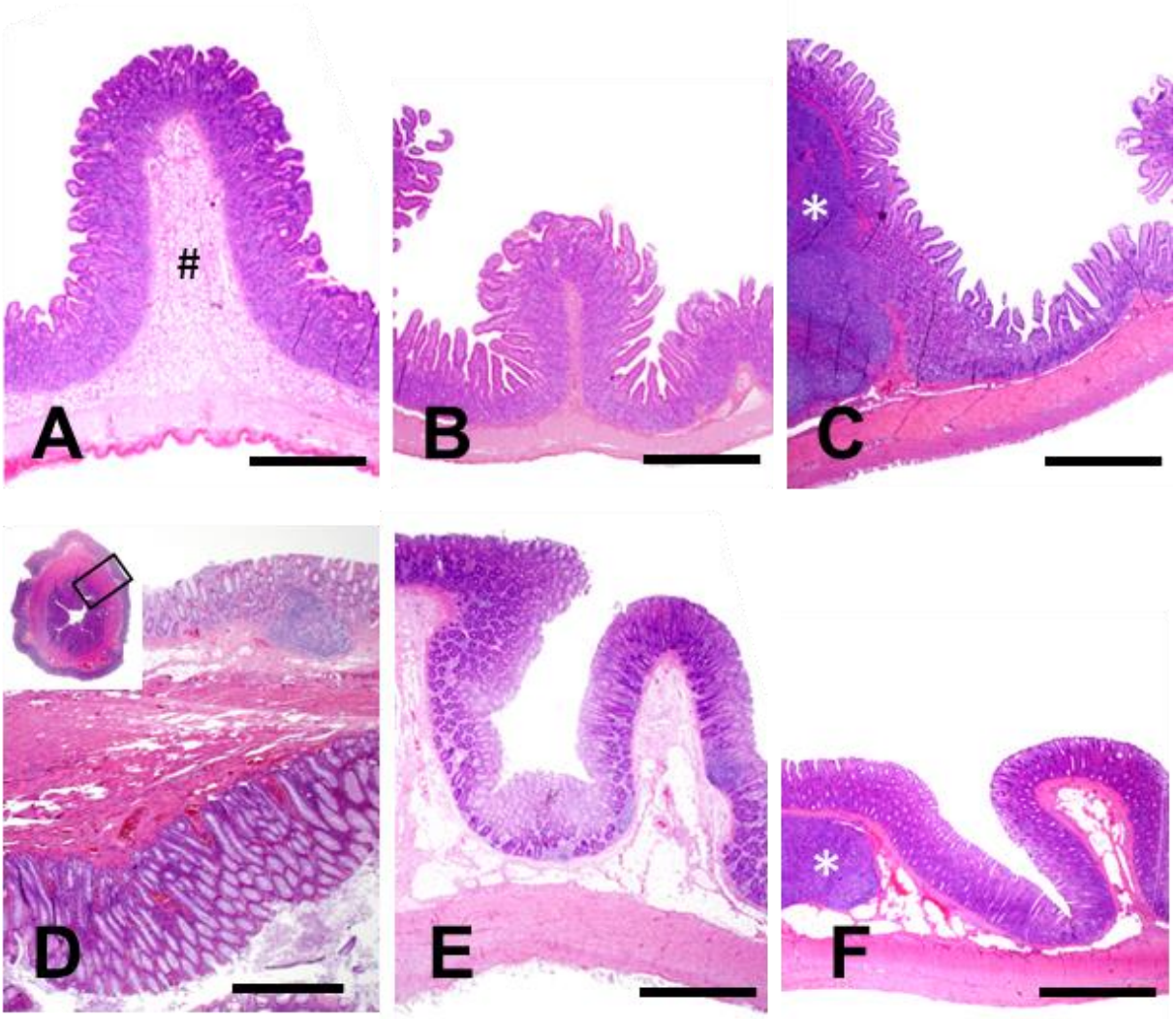


Fig. S202. Histology of intestinal segments *(cross sections).* ***A****: Duodenum with glands of Brunner (#).* ***B****: Jejunum.* ***C****: Ileum. The asterisk identifies gut-associated lymphoid tissue (GALT, Peyer's patch).* ***D****: Ileal papilla. Inset: Photo of histology slide. Note the ileal and cecal mucosa side of the ileal papilla.* ***E****: Cecum.* ***F****: Descending colon with GALT (asterisk). FF-PE. HE. Scale bars = 1 mm.*

2.10.2.2. Intestine samples in Type-II and Type-III studies

General sampling strategy

The sampling positions and the number of samples required are determined by a length weighted systematic random sampling procedure. The different intestinal segments are laid on the dissection table and measured as shown in Fig. S203-S209. Samples are taken in defined and equidistant intervals depending on the number of samples needed and the length of the respective intestinal segments. For example, if five locations are needed for a particular intestinal segment with the length l, the interval (i) would be i = l/5. The first sampling position is then defined randomly within the first interval using a random number table. Every subsequent location is then defined by the interval i (Fig. S204). This interval method is used for all animals in a specific study. Therefore, more locations may be sampled in animals with longer intestinal segments.

From each sampled location, a 3-4 cm long segment is obtained. From this segment, all subsamples for subsequent analyses are taken (Fig. S210 and Fig. S211).

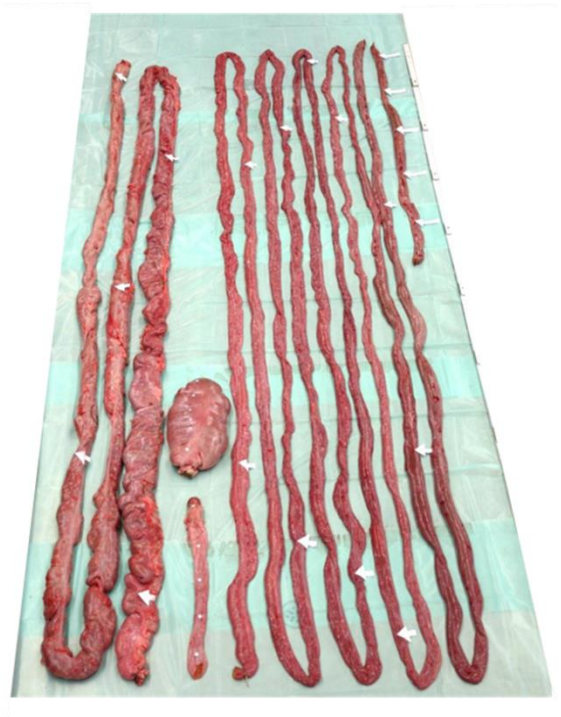


Fig. S203. *Intestinal segments with systematically randomly determined sampling locations are indicated by paper arrows (Type-II study).*

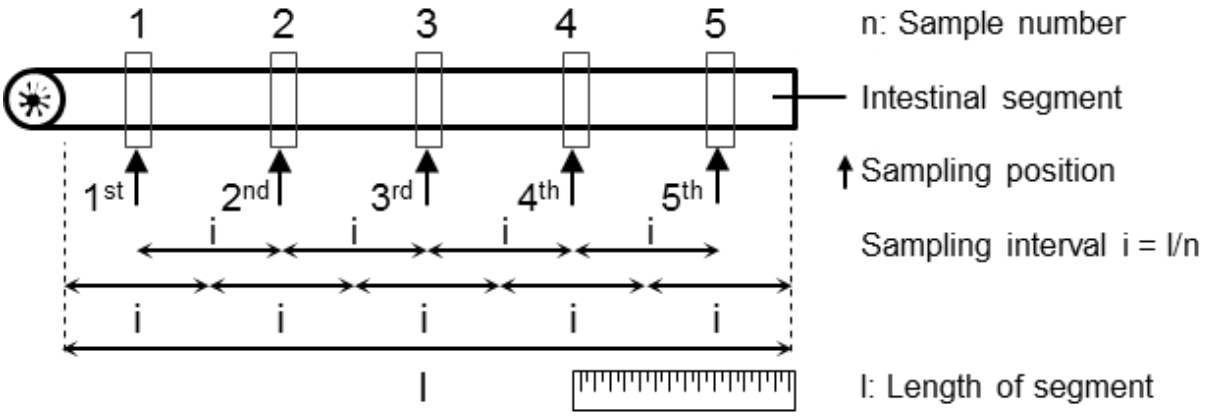


Fig. S204. *A schematic illustration of the general systematic random sampling scheme for intestinal segments. The first sampling position is determined randomly within the i interval. All subsequent samples are then taken at every i interval*.

Number of systematically randomly sampled locations in individual intestinal segments

Duodenum (Fig. S205)
Type-II studies: Three locations
Type-III studies: Five locations
(The Type-II/III-study sampling procedures described for the duodenum can also directly be applied for the sampling of the esophagus if required).

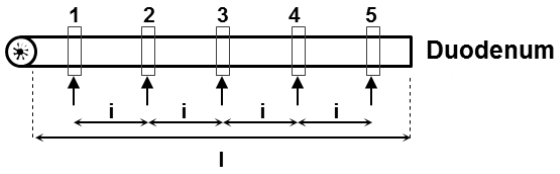


Fig. S205. *A schematic illustration of the systematic random sampling of the duodenum.*

Jejunum (Fig. S206)
Type-II studies: Five locations
Type-III studies: Ten locations

Remarks: The determination of sampling locations can be completed quickly if the number of loops of the intestine on the dissection table is the same as the number of samples to be obtained (Fig. S206). The length of a single loop is made equal to the sampling interval i. Therefore, after determining the position of the first sampling location (1^{st}) in the first loop, all subsequent samples are taken on the same level of the next following loop. This method can be used only if the intestinal lengths of the animals examined in a specific study do not differ significantly from each other.

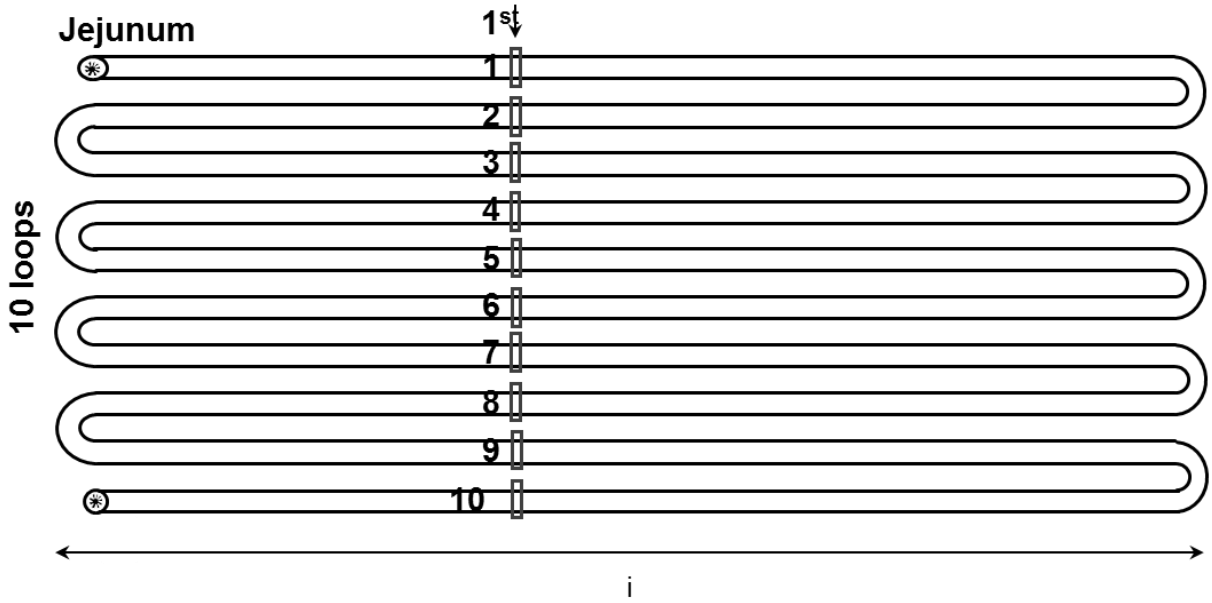


Fig. S206. *A schematic illustration of the systematic random sampling of the jejunum.*

Ileum (Fig. S207)
Type-II studies: Two locations
Type-III studies: Five locations

Remark: In addition to the systematically randomly sampled ileal locations, the ileal papilla is always sampled.

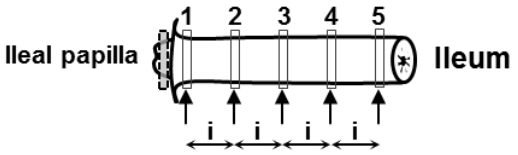


Fig. S207. *A schematic illustration of the systematic random sampling of the ileum.*

Cecum (Fig. S208)
Type-II studies: Two locations
Type-III studies: Five locations

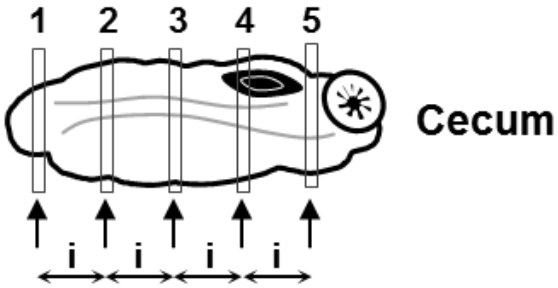


Fig. S208. *A schematic illustration of the systematic random sampling of the cecum.*

Colon (Fig. S209
Type-II studies: Three locations
Type-III studies: Five locations

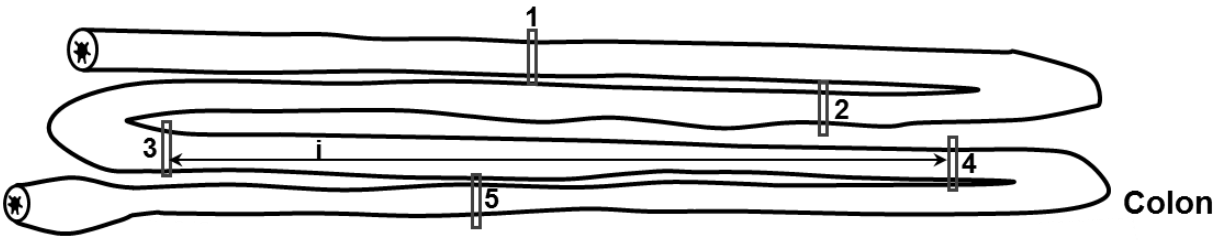


Fig. S209. *A schematic illustration of the systematic random sampling of the colon.*

Processing of samples for different subsequent analyses
All subsamples for subsequent analyses are obtained from each 3-4 cm long sampled specimen (Fig. S210 and Fig. S211) depending on the extent and type of recommended downstream analyses (Table S29).

Samples for histopathological examination

Paraffin histology samples (FF-PE, MTC-PE):
Cross sections are prepared from the respective intestinal segments. “Swiss rolls” are prepared if the samples of large diameter intestinal segments exceed the size of embedding cassettes (Fig. S200 and Fig. S201).
Downstream analyses: Histology, IHC, ISH, quantitative stereological parameters independent of embedding-related tissue shrinkage.

Samples for cryo-histology, GA-EE, and FF-PRE-VUR sections for quantitative stereological analyses:
These samples are smaller than a complete cross section of the intestine. Therefore, the specimens have to be randomly sampled from a strip-shaped piece of the intestinal wall (Fig. S210 and Fig. S211).

Samples for preparation of FF-PRE-VUR sections for quantitative stereological analyses (Fig. S210 and Fig. S211).

Depending on the intestinal segment, there are at least four possible sampling positions on a strip of gut for a FF-PRE-VUR sections: One from the antimesenteric side of the intestinal wall, two from the lateral intestinal wall, and one from the mesenteric side of the intestinal wall. One location is determined randomly from these three possible anatomical locations. In the example shown in Fig. S210, the lateral wall (location N° 2 or 4) is sampled. In this case, location 2 was chosen randomly. The round samples are taken with the mucosal surface facing up using a hole punch with an inner diameter of 1 cm and a hammer.

Processing: Samples are fixed in in a 4% formaldehyde solution and embedded in GMA/MMA.

Downstream analyses: Quantitative stereological analyses.

Cryo-histology samples (Fig. S210 and Fig. S211).

Cryo-histology samples are sampled in a strip of the intestinal wall in the same manner as described above.

Processing: Cryo-sections (cross sections).

Downstream analyses: Cryohistology, IHC, IF-IHC, and special stains, such as oil red O.

GA-EE samples (Fig. S210 and Fig. S211).

An approximately 1-2 mm wide strip of the intestinal wall is cut into 1-2x1-2x1-2 mm pieces. These are pieces are lined up in a row, and two (study Type-II) or five (study Type-III) samples are systematically randomly selected.

Processing: Sampled specimens are fixed in a 2.5% glutaraldehyde solution and then embedded in Epon.

Downstream analyses: Semithin section histology, electron microscopy, and quantitative stereological analyses.

Remarks: If necessary, ISECTOR samples can be prepared from Epon-embedded samples by re-embedding them in spherical casting molds.

Samples for molecular analyses (Fig. S210 and Fig. S211).

Sampling location: An approximately 3 mm wide strip of the intestinal wall is cut into 3x3x3 mm full-thickness pieces. These pieces are lined up in a row, and two (study Type-II) or five (study Type-III) samples are systematically randomly selected for molecular analyses.

Remarks: The mucosa is carefully washed (≡) in PBS buffer before obtaining the molecular analyses samples.

Processing: Samples are frozen on dry ice and stored at -80°C.

Downstream analyses: DNA, RNA, and protein analyses (OMICS profiling).

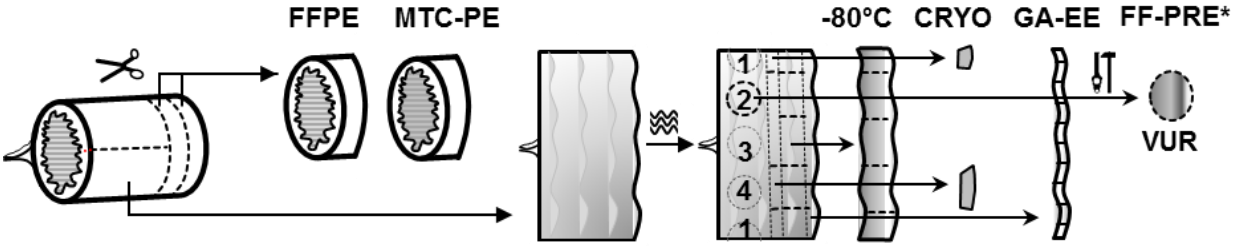


Fig. S210. *A schematic illustration of sample processing from each sampled location (Type-II/III studies). *PR = GMA/MMA.*

Table S29. The type and number of samples per systematically randomly sampled location of intestine for histopathological examination and molecular analyses in Type-II/III studies.

Sample type	Number and orientation of specimen per sample location Type-II studies	Type-III studies
FF-PE	*1 (cross)*	*1 (cross)*
MTC-PE	*-*	*1 (cross)*
CRYO	*1 (cross)*	*1 (cross)*
GA-EE	*1 (cross)*	*2 (cross)*
FF-PRE (VUR)	*1 (VUR)*	*1 (VUR)*
Molecular analyses (-80°C)	*2*	*2-5*

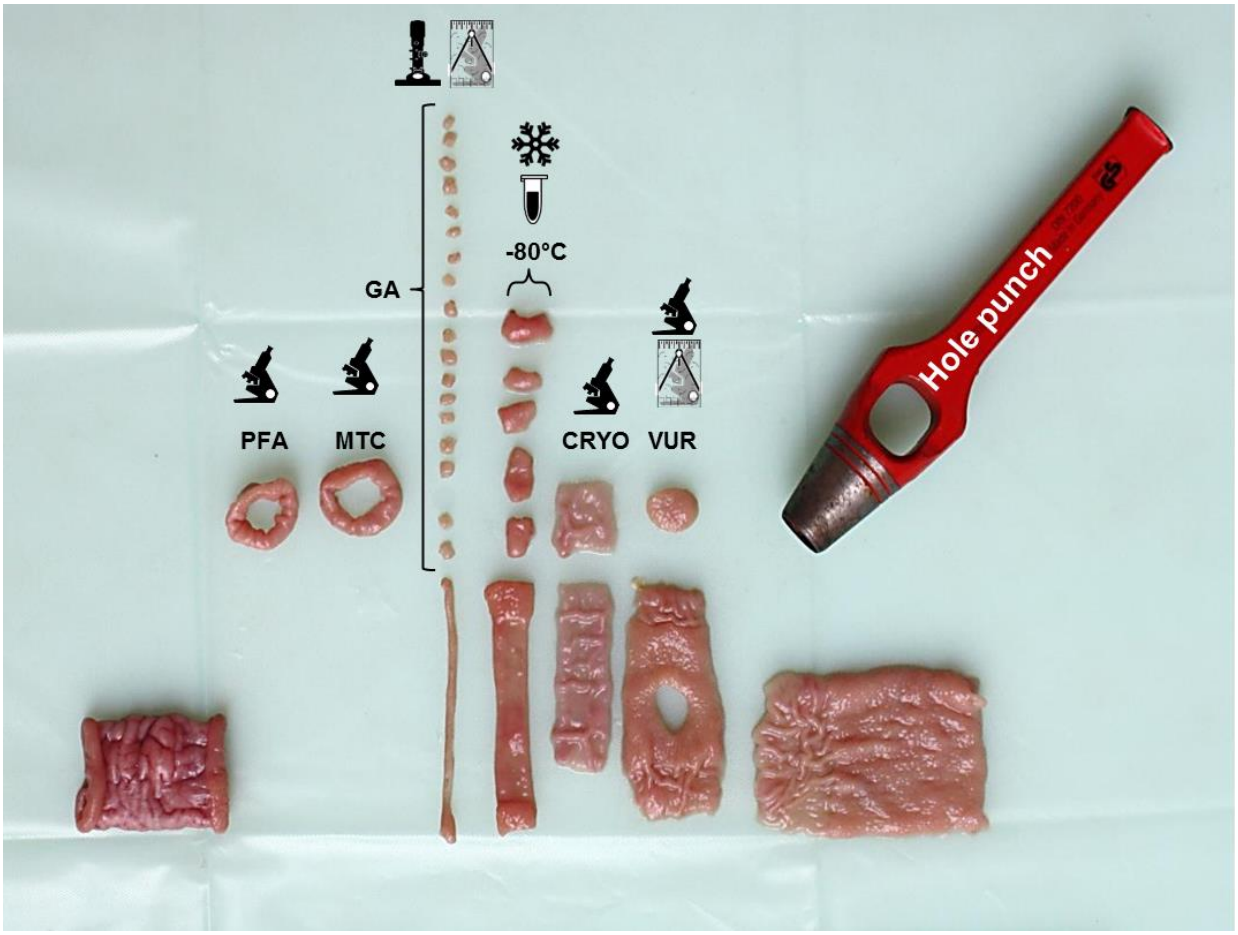


Fig. S211. The specimen processing from sampled locations of intestine (Type-II/III studies)*. See the text for details.*

Time and personnel requirements

At least 50 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and 2-3 assistants. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Bertram et al., 2013, Gelberg, 2012, Ghetie and Pastea, 1958, Nyengaard and Alwasel, 2014, Ruehl-Fehlert et al., 2003)

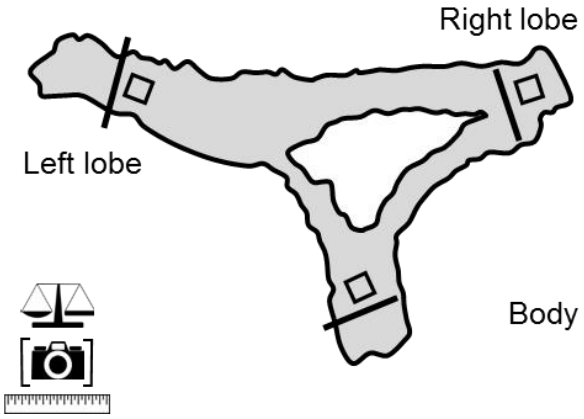
2.10.3. Pancreas

Relevant anatomical features/preparation

The gastrointestinal tract with the intestine, the stomach and pancreas is removed from the abdominal cavity *in toto*. Next, the pancreas is carefully removed (Fig. S213). The pancreas should quickly be harvested after the animal's death to prevent autolytic RNA and protein degradation. The adjacent peripancreatic adipose, omental and connective tissue is removed prior to the subsequent sampling steps.

General examination parameters

The organ size (dimensions) and weight are determined. The pancreas is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.



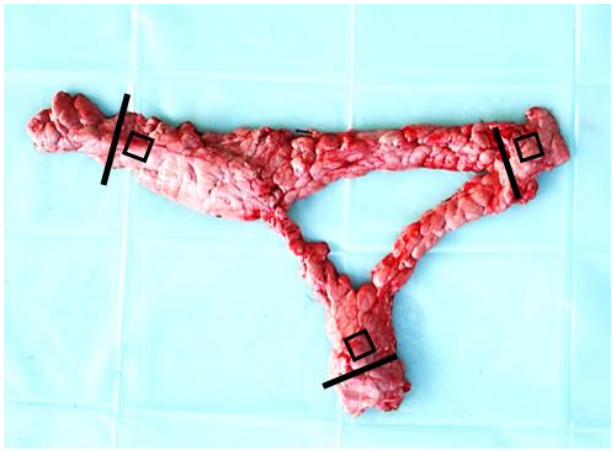


Fig. S212 (↑) A schematic illustration; Fig. S213 (→) Image of the pancreas. *The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*

2.10.3.1. Pancreas samples in Type-I studies

The density of endocrine pancreatic islets is not equal across the different pancreatic lobes. Therefore, samples from three parts of the organ are harvested. Histologically, the islets of Langerhans in the porcine pancreas are not as clearly delimited from the adjacent exocrine tissue as in rodents.

Samples for molecular analyses

Location:	Right and left lobe and body of the pancreas as indicated in Fig. S212 and Fig. Fig. S213.
Number of samples:	Three.
Remarks:	Homogeneous samples are cut from the parenchyma while avoiding specimens with nonhomogenous composition of different compartments, such as ducts, vessels, and parenchyma.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Sampling locations are indicated in Fig. S212 and Fig. S213. Next to the location of the histology samples, three cross sections are that contain pancreatic parenchyma, vessels and ducts are collected. One additional section is prepared from the pancreaticoduodenal lymph nodes if they can be identified.
Fixation/Embedding:	FF-PE (Fig. S214).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one longitudinal and horizontal section of the left lobe (Ruehl-Fehlert et al., 2003). Considering the size of the porcine pancreas, a comparable sampling technique is not applicable in pigs. For a thorough examination of the porcine pancreas, samples from separate locations of the organ must be evaluated. The three samples generated according to the Type-I-study sampling protocols allow for an adequate evaluation of the porcine pancreas in routine studies.

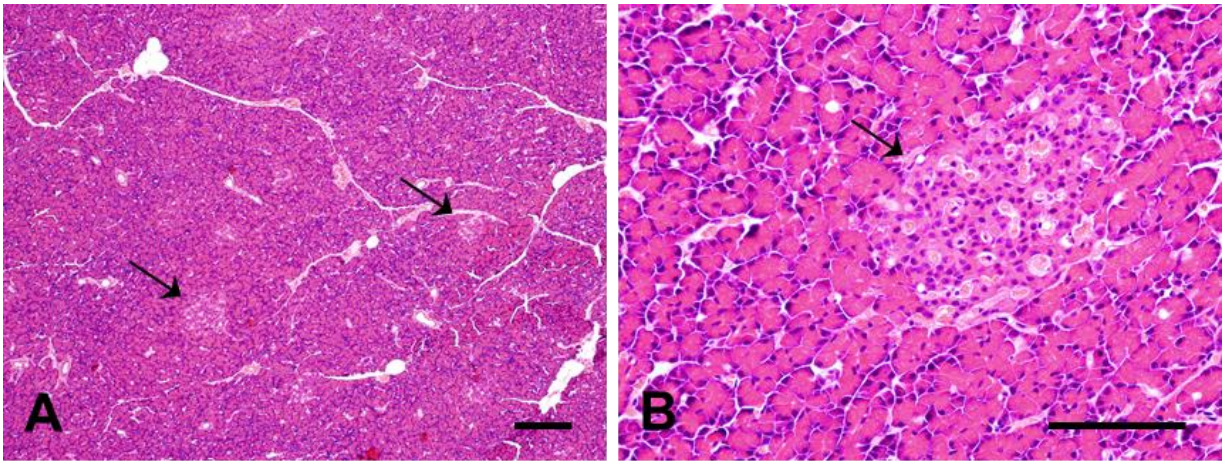


***Fig. S214. Pancreas histology**. **A**: Overview. **B**: Detailed enlargement. The arrows indicate pancreatic islets. FF-PE. HE. Scale bars = 1 mm (A), 100 µm (B).*

2.10.3.2. Pancreas samples in Type-II and Type-III studies

General systematic random sampling strategy

Sampling positions and organ size-dependent sample numbers are determined by systematic random sampling. The pancreas weight and length along the longitudinal axis are determined. The pancreatic parenchyma is cut between the body and the left lobe of the pancreas. The pancreas is then cut into parallel equidistant slabs orthogonal to the longitudinal axis of the organ with an approximate thickness of 20 mm. The first section is randomly positioned between 0 and 20 mm from the margin of the organ (Fig. S215 and Fig. S216). The tissue slabs are counted and placed on the same side on plastic transparencies. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-3 cm, depending on the organ size and number and area of slabs). The systematic random sampling of tissue locations is performed as described in section 1.4.The sampled locations can be marked by placing pieces of confetti paper on the respective positions. An approximately 1.5x1.5x1-cm tissue piece is excised from each of the sampled locations regardless of the structures contained in the sample. If the sampled location contains vascular structures or ducts, these structures must be included in the rest of the sample to maintain representativity. The excised tissue specimens are subdivided into slabs for downstream analyses (Fig. S217, Table S30 and Table S31).

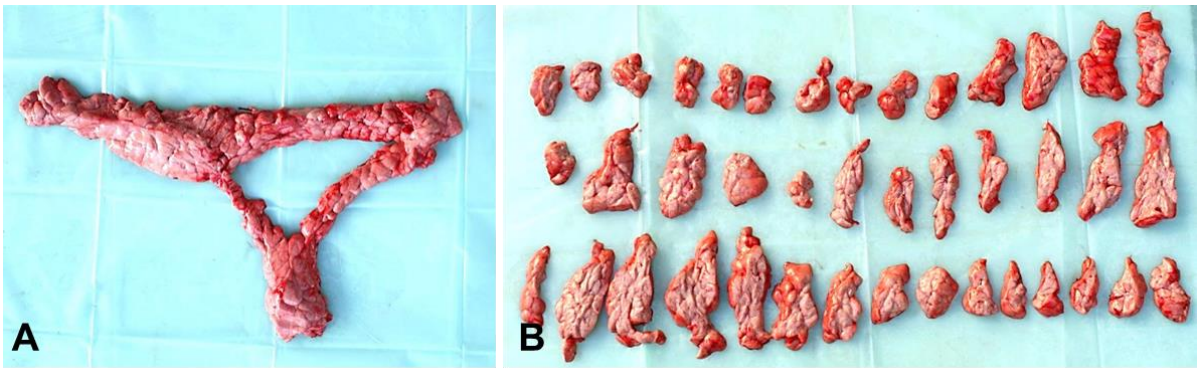


Fig. S215. *Excised (**A**) and serially sectioned (**B**) pancreas for systematic random sampling.*

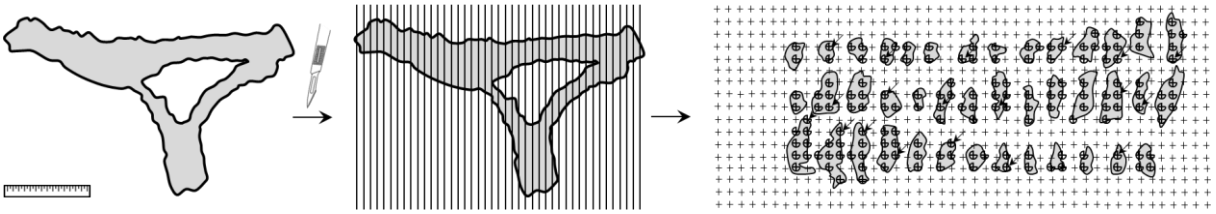


Fig. S216. *A schematic illustration of the systematic random sampling of the pancreas in Type-II/III studies.*

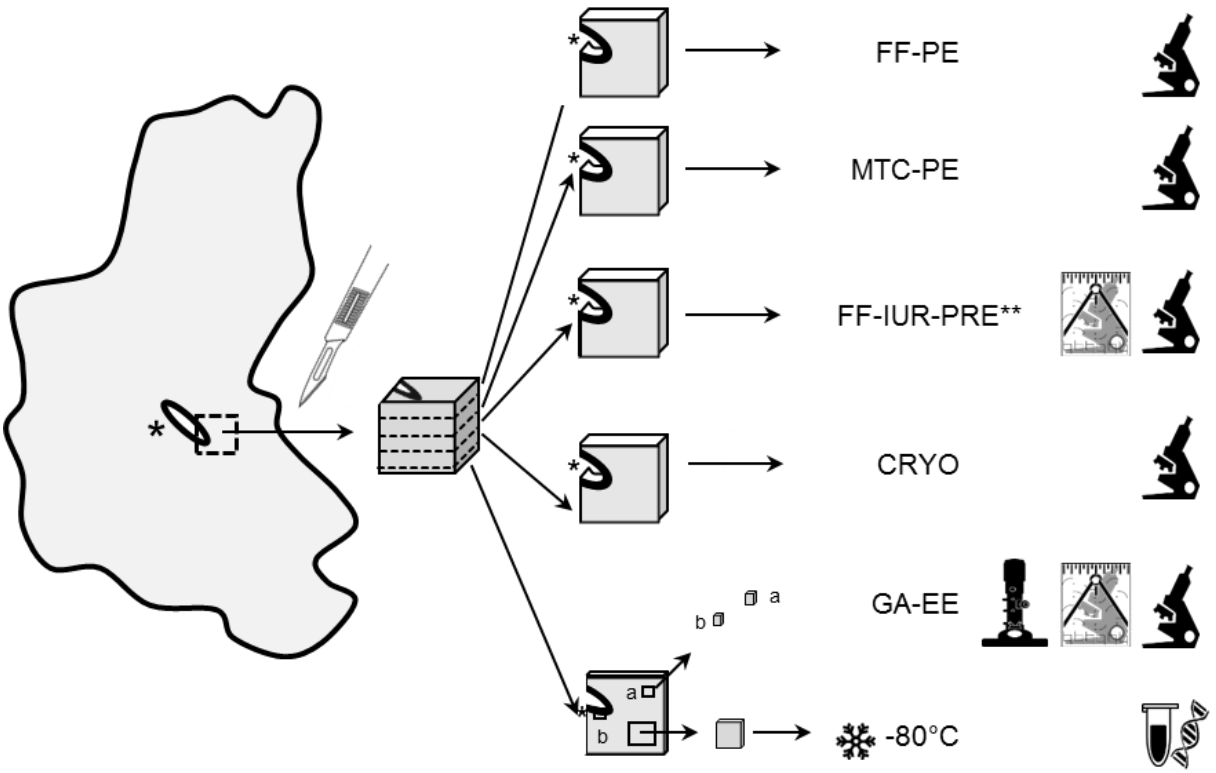


Fig. S217. *A schematic illustration of subsample preparation from one systematically randomly sampled location of pancreas tissue for downstream analyses (Type-II/III studies). *Duct section. [a]GA-EE sample of pancreas parenchyma; [b]GA-EE sample of duct epithelium (optional). **PR = GMA/MMA.*

Table S30. The type and number of pancreas samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

Sample type	Number of sampled locations/samples per location Type-II studies	Type-III studies
Sampled locations	*10*	*20*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 out of every 2nd location
CRYO	1 out of every 3rd location	1 out of every 4th location
GA-EE	2 out of every 2nd location	1 out of every 2nd location
FF-PRE (IUR)	1 out of every 2nd location	1 out of every 2nd location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

Remarks

The subsamples for paraffin histology and PRE consist of ~5-mm-thick slabs taken from each of the systematically randomly sampled tissues (Fig. S217).

An additional thinner ~2-mm-thick slab is taken from each of the systematically randomly sampled tissue samples (Fig. S217, Table S30). From this slab, the subsamples for molecular analyses and GA fixation and Epon embedding are excised from areas of homogeneous pancreas parenchyma (avoid sampling specimens with a nonhomogenous composition of different tissue compartments, such as ducts, large vessels, and parenchyma).

For qualitative histological, ultrastructural, and molecular analyses of specific pancreas tissue structures, additional samples of ducts may optionally be taken from the systematically randomly sampled locations of the pancreas (Fig. S217 and Table S31*).* If duct section profiles are present in more locations than needed for the recommended number of samples for GA-EE and molecular analyses, the tissue slabs are sampled by systematic random sampling. For example, six GA-EE samples of pancreatic duct epithelium are needed for a Type-III study, and 18 out of 20 systematically randomly sampled pancreas tissue locations have duct section profiles. Thus, one ductal GA-EE sample is taken from every third sampling location (18/6 = 3), and the first location is randomly determined in between locations 1 and 3.

Table S31. Additional pancreas samples (molecular analysis samples and GA-EE samples).

Location	Sample type	Total number of samples Type-II studies	Type-III studies
Pancreatic duct (epithelium)	**GA-EE**	*3*	*6*
	Molecular analyses (-80°C)	*3*	*6*

Time and personnel requirements

At least 25 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Bock et al., 2005, Cullen and Brown, 2012, Herbach et al., 2011, Herbach et al., 2005, La Perle, 2012, Noorafshan et al., 2012, Renner et al., 2013, Renner et al., 2010, Rosol et al., 2013, Wallig and Sullivan, 2013)

2.10.4. Liver

Relevant anatomical features/preparation

In contrast to many other species, the porcine liver displays a prominent lobulation (Fig. S221) that is macroscopically visible. The liver is removed from the abdominal cavity after removal of the gastrointestinal tract. Attention should be paid not to puncture the gall bladder. In icteric animals, the liver should be removed with the duodenum to rule out the presence of a posthepatic icterus by testing the bile duct for blockage (press on the gall bladder to determine if bile can flow through the major duodenal papilla into the lumen of the duodenum).

General examination parameters

The organ size (dimensions) and weight are determined. The liver is examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

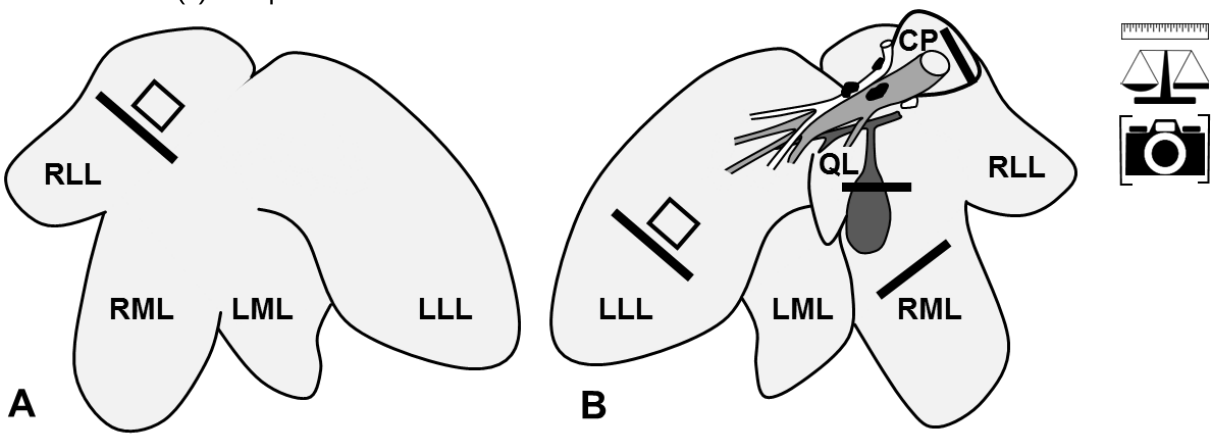


Fig. S218. *A schematic illustration of the liver.* ***A****: Diaphragmatic surface.* ***B****: Visceral surface. The different lobes of the liver are indicated: Quadrate lobe (QL), caudate process (CP), left lateral lobe (LLL), left medial lobe (LML), right lateral lobe (RLL), and right medial lobe (RML). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*

2.10.4.1. Liver samples in Type-I studies

Samples for molecular analyses

Location:	Left and right lateral main lobes as indicated in Fig. S218 and Fig. S219.
Number of samples:	Two.
Remarks:	Homogeneous samples are cut from the liver parenchyma while avoiding specimens with a nonhomogenous composition of liver tissue compartments, such as bile ducts, vessels, and parenchyma.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Sections of the right lateral (one section) and right medial (one section) lobe, left lateral lobe (one section), caudate process (one section), and gall bladder, including the underlying liver parenchyma (one section), are prepared. The sampling locations and orientations are indicated in Fig. S218 and Fig. S219. The sections should contain the capsule, liver parenchyma, vessels and bile ducts.
Fixation/Embedding:	FF-PE (Fig. S220 and Fig. S221).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in mice and rats recommend the preparation of each one transverse section from the left lateral lobe, right medial lobe (in rats), and caudate process (optionally) and one longitudinal-vertical section through both the left and right medial lobe, including the gall bladder (Ruehl-Fehlert et al., 2003). The same sampling recommendations are described in an INHAND (International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice) publication on proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system (Thoolen et al., 2010). The generation of additional sections for histopathological examination is recommended in Type-I-study sampling protocols to allow for an adequate evaluation of the porcine liver.

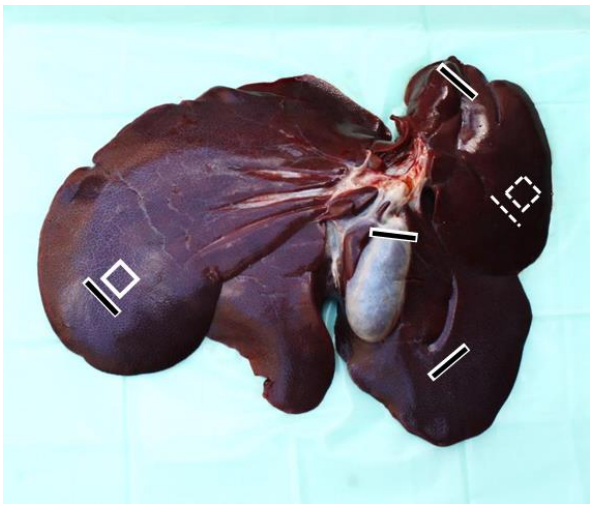


Fig. S219 (←). Liver *(visceral surface). The locations and orientations of the histology (lines) and molecular analyses (white rectangle) samples for Type-I studies are indicated. The sampling positions on the diaphragmatic surface are indicated by dotted lines.*

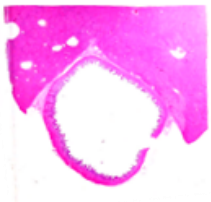


Fig. S220 (↑). *Histological slide with a cross section of the gall bladder and adjacent liver parenchyma. FF-PE. HE.*

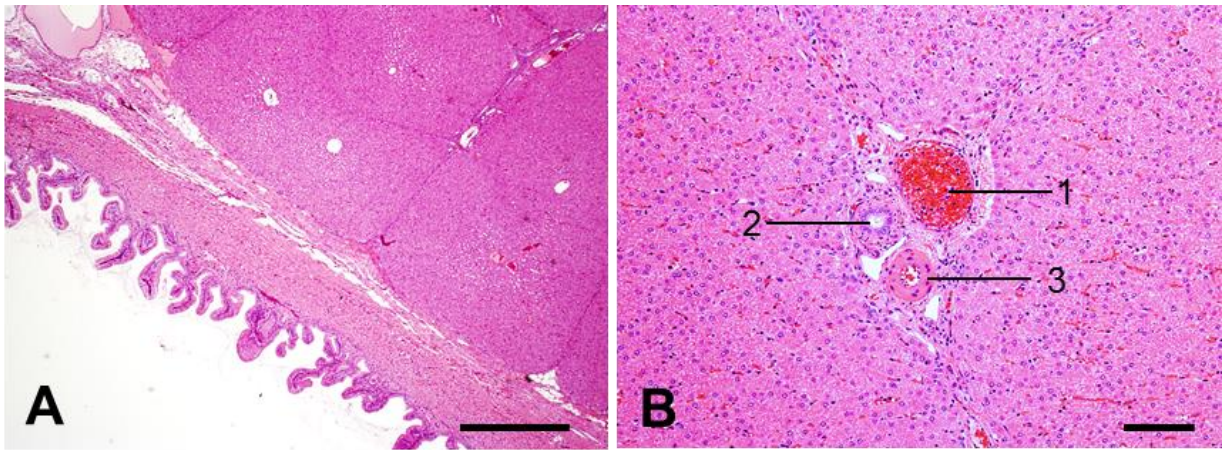


***Fig. S221. Liver histology**. **A**: Gall bladder wall with adjacent liver parenchyma. Note the separation of individual hepatic lobules. **B**: Portal tract with cross sections of the interlobular vein (**1**), interlobular bile duct (**2**), and interlobular artery (**3**). FF-PE. HE. Scale bars = 1 mm (A), 100 µm (B).*

2.10.4.2. Liver samples in Type-II and Type-III studies

General systematic random sampling strategy

The sampling positions and organ size-dependent sample numbers are determined by systematic random sampling. The weight and the dimensions of the liver are determined, and the liver is cut into parallel equidistant slabs approximately 20 mm thick orthogonal to the width axis of the organ. The first section is randomly positioned between 0 and 20 mm from the margin of the organ (Fig. S222). The organ slabs are placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-4 cm, depending on the organ size and number and area of slabs). The systematic random sampling of tissue locations is performed as described in section 1.4. and shown in Fig. S223. The sampled positions can be marked by placing pieces of confetti paper on the respective positions. An approximately 1.5x1.5x2-cm tissue piece is excised from each of the sampled locations regardless of the tissue structures contained in the sample. If the sampled location contains vascular structures or bile ducts, these structures must be excised together with the rest of the specimen to maintain representativity. The excised tissue specimens are subdivided for downstream analyses (Fig. S224, Table S32) while ensuring comparable tissue compositions, which is particularly advantageous if the tissue composition of samples has to be considered when interpreting the results of the molecular analyses.

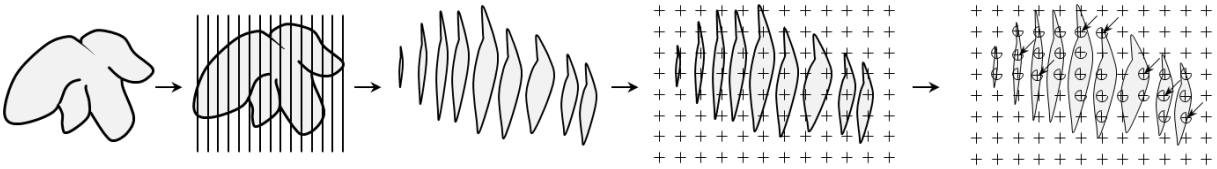


***Fig. S222.** A schematic illustration of liver sampling in Type-II/III studies.*

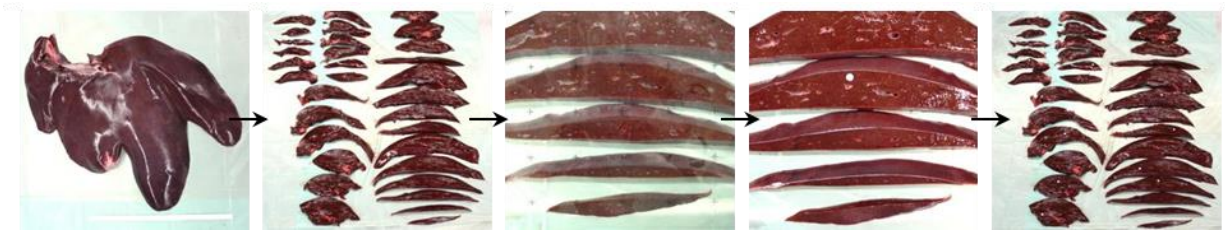


***Fig. S223.** Systematic random sampling of the liver. The sampled positions are marked using pieces of confetti paper.*

Table S32. The type and number of liver samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

Sample type	Number of sampled locations and samples per location Type-II studies	Type-III studies
Sampled locations (per liver)	*10*	*20*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 out of every 2nd location
EtOH*-PE	-	3 per liver
CRYO	1 out of every 3rd location	1 out of every 3rd location
GA-EE	2 out of every 2nd location	2 out of every 3rd location
FF-PRE (IUR)	1 out of every 2nd location	1 out of every 2nd location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

**96% ethyl alcohol*

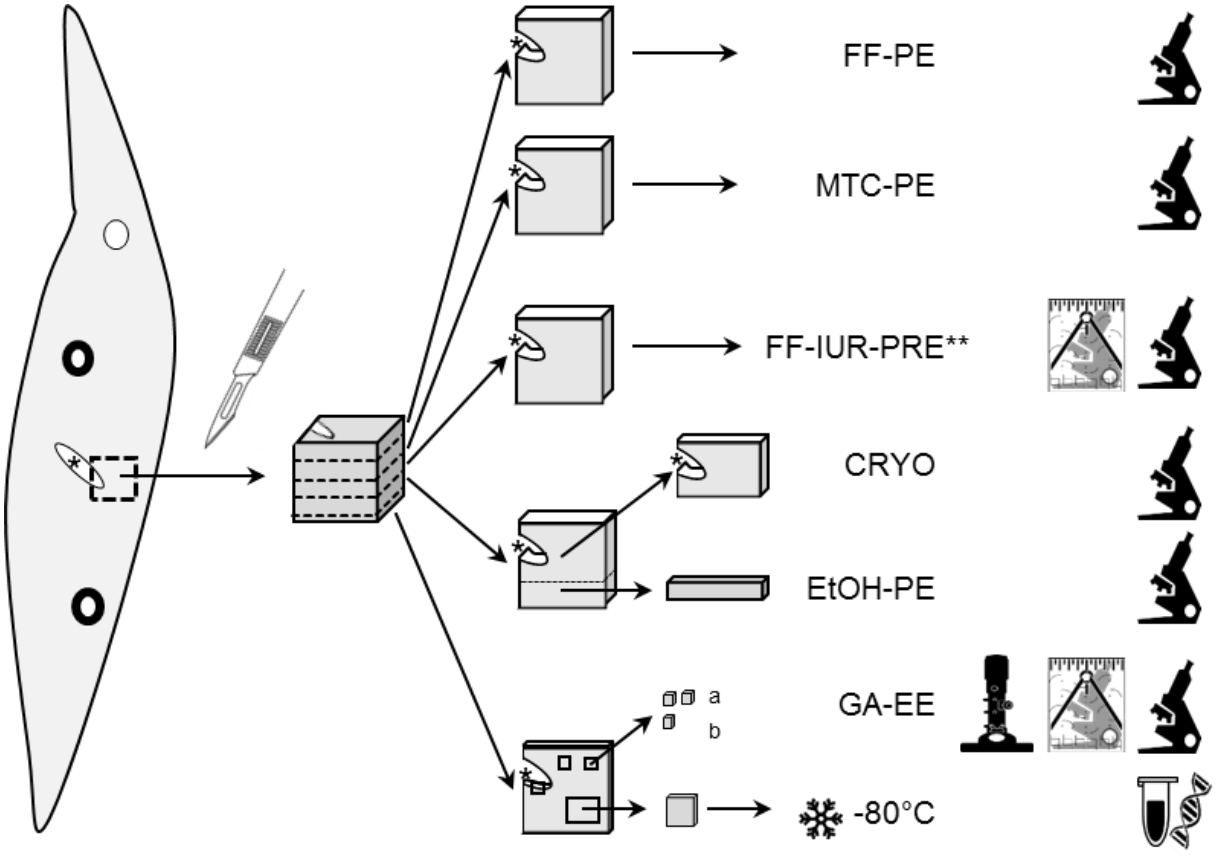


Fig. S224. *A schematic illustration of subsample preparation from one systematically randomly sampled location of liver tissue for downstream analyses (Type-II/III studies). *Bile duct section. [a]GA-EE samples of liver parenchyma; [b]GA-EE samples of bile duct epithelium. **PR = GMA/MMA.*

Remarks

The subsamples for paraffin histology and PRE consist of ~5-mm-thick slabs taken from each of the systematically randomly sampled liver tissue samples (Fig. S224).

An additional thinner ~2-mm-thick slab is taken from each of the systematically randomly sampled liver tissue specimens (Fig. S224). From this slab, the subsamples for molecular analyses and GA fixation and Epon embedding are excised from areas of homogeneous liver parenchyma while avoiding specimens with a nonhomogenous composition of liver tissue compartments, such as bile ducts, vessels, parenchyma.

For qualitative histological, ultrastructural, and molecular analyses of specific liver tissue structures, additional samples of bile ducts and the gall bladder wall are taken from the systematically randomly sampled locations of liver tissue (Fig. S224 and Table S33). If the bile duct section profiles are present in more locations than required for GA-EE and molecular analyses, the tissue samples are chosen by systematic random sampling. For example, six GA-EE bile duct samples are generated in a Type-III study, but bile duct section profiles are present in 18 out of 20 systematically randomly sampled liver tissue locations. Therefore, one bile duct GA-EE sample is taken from every third sampling location (18/6 = 3), and the first location is randomly determined in the interval between 1 and 3.

Table S33. Additional samples for molecular analyses and GA fixation and Epon embedding.

		Total number of samples	
Location	**Sample type**	**Type-II studies**	**Type-III studies**
Bile ducts (epithelium)	**GA-EE**	*6*	*6*
	Molecular analyses (-80°C)	*3*	*6*
Gall bladder wall (adjacent to the base of the gall bladder)	**GA-EE**	*6*	*6*
	Molecular analyses (-80°C)	3	6

Time and personnel requirements

At least 20 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Cattely and Cullen, 2013, Cullen and Brown, 2012, Marcos et al., 2012, Ruehl-Fehlert et al., 2003, Thoolen et al., 2010)

2.10.5. Spleen

Relevant anatomical features/preparation
After removal of the gastrointestinal tract from the abdominal cavity, the spleen is carefully removed from the omentum using scissors.

General examination parameters
The organ size (dimensions) and weight are determined. The spleen is examined for pathological alterations and reactive hyperplasia of the white or red pulp. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

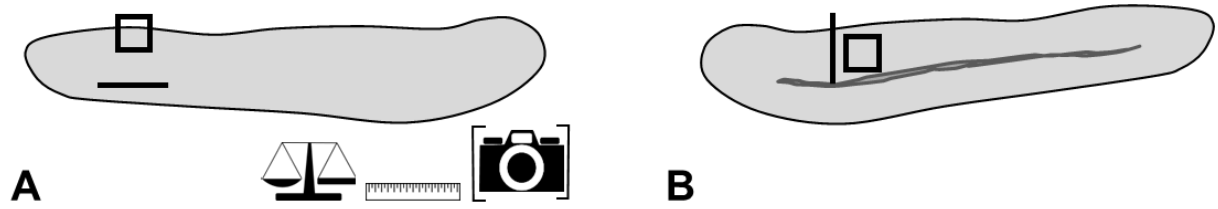
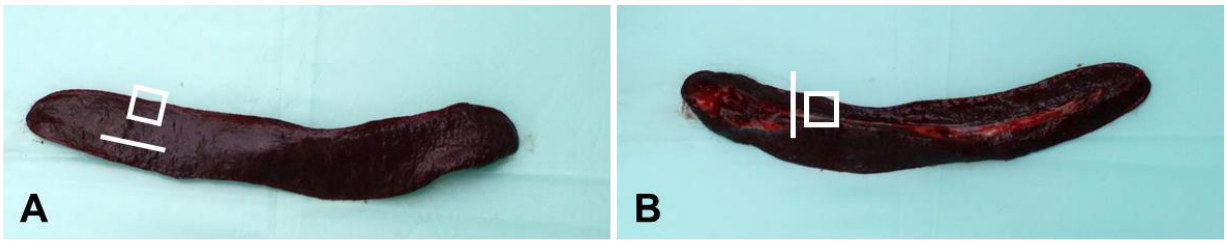


Fig. S225 (↑) and Fig. S226 (↓). *A schematic illustration and image of the spleen.* ***A:*** *Parietal side.* ***B: V****isceral side. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*



2.10.5.1. Spleen samples in Type-I studies

Samples for molecular analyses

Location:	Sampling locations are indicated in Fig. S225 and Fig. S226.
Number of samples:	Two.
Remarks:	Homogeneous samples are cut from the spleen parenchyma while avoiding specimens with a nonhomogenous composition of splenic capsule and trabecula.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Samples are taken next to the location of the molecular analyses samples as indicated in Fig. S225 and Fig. S226. One cross section and one longitudinal section are prepared. Each section should include the capsule and spleen parenchyma (Fig. S225 and Fig. S226).
Fixation/Embedding:	FF-PE (Fig. S227).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species

The Immunotoxicity Screening Working Group (STP IWG) of the Society of Toxicologic Pathology (STP) recommends the routine histopathological evaluation of the spleen using sections containing all separate spleen compartments (Haley et al., 2005).

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one transverse section at the largest extension of the spleen (Morawietz et al., 2004).

Considering the size of the porcine spleen, a comparable sampling technique is not applicable in pigs.

The spleen is a homogeneous organ; thus, the Type-I-study sampling protocols recommend the generation of two samples from the spleen for an adequate evaluation of the porcine spleen in routine studies.

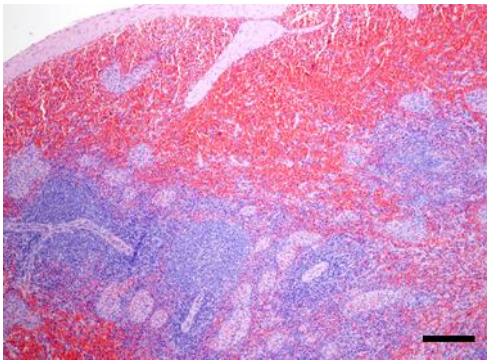


Fig. S227. Spleen histology. *FF-PE. HE. Scale bar = 100 µm.*

2.10.5.2. Spleen samples in Type-II and Type-III studies

General systematic random sampling strategy

The sampling positions and organ size-dependent sample numbers are determined by systematic random sampling. The spleen weight and length is determined. Next, the organ is cut into parallel equidistant slabs of 20 mm thickness orthogonal to the longitudinal axis of the organ. The first section is randomly positioned between 0 and 20 mm from the margin of the organ (Fig. S228 and Fig. S229). The organ slabs are then placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 2-4 cm, depending on the organ size and number and area of slabs). The systematic random sampling of tissue locations is performed as described in section 1.4. The sampled positions can be marked by placing pieces of confetti paper on the respective positions (Fig. S229). An approximately 1.5x1.5x2-cm tissue piece is excised from each of the sampled locations, regardless of the structures contained in the sample. If the sampled location contains large vascular structures or trabeculae, these structures must be excised together with the rest of the specimen to maintain representativity. The excised tissue specimens are subdivided for downstream analyses (Fig. S230, Table S34).

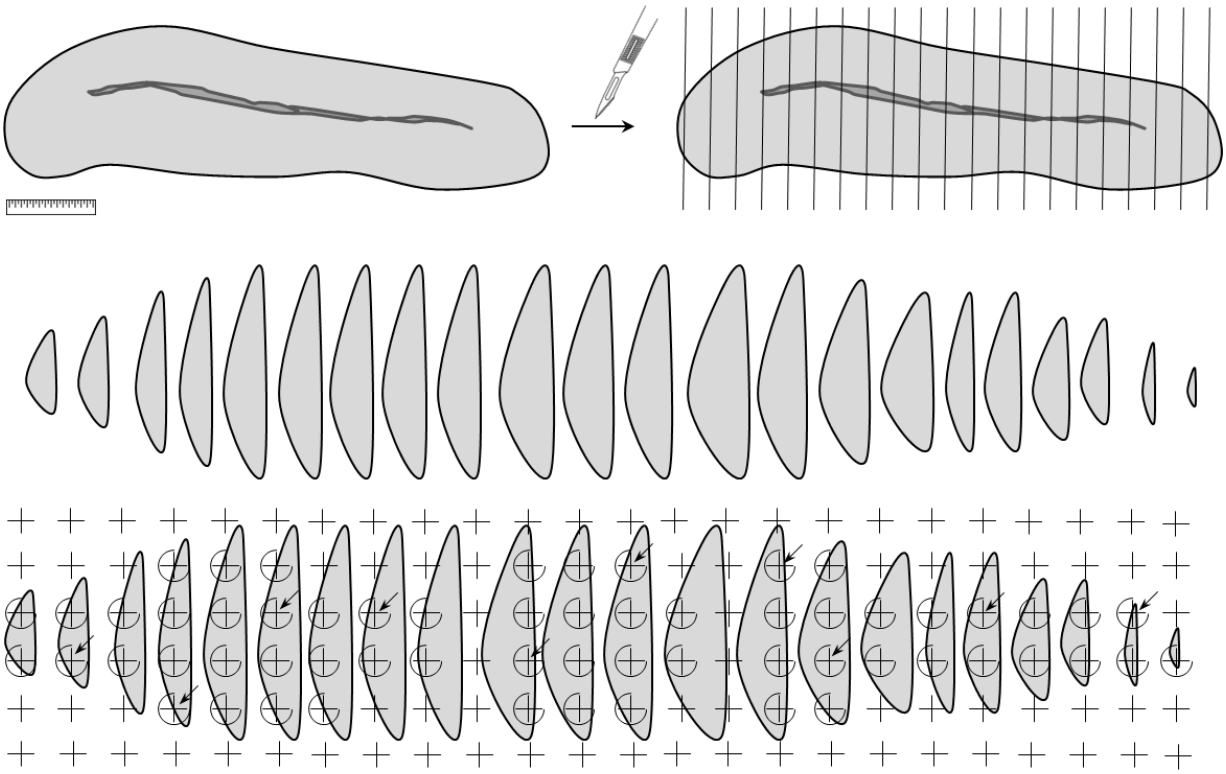


Fig. S228. *A schematic illustration of the systematic random sampling of the spleen in Type-II/III studies.*

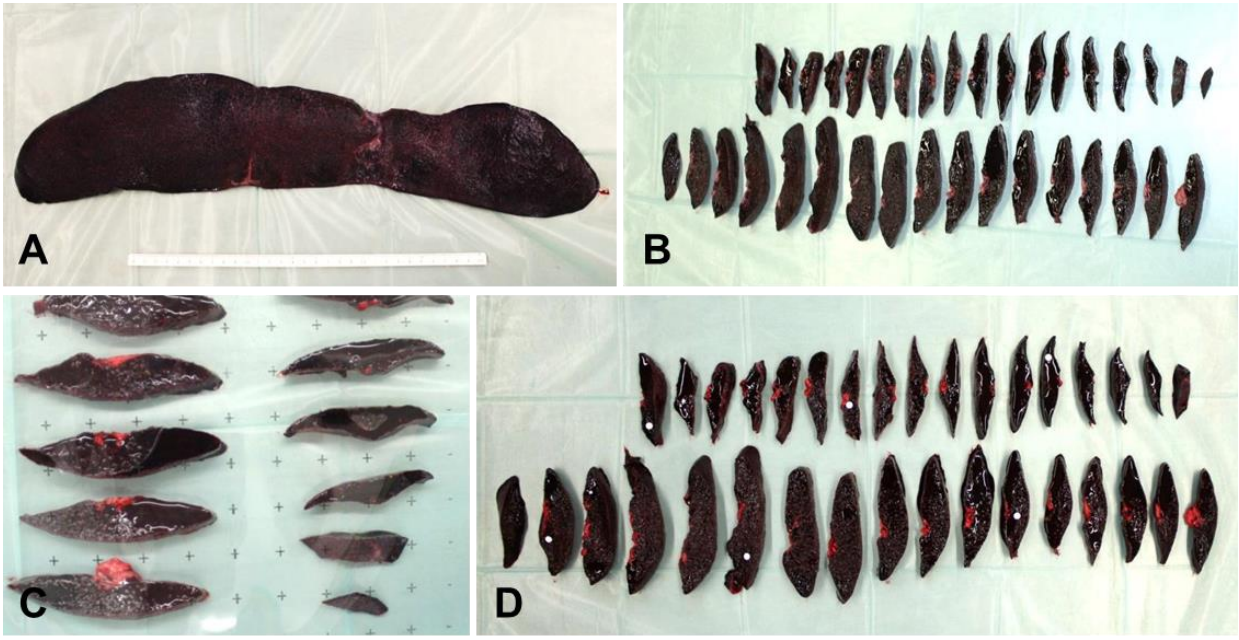


Fig. S229. *Systematic random sampling of the spleen.* ***A****: Excised spleen.* ***B****: Serially sectioned spleen.* ***C****: Systematic random sampling with overlaid cross grid transparency.* ***D****: The sampled positions are marked with pieces of confetti paper.*

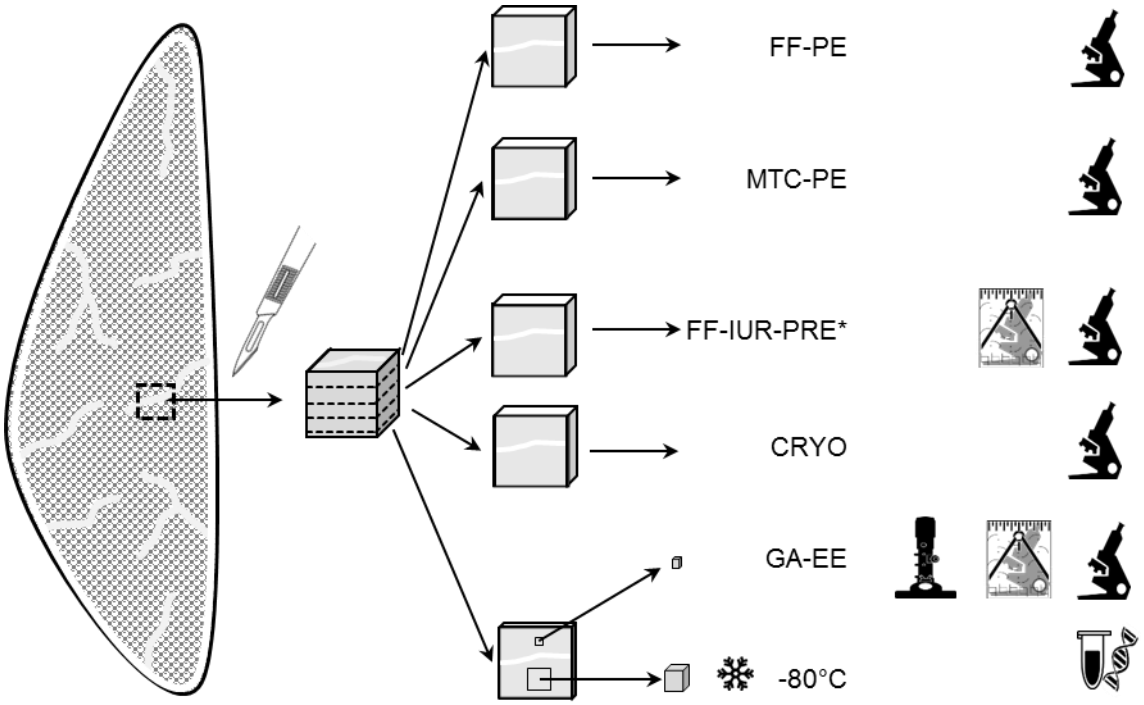


Fig. S230. *A schematic illustration of subsample preparation from one systematically randomly sampled location of spleen tissue for downstream analyses (Type-II/III studies). *PR = GMA/MMA.*

Table S34. The type and number of spleen samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

	Number of locations/samples	
Sample type	**Type-II studies**	**Type-III studies**
Sampled locations (per spleen)	*6*	*10*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 out of every 3rd location
CRYO	1 out of every 3rd location	1 out of every 3rd location
GA-EE	1 per sampled location	1 per sampled location
FF-PRE (IUR)	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

Remarks

The subsamples for paraffin histology and PRE consist of ~5-mm-thick slabs taken from each of the systematically randomly sampled spleen tissue samples (Fig. S230).

An additional thinner ~2-mm-thick slab is taken from each of the systematically randomly sampled spleen tissue specimens (Fig. S230). From this slab, the subsamples for molecular analyses and GA fixation and Epon embedding are excised.

Time and personnel requirements

At least 20 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Docampo et al., 2014, Fry and McGavin, 2012, Haley et al., 2005, Kuper et al., 2013, Morawietz et al., 2004)

2.10.6. Kidneys

Relevant anatomical features/preparation

Similar to humans, porcine kidneys have a smooth surface and multiple renal papillae. The kidneys are removed from the abdominal cavity after the adrenal glands and the gastrointestinal tract.

Prior to sampling, the kidneys are decapsulated. However, if one kidney is to be fixed by vascular perfusion for advanced analyses (section 2.10.6.2., Fig. S234 and Fig. Fig. S235), the organ is taken from the body directly after euthanasia of the pig. The proximal portion of the ureters and the renal arteries are removed with the kidneys.

General examination parameters

The decapsulated kidneys are evaluated for organ symmetry. The length of the longitudinal axis of each organ is measured, and the weight of each kidney is determined. The kidneys are inspected for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

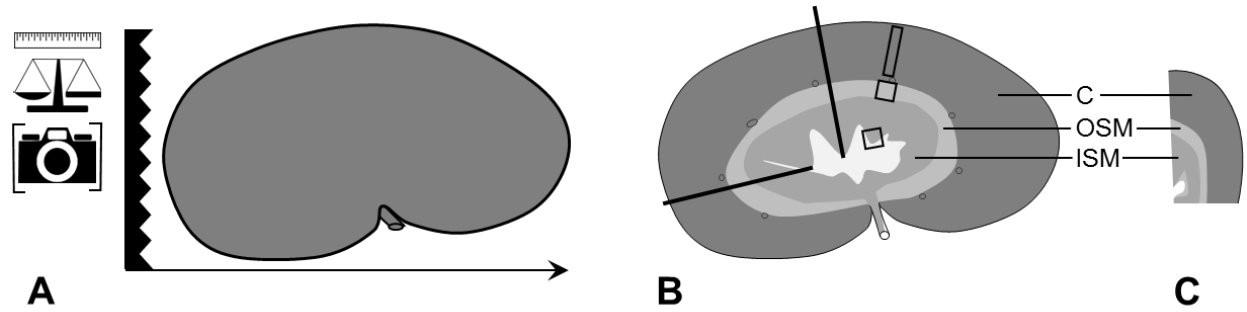


Fig. S231. *A schematic illustration of a kidney after decapsulation (**A**) and after longitudinal sectioning (**B**). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. **C**: Kidney tissue sample for FF-PE histology containing cortex (C), outer zone of the medulla (OZM), inner zone of the medulla (IZM including papilla), and renal pelvis.*

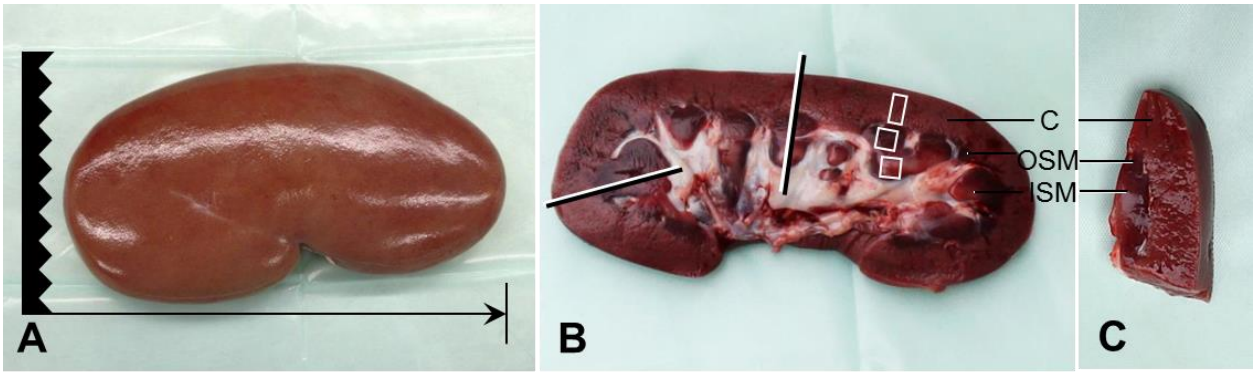


Fig. S232. *Image of one kidney after decapsulation (**A**) and after longitudinal sectioning (**B**). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. **C**: Kidney tissue sample for FF-PE histology containing cortex (C), outer zone of the medulla (OZM), inner zone of the medulla (IZM including papilla), and renal pelvis.*

2.10.6.1. Kidney samples in Type-I studies

Samples for molecular analyses

Location and number of samples:	For each kidney, three specimens are taken: Cortex (C), outer zone of medulla (OZM), and inner zone of the medulla (IZM, including the papilla). The sampling locations are indicated in Fig. S231 and Fig. S232.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number and orientation of sections:	Two sections (middle of the organ and caudal pole) are taken per kidney as indicated in Fig. S231 and Fig. S232. Kidney tissue samples for FF-PE histology should contain the cortex (C), outer zone of the medulla (OZM), inner zone of the medulla (IZM including papilla), and renal pelvis.
Fixation/Embedding:	FF-PE (Fig. S233).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one median longitudinal section through one kidney and one median transverse section through the other kidney. Both sections should include the renal pelvis and papilla (Morawietz et al., 2004).

Considering the size of the porcine kidneys, a comparable sampling technique is not applicable in pigs. The Type-I-study sampling protocols recommend the generation of samples from all kidney compartments from two different locations per organ. This sampling method is considered sufficient for an adequate evaluation of the porcine kidney in routine studies.

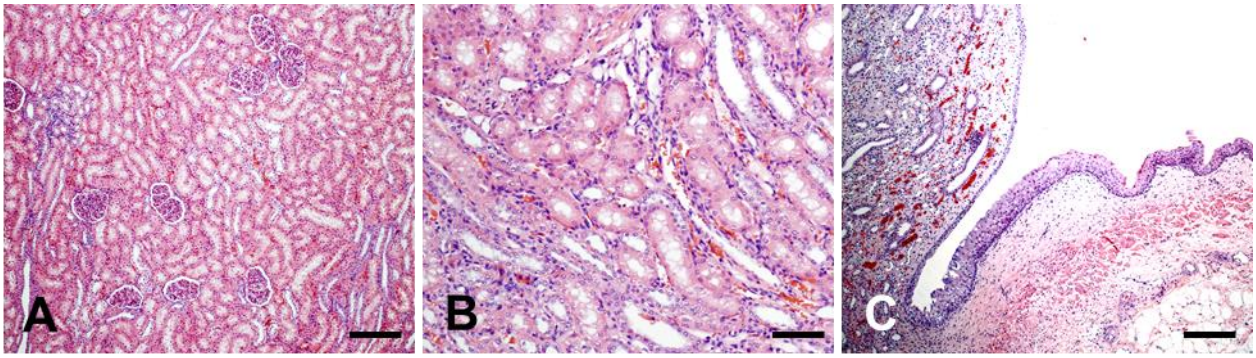


***Fig. S233. Kidney histology. A**: Cortex. **B**: Outer zone of medulla. **C**: Inner zone of medulla and renal pelvis. FF-PE. HE. Scale bars = 100 µm.*

2.10.6.2. Kidney samples in Type-II and Type-III studies

General sampling strategy

The kidney is a bilateral organ, and the size of a pig kidney provides sufficient tissue for virtually unlimited sample numbers. Thus, one kidney can be preserved with vascular perfusion for subsequent qualitative and quantitative histopathological analyses. Samples for molecular analyses and special fixation/embedding techniques are collected from the unfixed fresh kidney. Perfusion fixation is advantageous for quantitative stereological analysis because the organ is nearly fixed “in situ” while maintaining the morphology of structures, such as glomeruli (Blutke et al., 2014, Herbach et al., 2009, Wanke et al., 2001, Renner et al., 2013). If no perfusion fixation is to be performed, both kidneys are sampled as fresh unfixed kidneys.

Vascular perfusion fixation (one kidney)

Allocation of the right or the left kidney for perfusion fixation is determined randomly. Prior to perfusion, the perfusion equipment is prepared. Warmed PBS buffer and a 4% neutrally buffered formaldehyde (FA) or paraformaldehyde solution (PFA) are put into separate containers, such as 2.0 liter plastic bottles with cut off bottoms, and hung approximately 1.5 m above the level of the kidney. The containers are each connected with infusion tubes and mounted infusion flow controllers. The buffer and the fixative tube are connected with a three-way valve and a single downstream perfusion tube optionally connected to a perfusion cannula (Fig. S234).

For vascular down draught perfusion fixation, one kidney is removed from the body directly after the euthanasia of the pig. The proximal portion of the ureter and renal artery are removed with the kidney (Fig. S235). Note that the renal artery usually splits into two main branches before entering the renal hilum. For a successful perfusion, it is advantageous to cut the renal artery proximal to its branch point (Fig. S235). Directly after removal of the organ, an appropriately sized plastic tube from an infusion kit or a large diameter infusion cannula is inserted in the renal artery or the main artery branches if the common origin of the vessel was not recovered. Next, the kidney is perfused with warm PBS buffer until the organ color turns a pale and the outflow from the renal vein is clear, which can take approximately two minutes. For support, the kidney can gently be patted with the hand. Next, the perfusion solution is changed to FA (PFA) for approximately six minutes. The perfused kidney is then post-fixed by immersion in 4% FA (or PFA) for 12-24 hours at RT until further processing.

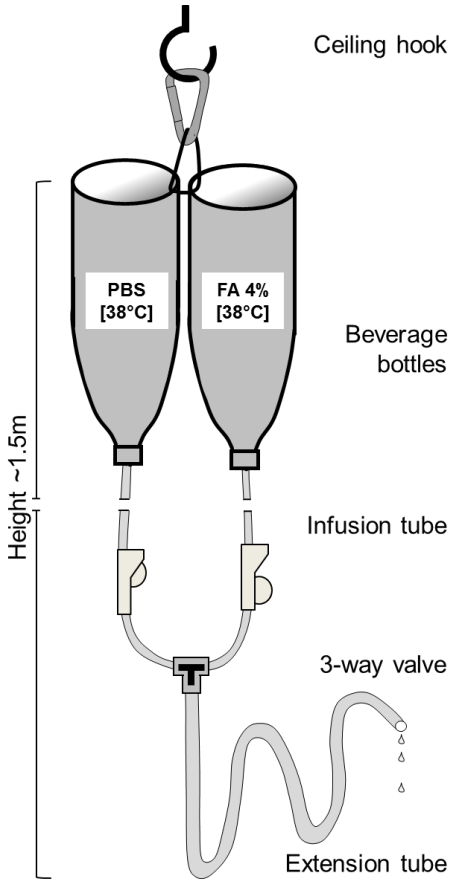


Fig. S234. A schematic illustration of the perfusion equipment.

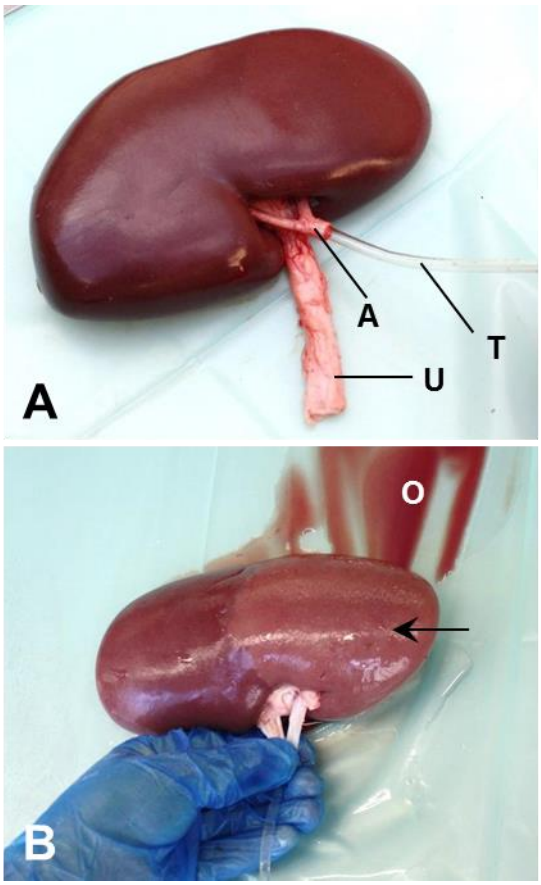


Fig. S235. Vascular perfusion fixation of a kidney. A*: Renal artery (A), ureter (U), perfusion tube (T).* ***B****: Following perfusion of one branch of the renal artery, the corresponding portion of the kidney turns a pale color. Outflow (O) of the renal vein.*

Samples of fresh unfixed kidney tissue (Type-II and -III studies)

The sampling of the fresh kidney is performed as outlined below and indicated in Fig. S236-S240. The weight of the unfixed fresh kidney and the length of its longitudinal axis are determined. The kidney is cut into parallel equidistant slabs approximately 15 mm in thickness orthogonal to the longitudinal axis of the organ. The first section is randomly positioned between 0 and 15 mm from the margin of the organ (Fig. S236 and Fig. S238). The tissue slabs are counted and placed on the same side. Systematic random sampling is separately performed for the kidney cortex (C), outer zone of the medulla (OZM), and inner zone of the medulla (IZM, including the papilla). These regions can be differentiated macroscopically with an adequate degree of certainty (Fig. S238). The kidney slabs are overlaid with a cross grid printed on a transparency (grid size: 2-3 cm, depending on the organ size, number and area of slabs, and the respective kidney compartment to be sampled). The sampled locations can be marked by placing pieces of confetti paper on the respective positions (Fig. S238). An approximately 1x1x1.5-cm tissue piece is excised from each of the sampled locations. The excised tissue specimens are subdivided for downstream analyses (Fig. S239 and Table S35).

In addition, three larger pieces of kidney tissue containing the cortex, OZM and IZM are collected for FF-PE (Fig. S240). The type and number of fresh kidney samples for histopathological examination and molecular analyses in Type-II and -III studies are listed in Table S35.

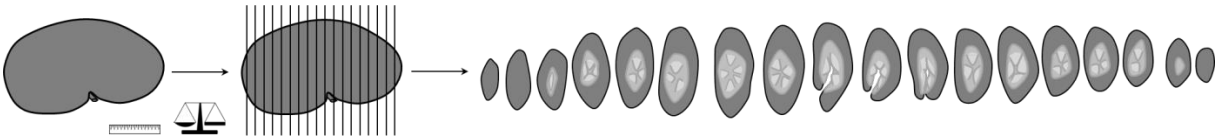


Fig. S236. *Serial sectioning of the kidney for systematic random sampling: Equidistant (~1.5 cm) sections are cut orthogonal to the longitudinal axis of the organ. The resulting kidney slices are counted and laid on the same side.*

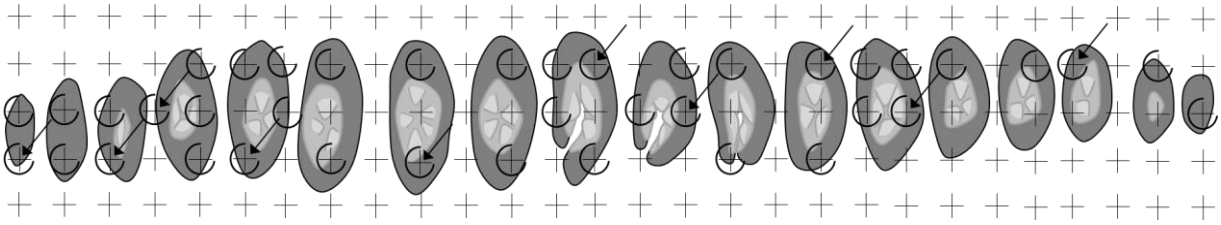


Fig. S237. *A schematic illustration of the systematic random sampling of the renal cortex: The kidney slices are overlaid with a cross grid (2-4 cm) printed on a plastic transparency. In the present example, 40 crosses hit the renal cortex (circled crosses). Every fourth point is sampled (arrows), and the first sampling position is randomly determined between locations 1 and 4.*

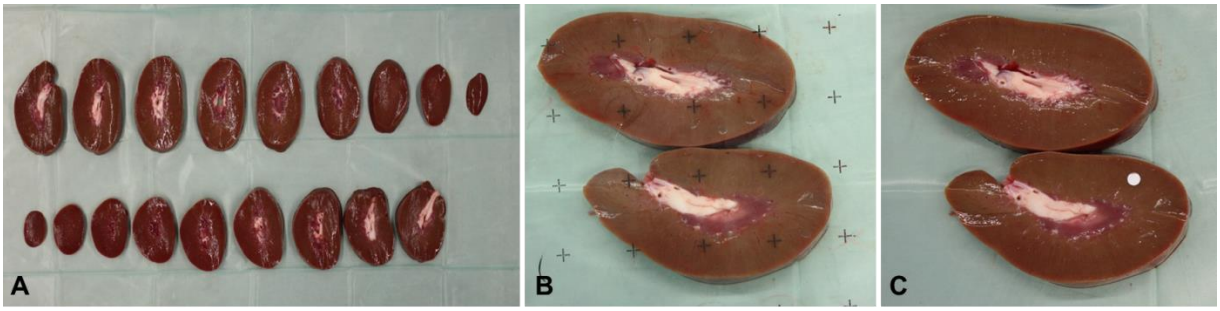
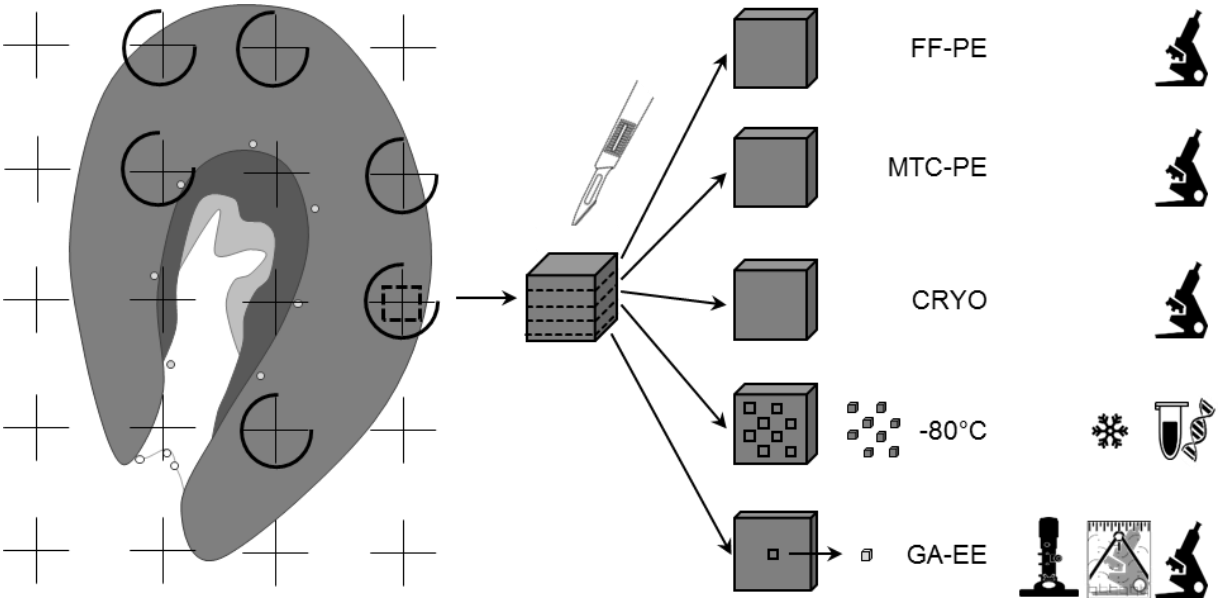


Fig. S238. *Systematic random sampling of the renal cortex: The kidney slices (**A**) are overlaid with a cross grid (2-4 cm) printed on a plastic transparency. **B**: Detailed enlargement of A. The sampled positions can be marked by small pieces of confetti paper (**C**).*



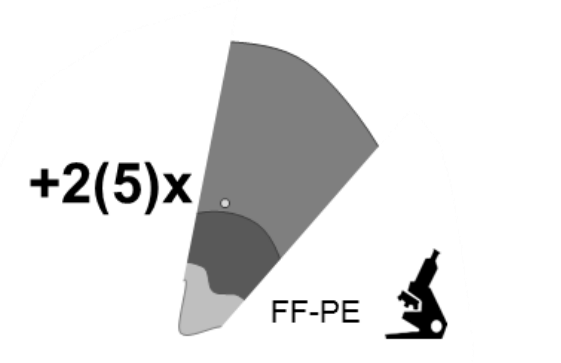


Fig. S239 (↑). *Processing of systematically randomly sampled kidney tissue specimens* ***of the unfixed (fresh) kidney*** *(e.g., renal cortex). From each sampled location (~1x1x1.5 cm), subsamples are generated for FF-PE, MTC-PE, cryo-histology, GA-EE, and molecular analyses.*

Fig. S240 (←). *In addition, two (Type-II studies) or five (Type-III studies) larger pieces of kidney tissue containing cortex, OZM and IZM are collected for FF-PE.*

Table S35. The type and number of samples of the unfixed fresh kidney for histopathological examination and molecular analyses in Type-II and Type-III studies.

Renal compartment Sample type	Number of sampled locations/samples per location Type-II studies	 Type-III studies
Cortex	*6 locations*	*10 locations*
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 per sampled location
CRYO	1 out of every 2nd location	1 per sampled location
GA-EE	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location
OZM	*3 locations*	*5 locations*
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 per sampled location
CRYO	1 per sampled location	1 per sampled location
GA-EE	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location
IZM	*3 locations*	*5 locations*
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 per sampled location
CRYO	1 per sampled location	1 per sampled location
GA-EE	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location
Cortex, OZM & IZM (FF-PE)	2	5

OZM: Outer zone of the medulla; IZM: Inner zone of the medulla (including the renal papilla)

Remarks

If the second kidney is not perfusion fixed, it is sampled in the same manner as the first kidney. Half of the number of samples are taken per kidney. If necessary, one additional sample from each sampled location is taken for FF-PRE (GMA/MMA) and processed for IUR sectioning (ORIENTATOR).

Samples of the perfusion fixed kidney (Type-II and -III studies)

After immersion post-fixation for 12-24 hours, the length of the longitudinal axis of the perfusion fixed kidney is determined. The volume of the kidney can be determined directly via volume displacement (Fig. S241). The perfused kidney is then serially sectioned and systematically randomly sampled as described for the unfixed fresh kidney. One subsample for paraffin embedding for subsequent ISH and IHC analyses, one subsample for FF-GMA/MMA embedding (Fig. S242), and one subsample for Epon embedding is taken from each of the sampled locations (Fig. S243). The specimens designated for GMA-MMA embedding are processed for IUR sections using the ORIENTATOR technique (section 1.6). In addition, three larger pieces of kidney tissue containing the cortex, OZM and IZM are collected for FF-PE (Fig. S243). The type and number of samples generated from the perfusion fixed kidney in Type-II and Type-III studies are listed in Table S36.

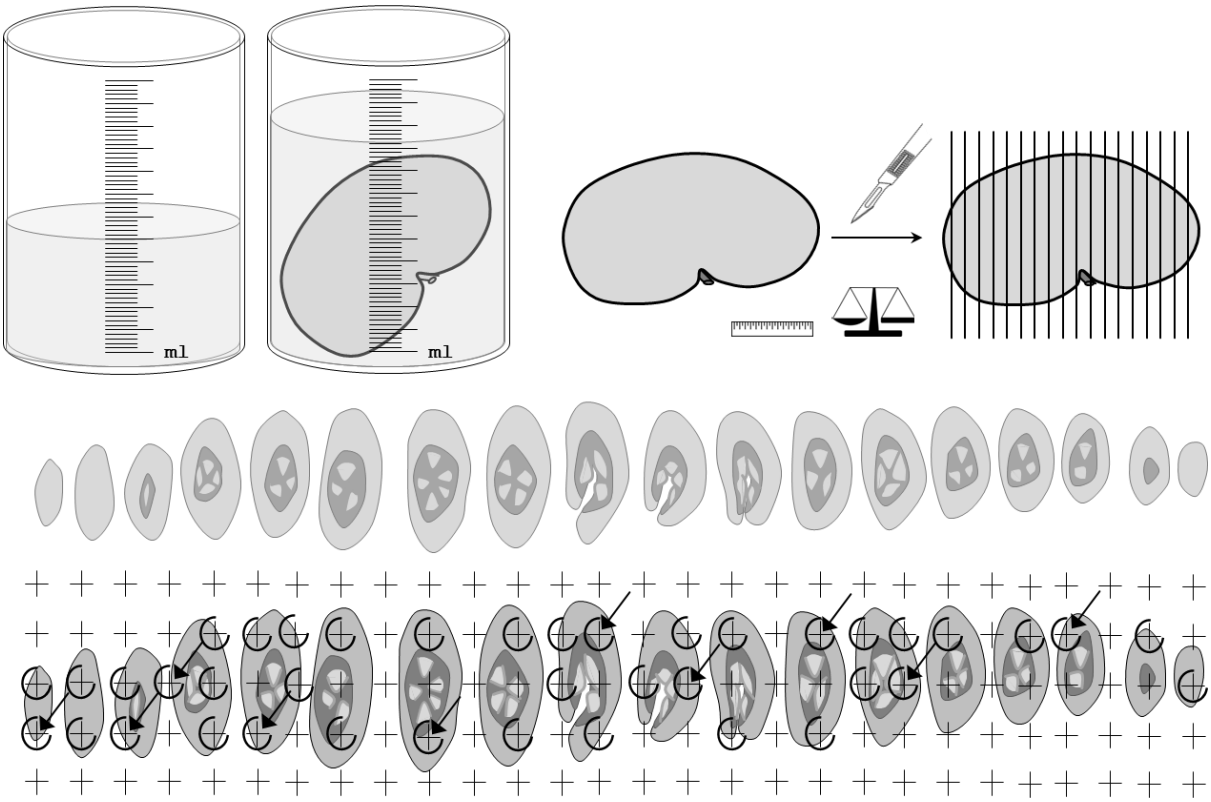


Fig. S241. *A schematic illustration of volumetry of the perfusion-fixed kidney, serial sectioning of the kidney, and systematic random sampling (shown for the cortical renal compartment).*

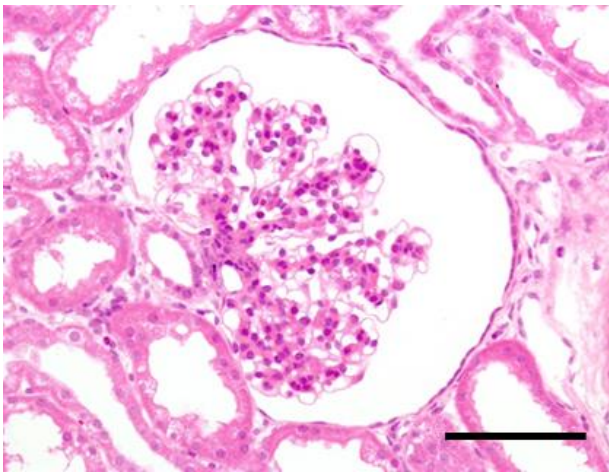
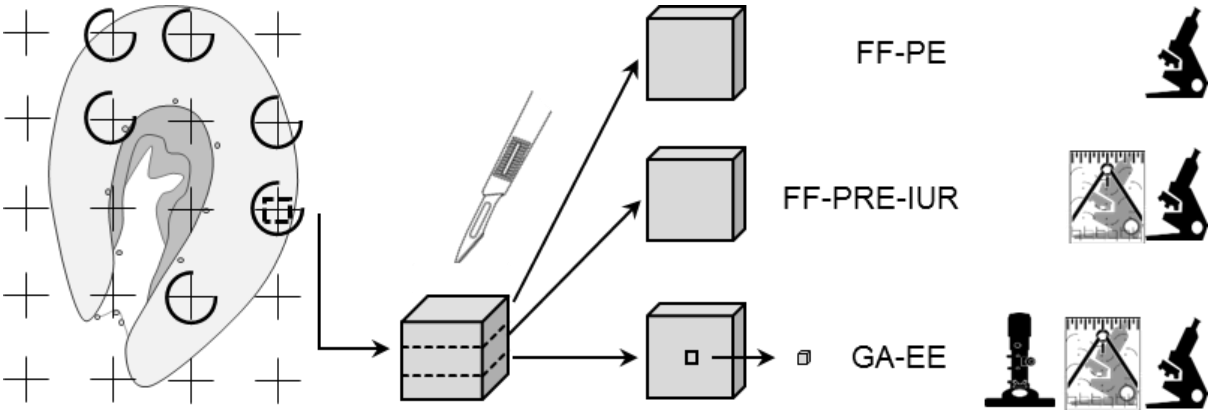


Fig. S242. Glomerular histology after vascular perfusion fixation*. Note the absence of blood cells in the widely dilated glomerular capillaries. GMA/MMA. HE. Scale bar = 100 µm.*



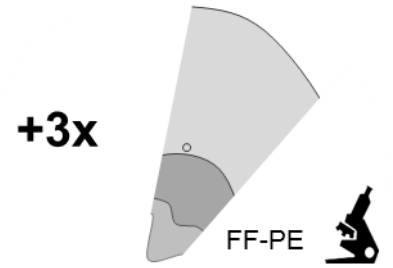


Fig. S243 (↑). *Processing of systematically randomly sampled kidney tissue specimens* ***from the perfusion-fixed kidney*** *(e.g., renal cortex). From each sampled location (~1x1x1.5 cm), subsamples for FF-PE, FF-PRE (GMA/MMA)-IUR, and GA-EE are generated.*

Fig. S244 (←). *In addition, three larger pieces of kidney tissue containing cortex, OZM and IZM are collected for FF-PE.*

Table S36. The type and number of samples of the perfusion fixed kidney for histopathological examination in Type-II and Type-III studies.

Renal compartment Sample type	Number of sampled locations/samples per location Type-II and Type-III studies
Cortex	*10 locations*
FF-PE	1 per sampled location
FF-PRE (GMA/MMA)-IUR	1 per sampled location
GA-EE	1 per sampled location
OZM	*5 locations*
FF-PE	1 per sampled location
FF-PRE (GMA/MMA)-IUR	1 per sampled location
GA-EE	1 per sampled location
IZM	*5 locations*
FF-PE	1 per sampled location
FF-PRE (GMA/MMA)-IUR	1 per sampled location
GA-EE	1 per sampled location
Cortex, OZM & IZM (FF-PE)	3

OZM: Outer zone of the medulla; IZM: Inner zone of the medulla (including the renal papilla)

Time and personnel requirements

At least 20 minutes (35 minutes with perfusion fixation of one kidney) must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator and one assistant. The indicated time does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Frazier et al., 2012, Herbach et al., 2009, Khan et al., 2013, Morawietz et al., 2004, Newman, 2012, Nyengaard, 1999, Wanke et al., 2001)

2.10.7. Ureters, urinary bladder and urethra

Relevant anatomical features/preparation

After removal of the gastrointestinal tract, the bladder is removed from the abdominal cavity with the kidneys, ureters, urethra, accessory sexual glands, and the penis or the ovaries, Fallopian tubes, uterus, cervix, and vagina. Prior to further dissection of the bladder, a sample of urine may be taken by cystocentesis. The cystocentesis urine is frozen at -80°C until further analysis. Subsequent urine analyses can include protein, electrolyte, and metabolite analyses, as well as freezing point depression osmolality analysis. The bladder is carefully opened to avoid damage of the mucosa. The incision lines are indicated in Fig. S246.

Fig. S245. *Refractometric determination of the urine density.*

General examination parameters

The organ size (dimensions) and weight (full and emptied) of the bladder and the volume and color of the urine are determined. The density of the urine is measured using a refractometer (Fig. S245). If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

2.10.7.1. Ureter, urinary bladder, and urethra samples in Type-I studies

Samples for molecular analyses

Location and number of samples:	Urinary bladder mucosa (one sample), ureters (one per side), and mucosa of the proximal urethra (one sample) in male and in female pigs as indicated in Fig. S246 and Fig. S247.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, protein, OMICS profiling.

Histology samples

Location, number and orientation of sections:	The sampling locations are indicated in Fig. S246 and Fig. S247. The samples include one longitudinal and one cross section (full thickness) of the dorsal bladder wall, two sections of the trigone of the bladder at the entry of the ureters into the bladder, two cross sections of the ureter (right and left) and one cross section of the urethra distal to the urinary bladder (in male and in female pigs).
Fixation/Embedding:	FF-PE (Fig. S248 and Fig. S250).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice (Morawietz et al., 2004) recommend the preparation of one longitudinal vertical section through the entire urinary bladder after the instillation of fixative (0.05 ml in mice and 0.2 ml in rats). Transverse or longitudinal sections are prepared from both ureters (Morawietz et al., 2004). The urethra is typically present in longitudinal and horizontal sections of the prostate gland in male mice (Kittel et al., 2004).

Considering the larger size of the porcine urinary bladder and the necessity to incorporate the collection of samples for molecular analyses into the sampling protocol, a comparable sampling technique is not applicable in pigs. The Type-I-study sampling protocols recommend the generation of samples from three different locations of the urinary bladder and cross sections of both ureters and the proximal urethra to allow for an adequate evaluation of the porcine lower urinary tract in routine studies.

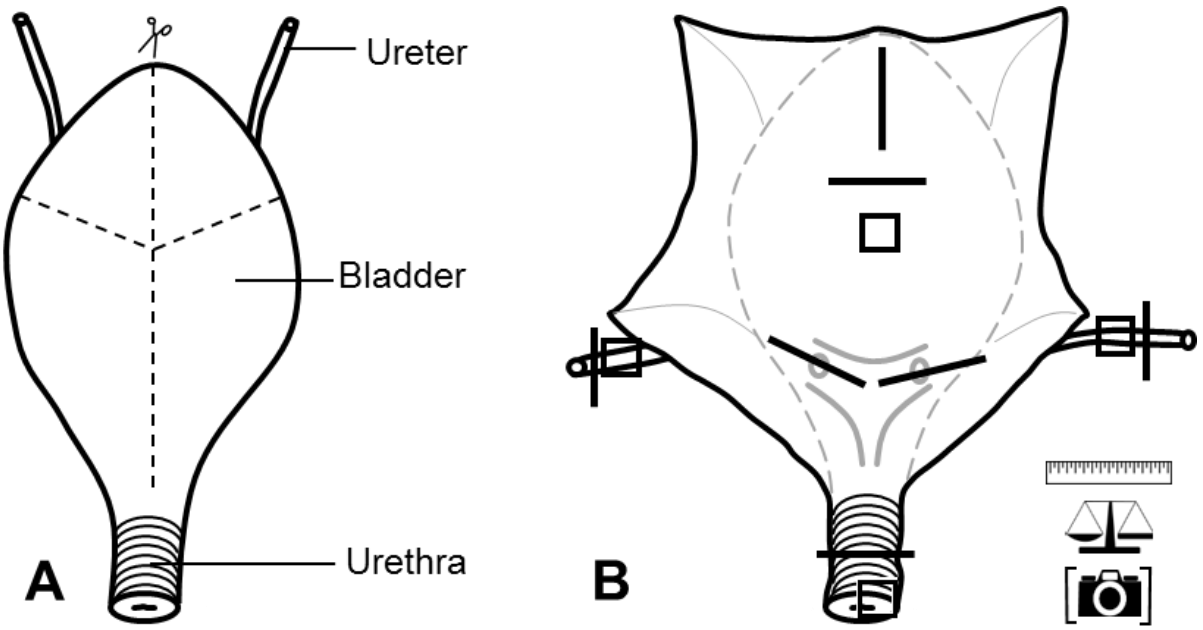


Fig. S246. *A schematic illustration of the urinary bladder with incision directions indicated (**A**). **B**: The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*

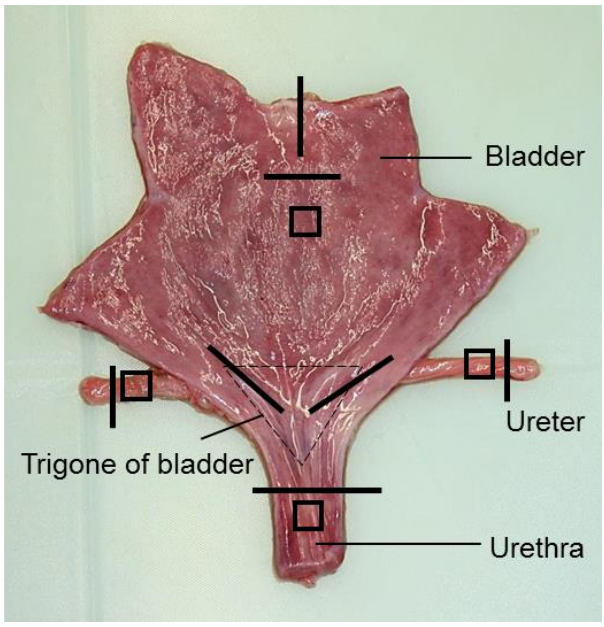


Fig. S247. *Opened urinary bladder. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*

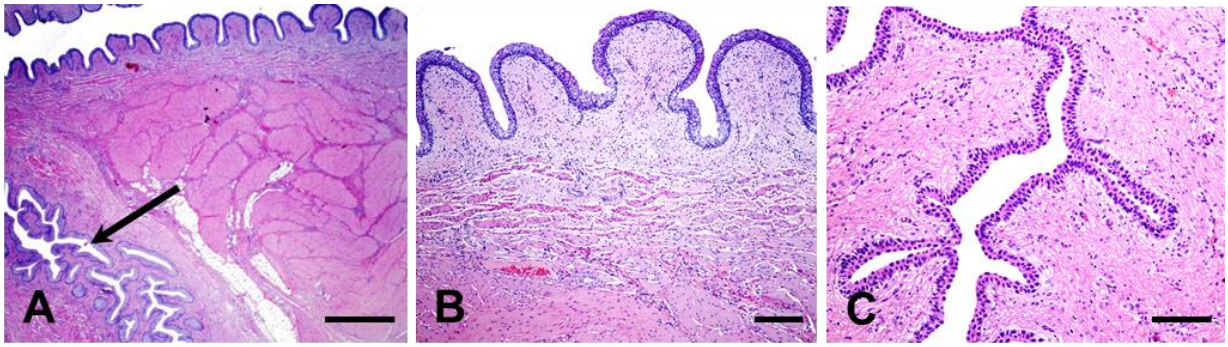


Fig. S248. *Histology of the urinary bladder and the urethra.* ***A****: End of the ureter (arrow) at the trigone of the bladder with adjacent urinary bladder wall tissue.* ***B****: Urinary bladder epithelium.* ***C****: Urethral mucosa. FF-PE. HE. Scale bars = 1 mm (A), 100 µm (B, C).*

2.10.7.2. Urinary bladder samples in Type-II and Type-III studies

This sampling guide does not recommend the generation of additional specimens from the ureters and the urethra for Type-II/III studies.

General systematic random sampling strategy

The sampling positions and organ size-dependent sample numbers are determined by systematic random sampling. The weight and the dimensions of the bladder are determined. The bladder is opened as shown above. The Type-I study histology samples are taken. Next, the opened bladder is overlaid with a cross grid printed on a transparency (grid size: 2-4 cm, depending on the size/area of the bladder). The systematic random sampling of six tissue locations is performed as described in section 1.4. and shown in Fig. S249. While avoiding damage the sensitive mucosa, the sampled positions can be marked by placing pre-moistened pieces of confetti paper on the respective positions. From each of the sampled locations, an approximately 1.5x1.5 cm sized full-thickness tissue piece is excised. The excised tissue specimens are subdivided for downstream analyses (Fig. S251). For generation of VUR-sectionable samples, round specimens are punched out from the urinary bladder with the mucosal side up near the systematically randomly sampled locations using a hole punch and hammer (Fig. S249 and Fig. S251). The type and number of urinary bladder samples for histopathological examination and molecular analyses in Type-II and Type-III studies are listed in Table S37.

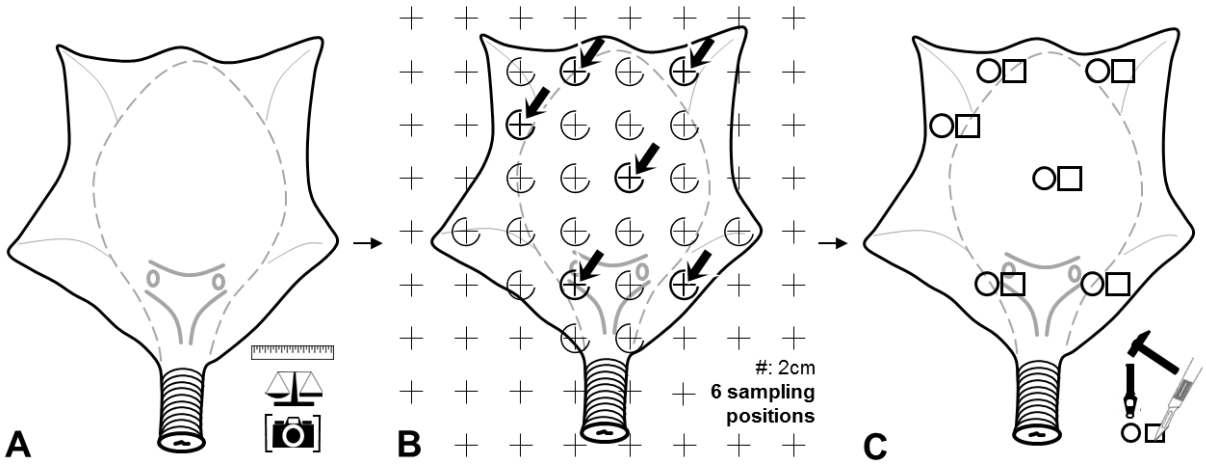


Fig. S249. *A schematic illustration of urinary bladder sampling in Type-II/III studies.*

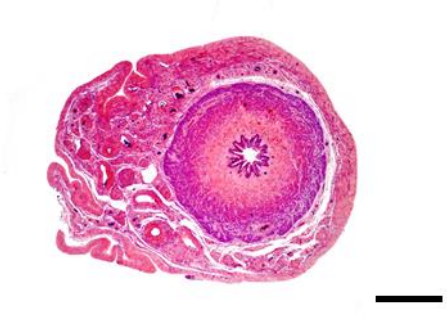


Fig. S250. Histology of the ureter *(distal portion). FF-PE. HE. Scale bar = 1 mm.*

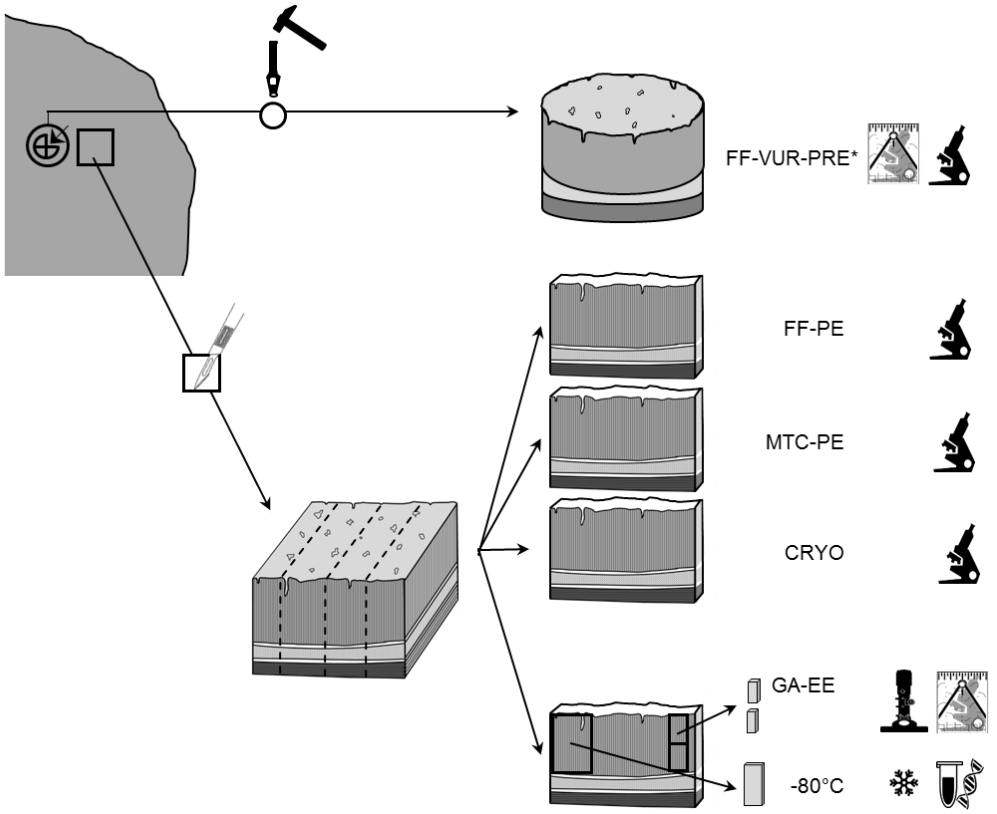


Fig. S251. *A schematic illustration of subsample preparation from one systematically randomly sampled location of urinary bladder for downstream analyses (Type-II/III studies). *PR = GMA/MMA.*

Table S37. The type and number of urinary bladder samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

	Number of sampled locations/samples per location	
Sample type	**Type-II studies**	**Type-III studies**
Sampled locations	*6*	*6*
	Number of samples	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 per sampled location
CRYO	1 out of every 3rd location	1 per sampled location
GA-EE	1 per sampled location	2 per sampled location
FF-PRE (IUR)	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

Remarks

The subsamples for molecular analyses are excised from the urinary bladder mucosa.

Time and personnel requirements

At least 20 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator. This estimate includes the time from the removal of the organ/tissue to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Cohen, 2013, Frazier et al., 2012, Morawietz et al., 2004, Newman, 2012, Vovk et al., 2009)

2.10.8. Adrenal glands

Relevant anatomical features/preparation

The adrenal glands are located cranio-medial to the renal hilum. Typically, the adrenal glands are quite difficult to directly identify because they are largely embedded in adipose tissue. It is advantageous to localize and collect the adrenal glands before the kidneys are removed from the body. The right adrenal gland is directly attached to the wall of the caudal caval vein and is generally slightly larger, and thus easier to find, than the left gland.

General examination parameters

The sizes (dimensions) and weights of both adrenal glands are determined. Here, special attention is given to the evaluation of symmetry, size, presence of gross pathological alterations and to the cortical/medullary sectional area ratio in transverse and mid-longitudinal sections. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

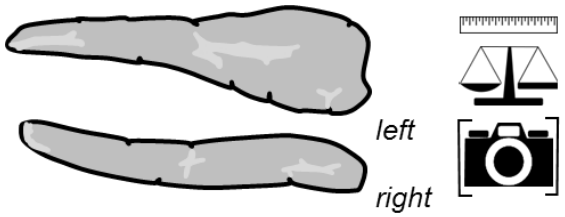


Fig. S252. *Adrenal glands, schematic.*

2.10.8.1. Adrenal gland samples in Type-I studies

Samples for molecular analyses

Location:	Separate samples of the cortex and of the medulla of both glands are taken. Sampling locations are indicated in Fig. S253-S255.
Number of samples:	Two per side.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).
Remarks:	Cortical samples are cut from cross-sectioned slabs of the glands as indicated in Fig. S253 and Fig. S255.

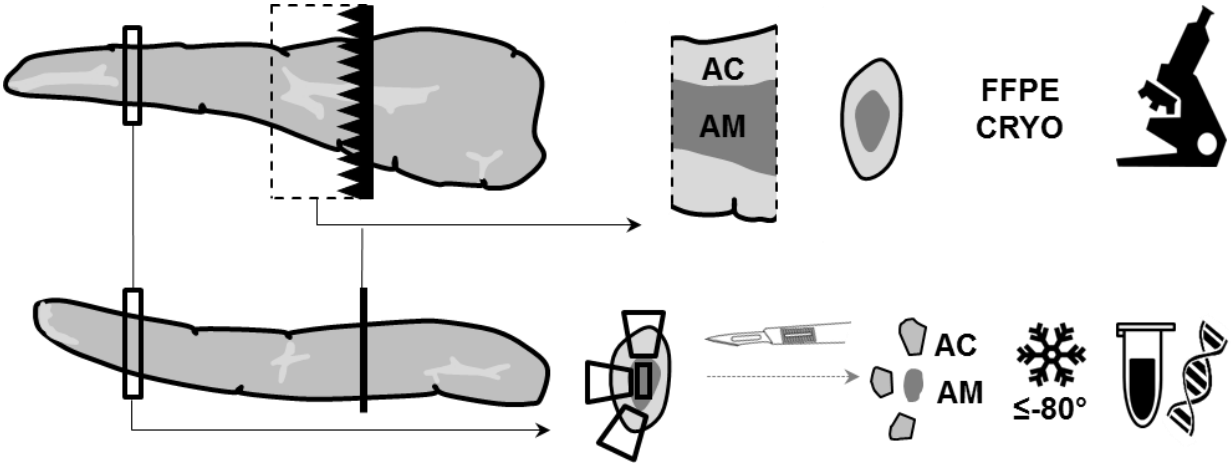


Fig. S253. *Adrenal gland sampling scheme for routine screenings (Type-I studies). The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples are indicated. AC: Adrenal cortex. AM: Adrenal medulla.*

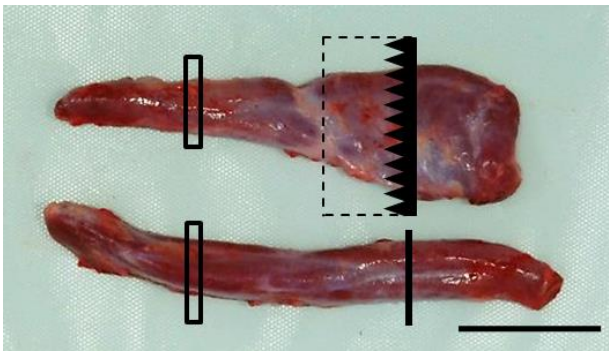


Fig. S254. Adrenal glands. *The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. Scale bar = 1 cm.*

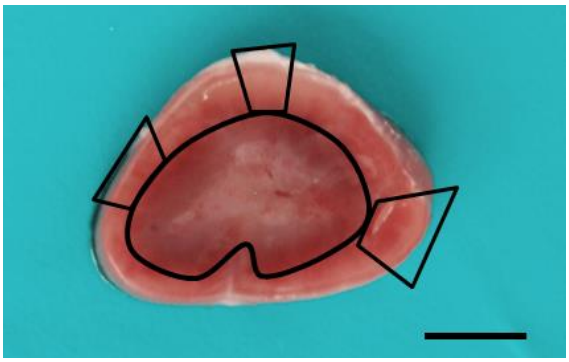


Fig. S255. Adrenal gland cross section (fresh tissue). *The cortex and medulla are clearly distinguishable. The excision borders of cortical and medullary tissue for the generation of samples for molecular analyses are indicated. Scale bar = 1 cm.*

Histology samples

Location, number and orientation of sections:

For each gland, two sections including the adrenal cortex and the medulla are prepared as indicated in Fig. S253. A horizontal section from the left middle portion of the adrenal gland and one cross section from the middle of the right adrenal gland is prepared as indicated in Fig. S253 and Fig. S254.

Fixation/Embedding:

FF-PE (Fig. S256).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the embedding of both organs *in toto* and the subsequent preparation of longitudinal sections at the level of the largest cut surface (Kittel et al., 2004).

Considering the larger size of porcine adrenal glands, a comparable sampling technique is not applicable in pigs. The Type-I-study sampling protocols recommend the generation of differentially orientated sections from both organs that include all adrenal gland compartments, for an adequate evaluation of the porcine adrenal gland in routine studies.

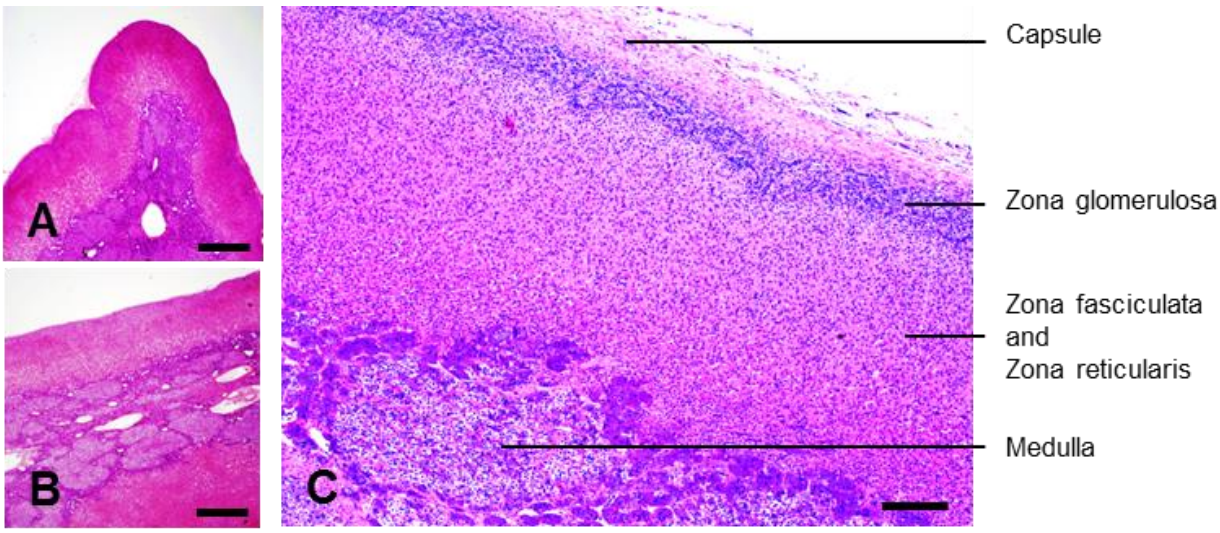


Fig. S256. Adrenal gland histology. A*: Cross section.* ***B****: Longitudinal section. C: Detailed enlargement. FF-PE. HE. Scale bars = 1 mm (A, B), 100 µm (C).*

2.10.8.2. Adrenal gland samples in Type-II and Type-III studies

General sampling strategy

The sampling positions and sample numbers are determined by systematic random sampling. The weights and lengths of both adrenal glands are determined. Next, the organs are cut into parallel slabs approximately 5 mm thick orthogonal to the longitudinal axis of the organ. The first section is randomly positioned between 0 and 5 mm from the margin of the organ (Fig. S257 and Fig. S258). The slabs are placed on the same side on a transparency. To determine the total volume of the adrenal gland(s), separated cortical and medullary volume of the adrenal glands by Cavalieri volumetry, and embedding-related tissue shrinkage, the section surfaces of the adrenal gland slabs are scanned in conjunction with a scale. Fig. S257 shows an example of extensive sampling of the adrenal gland (Type-III study).

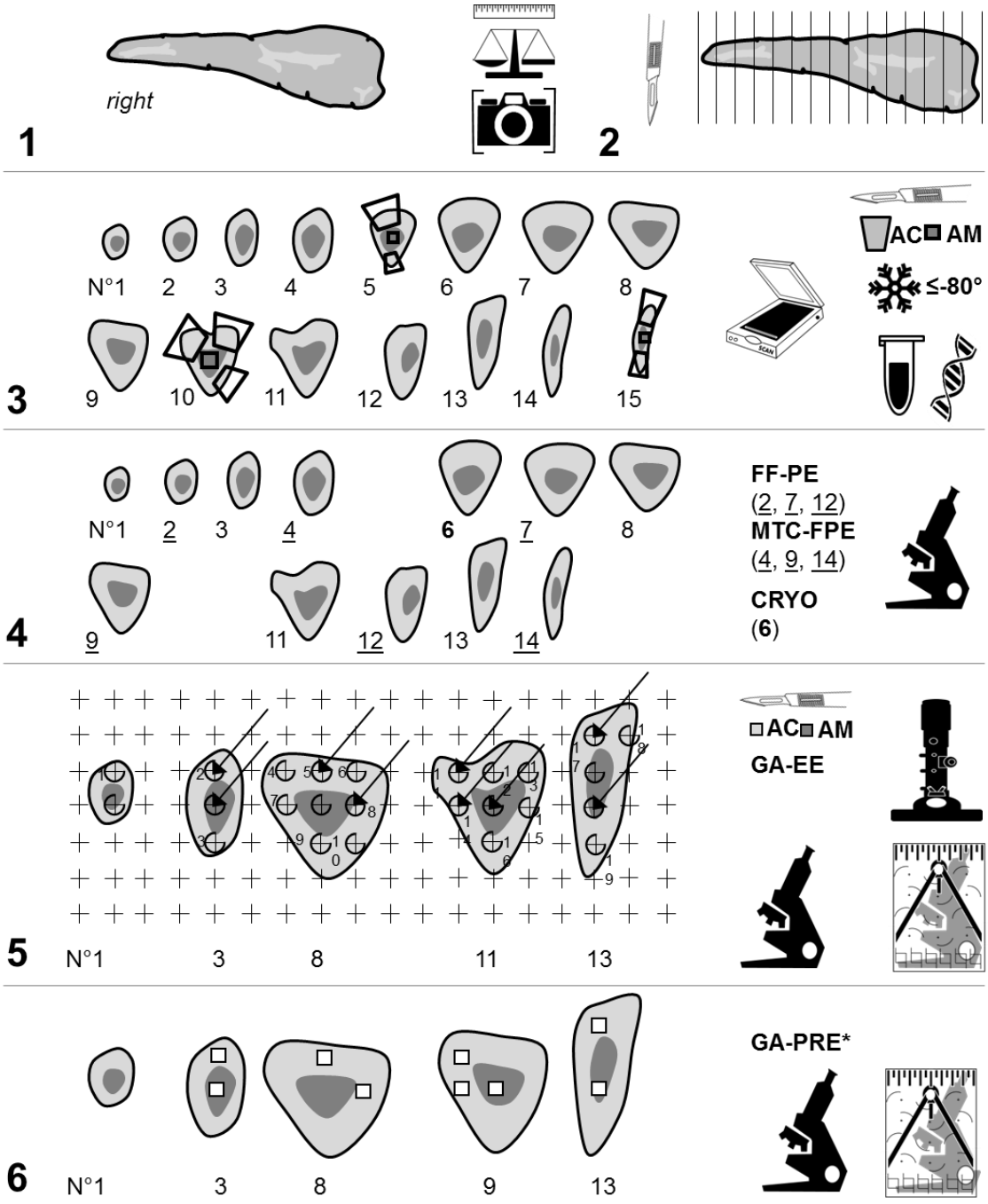


Fig. S257. *A schematic illustration of the sampling of the right adrenal gland in Type-II/III studies.*PR = GMA/MMA. AC: Adrenal cortex. AM: Adrenal medulla.*

Samples for molecular analyses

Location:	Separate samples of the adrenal cortex and adrenal medulla are taken from 2-3 systematically sampled slabs of the left and right adrenal gland. In the example shown in Fig. S257, three slabs are sampled. From 15 slabs, every third (N° 5, 10, and 15) specimen is sampled, and the first section (N° 5) is randomly selected.
Number of samples:	Three (study Type-II) or ten (study Type-III) individual cortical and medullary samples are collected per gland.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).
Remarks:	Cortical samples are cut from cross-sectioned slabs of the glands as indicated in Fig. S255 and Fig. S257.

Fig. S258. *Sliced fresh adrenal glands for subsequent sampling (studies Type-II and -III). Scale bar = 2 cm.*

Samples for histopathological examination

Paraffin embedded specimen

Locations, numbers and sizes of samples, fixation:	From the remaining slabs, every second slab is systematically sampled (N° 2, 4, 7, 9, 12, 14). From these slabs, every second specimen is fixed with a 4% formaldehyde solution (N° 2, 7, 12) or MTC (N° 4, 9, 14) prior to paraffin embedding (Fig. S257).
Downstream analyses:	Histology, IHC, ISH, and quantitative stereological parameters independent of embedding-related tissue shrinkage.

Specimen for cryo-histology

Location, number and size of samples, fixation:	From the remaining slabs, one slab (per side) containing both cortical and medullary compartments is deliberately selected (N° 6) and processed for cryo-histology. (Fig. S257).
Downstream analyses:	Cryohistology, IHC, IF-IHC, and special stains, such as oil red O.

Specimen for plastic resin embedding for semithin section histology, electron microscopy, and quantitative stereological analyses

Location of samples:	From the remaining tissue slabs (N° 1, 3, 8, 11, 13), six cortical and three medullary samples are taken per gland. The sampling positions are determined by systematic random sampling. The tissue slabs are overlaid with a 5-10 mm cross grid transparency. Points hitting the respective compartment (adrenal cortex or medulla) are counted. For example, 19 points hit the adrenal cortex in the example provided in Fig. S257. Therefore, every third location is sampled for collection of six specimens (19:3 = 6.33). The first location is randomly between locations 1 and 3 using a random number table. In the example, locations 2, 5, 8, 11, 14, and 17 are sampled. Cortical specimens of ~2x2x2 mm size are cut from the tissue using a scalpel or razor blade. The remaining tissue is processed for GMA/MMA embedding (Fig. S257).
Number of samples:	For each gland, six cortical and three medullary samples are taken for Epon embedding, and 3-5 tissue slabs are collected for GMA/MMA-embedding per gland.
Processing:	Sampled specimens are fixed in a 2.5% glutaraldehyde solution and embedded in Epon. The remaining tissue slabs are fixed in a 4% formaldehyde solution and embedded in GMA/MMA.
Downstream analyses:	Plastic histology, semithin section histology, electron microscopy, and quantitative stereological analyses.
Remarks:	If necessary, IUR sectionable samples can be prepared from Epon-embedded or GMA/MMA-embedded tissue samples by re-embedding the samples in spherical casting molds (ISECTOR).

Time and personnel requirements

At least 20 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator. This estimate includes the time from the removal of the organs to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Blutke et al., 2015, Hoeflich et al., 2002, Kittel et al., 2004, La Perle, 2012, Rosol et al., 2013)

2.11. Genital system

2.11.1. Male genital tract

2.11.1.1. Testes, epididymides and spermatic cords

Relevant anatomical features/preparation

After careful incision of the internal spermatic fascia, the testes are removed from the cavum vaginale in the scrotum with the epididymides and spermatic cords. The testes, epididymides, and spermatic cords are then separated and sectioned as shown in Fig. S259 and Fig. S260.

General examination parameters

The dimensions and weights of the testes and the epididymides of both sides are determined. The testes, epididymides and spermatic cords are examined for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required. A detailed summary of the examination of testes, epididymides, and male accessory sex glands recommended by different regulatory guidelines is provided by Lanning et al. (2002).

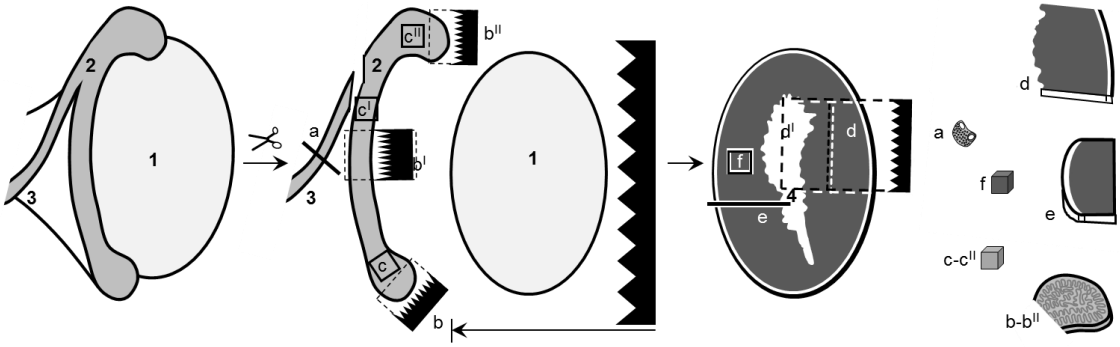
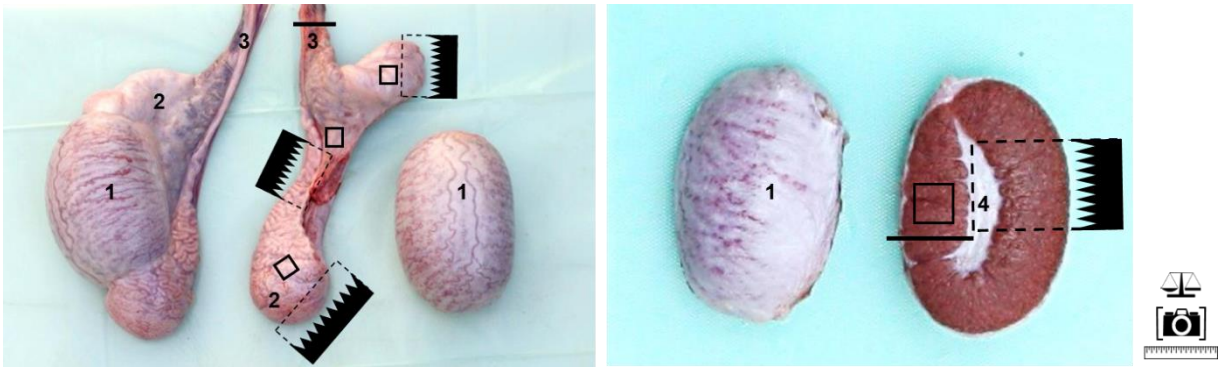


Fig. S259 (↑) and Fig. S260 (↓). *A schematic illustration and image of the testes (**1**), epididymides (**2**), spermatic cords (**3**),and the rete testis (**4**). The indicated sampling locations (a-f) for Type-I studies refer to section 2.11.1.1.1. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated.*



2.11.1.1.1. Testis, epididymis, and spermatic cord samples in Type-I studies

Samples for molecular analyses (testes and epididymides)

Location and number of samples:	Both testes, epididymides, and spermatic cords are sampled. The sampling locations and section directions are indicated in Fig. S259 and Fig. S260. Per side, one sample of the testicular parenchyma (f), one sample of the spermatic cord (a), and one sample of the head, body, and tail of the epididymis (c-c^{II}) are collected.
Remarks:	Homogeneous samples are cut from the testicular parenchyma while avoiding specimens with a heterogenous composition of the capsule and rete.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

Histology samples

Location, number, and orientation of sections:	After collection of the samples for molecular analyses, the right and left testes, epididymides and spermatic cords are fixed and subsequently trimmed. The sampling locations and section directions are indicated in Fig. S259 and Fig. S260. **Testes**: One horizontal (d) and one transverse section (e) are prepared per side. Testicular sections should contain the capsule, parenchyma (d) and the rete testis (d'). If the samples are too large to fit into standard embedding cassettes, the samples are cut into smaller pieces (d, d'). **Epididymides**: One longitudinal section of the head, body, and tail (b-b^{II}). **Spermatic cord**: One cross section per side (a).
Fixation/Embedding:	Modified Davidson's fixative (30% of a 37-40% solution of formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled H_2O) is recommended for histology of the testes and epididymides, particularly in fertility studies. Small piglet testes may be fixed *in toto*. For fixation of large testes from adult boars, the tissue is sectioned into 1-2 cm thick slices prior to fixation. After ~48 hours of fixation in modified Davidson's fixative, the samples can be trimmed and embedded in paraffin (Fig. S261), or transferred into a 4% neutrally buffered formaldehyde solution for storage.

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodent and non-rodent species The Recommended Approaches for the Evaluation of Testicular and Epididymal Toxicity recommend the sampling of both testes and epididymides in all species (Lanning et al., 2002). Modified Davidson's fixative is recommended for fixation. Transverse and longitudinal sections from the testes, including the rete testis, and longitudinal sections of the head, body, and tail of the epididymis are prepared. For routine studies, paraffin embedding is sufficient. For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of one transverse section of both testes close to the rete testis. Both epididymides are completely sectioned in a longitudinal-vertical orientation (Kittel et al., 2004). The Type-I-study sampling protocols recommend the generation of comparable samples for the adequate evaluation of porcine testes and epididymides in routine studies.

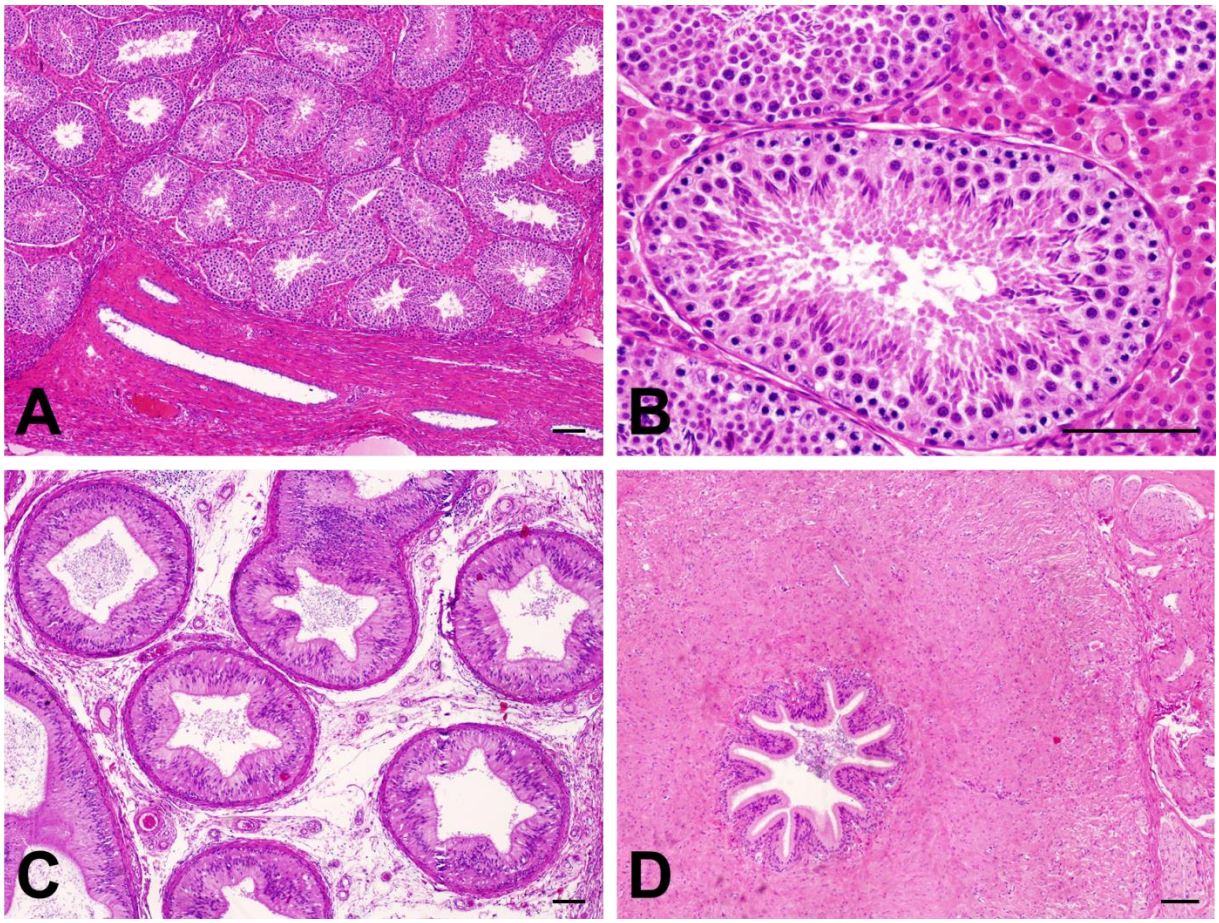


Fig. S261. *Histology of the adult testis (**A**, **B**), epididymis (**C**), and the ductus deferens (**D**). Fixation: Modified Davidson's fixative. Paraffin sections. HE. Scale bars = 100 µm.*

2.11.1.1.2. Testis, epididymis, and spermatic cord samples in Type-II and Type-III studies

General systematic random sampling strategy

The sampling positions and organ size-dependent sample numbers are determined by systematic random sampling. Testicular and epididymal weights and lengths are determined. Next, the testes and the epididymides are cut into parallel equidistant slabs of 1-2 cm thickness orthogonal to the longitudinal axis of the organ. The first section is randomly positioned between 0 and 10-20 mm from the margin of the organ (Fig. S263 and Fig. S264). The organ slabs are then placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 1-2 cm, depending on the organ size and number and area of slabs). The systematic random sampling of tissue locations is performed as described in section 1.4. and indicated in Fig. S264. The sampled positions can be marked by placing pieces of confetti paper on the respective positions.

An approximately 1.5x1.5x2-cm tissue piece, or a smaller sample in younger pigs with smaller genital organs, is excised from each of the sampled locations regardless of the structures contained in the tissue. If the sampled location contains large amounts of the rete testis, this must be excised together with the rest of the specimen to maintain representativity. The excised tissue specimens are subdivided for downstream analyses (Fig. S264). The type and number of testicular and epididymal samples for histopathological examination and molecular analyses in Type-II and -III studies are listed in Table S38. Depending on the experimental design of the specific study, additional samples can be harvested in advance for the evaluation of fertility/sperm motility or the isolation of sperm.

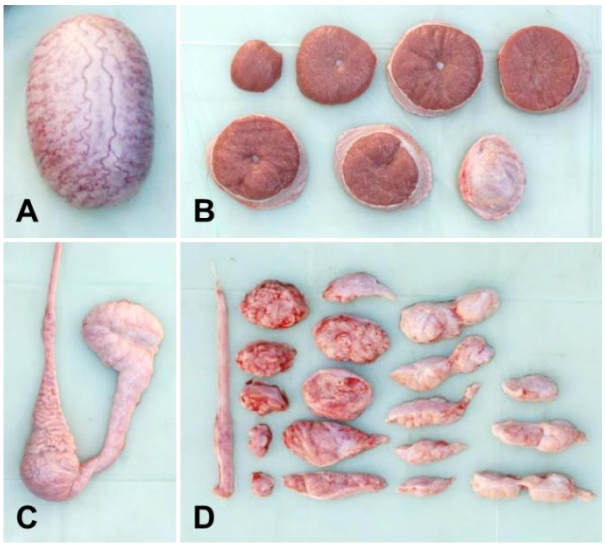


Fig. S262. *Excised and serially sliced testis (**A**, **B**) and epididymis (**C**, **D**) for systematic random sampling.*

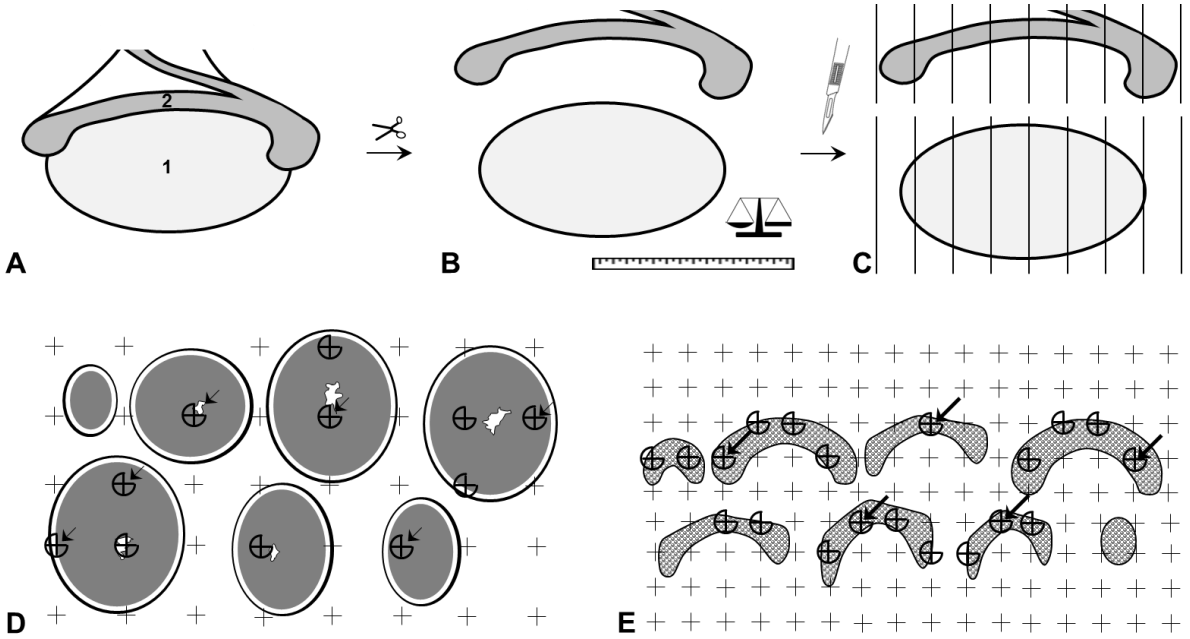


Fig. S263. *A schematic illustration of the systematic random sampling of the testes and epididymides in Type-II/III studies. **A**: Excised testis and epididymis. **B**: The separated testis and epididymis are weighed, measured, and serially sliced (**C**). Systematic random sampling using cross grid transparencies (**D**, **E**).*

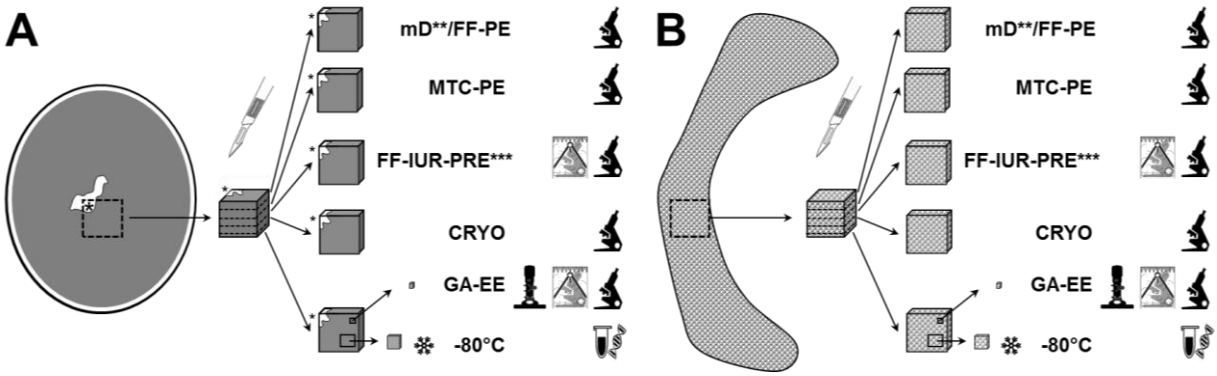


Fig. S264. *A schematic illustration of subsample preparation from one systematically randomly sampled location of testicular (**A**) or epididymal (**B**) tissue for downstream analyses (Type-II/III studies). *Rete testis; **mD = Modified Davidson's fixative; ***PR = GMA/MMA.*

Table S38. The type and number of testicular and epididymal samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

Sample type	Number of sampled locations per side and of samples per location Type-II studies	Type-III studies
Sampled locations per side	*5/6*	*5/6*
	Number of samples	
mD*/FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 out of every 2nd location
CRYO	1 out of every 3rd location	1 out of every 2nd location
GA-EE**	1 out of every 3rd location	1 out of every 2nd location
FF*-PRE (IUR)	-	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

**Modified Davidson's fixative (preferred). **If appropriate, IUR-sectionable ISECTOR samples can be prepared from GA-EE samples. for histology of the testes and epididymides*

Remarks

The subsamples for paraffin histology and PRE consist of ~5-mm-thick slabs taken from each of the systematically randomly sampled tissue samples (Fig. S264).

An additional thinner ~2-mm-thick slab is taken from each of the systematically randomly sampled tissue specimens (Fig. S264). From this slab, the subsamples for molecular analyses and GA fixation and Epon embedding are excised from areas of homogeneous parenchyma (avoid sampling specimens with a heterogenous volume composition of parenchyma, capsule and rete testis).

Time and personnel requirements

At least 30 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator. This estimate includes the time from the removal of the organs/tissues to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature

(Almeida et al., 2006, Creasy et al., 2012, Creasy and Chapin, 2013, Foster, 2012c, Kittel et al., 2004, Lanning et al., 2002, Ljungvall et al., 2008)

2.11.1.2. Male accessory sex glands

Relevant anatomical features/preparation

In male pigs, three major accessory sex glands are macroscopically identifiable and accessible for sampling. These samples include the paired, tan, soft vesicular glands (Glandula vesicularis), the body of the prostate gland, which is located directly adjacent to the beginning of the urethra, and the paired, firm, large bulbourethral glands, which are mostly covered by the bulboglandularis muscle. The accessory sex glands are removed from the body with the bladder. The location and anatomy of the male accessory sex glands is shown in Fig. S265 and Fig. S266. The glands are carefully dissected and isolated. The bulboglandularis muscle (Fig. S266) has to be removed prior to the sampling/weighing of the bulbourethral glands.

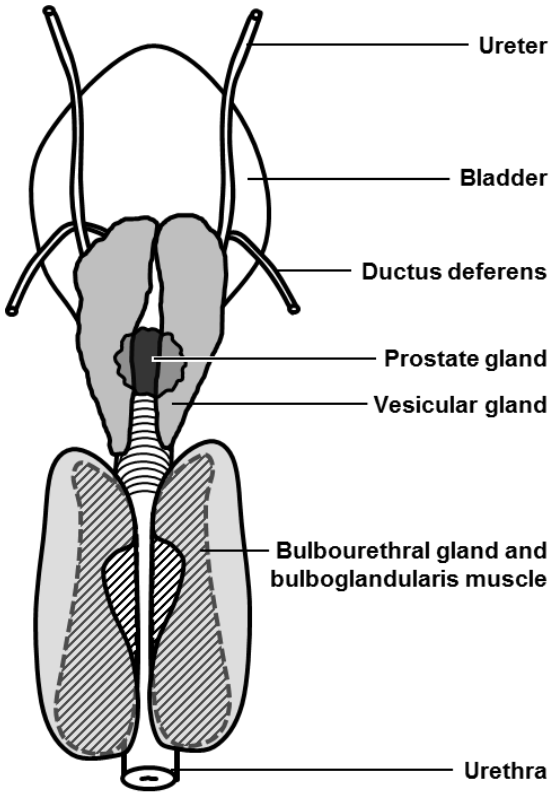


Fig. S265. *A schematic illustration of the accessory male sex glands. Note that most of the bulbourethral gland is covered by the bulboglandularis muscle.*

General examination parameters

The sizes (dimensions) and weights of the accessory sex glands are determined. The glands are then inspected for gross pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required. A detailed summary of the examination of testes, epididymides and male accessory sex glands recommended by different regulatory guidelines is provided by Lanning et al. (2002).

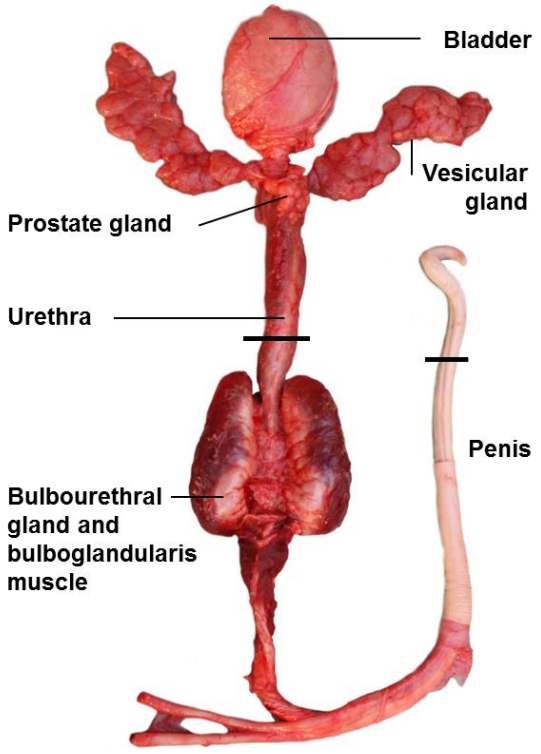


Fig. S266. *Male accessory sex glands. Study Type-I sampling locations for histology samples of the urethra and the penis are indicated by black lines (section 2.11.1.3.).*

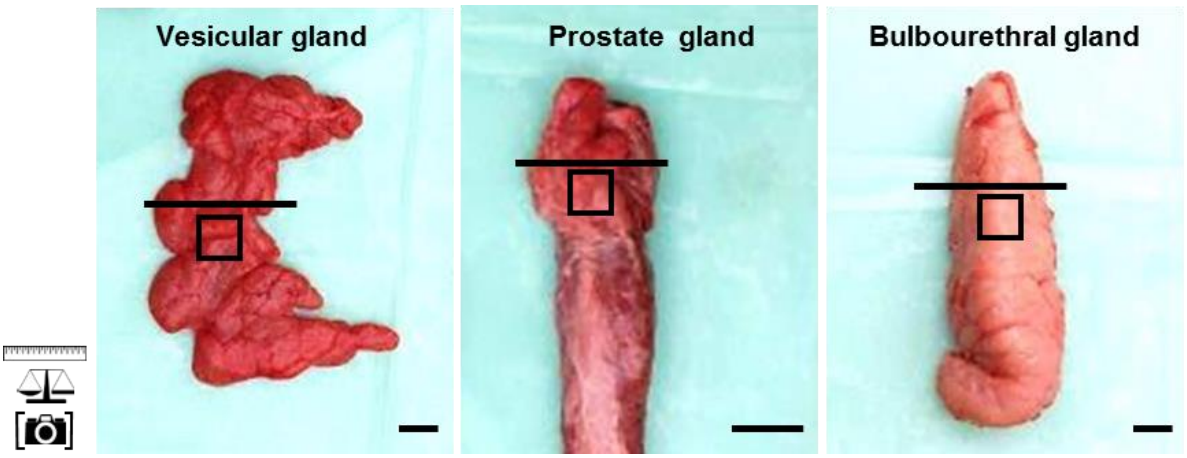


Fig. S267. Dissected and isolated male accessory sex glands. *The locations and orientation of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. Note that the bulboglandularis muscle was removed from the bulbourethral gland.*

2.11.1.2.1. Samples of male accessory sex glands in Type-I studies

Samples for molecular analyses

Location:	The body of the prostate gland, left vesicular gland, and left bulbourethral gland are sampled. One sample is taken from each accessory sex gland. The sampling locations are shown in Fig. S267.
Remarks:	The parenchyma of the bulbourethral gland contains a gelatinous viscous secretion that complicates the cutting and handling of the tissue sample.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses.

Histology samples

Location, number and orientation of sections:	The body of the prostate gland, left vesicular gland, and left bulbourethral gland are sampled. For each accessory sex gland, one sample is taken next to the positions of the samples for molecular analyses. The sampling locations are indicated in Fig. S267.
Fixation/Embedding:	FF-PE (Fig. S268).
Remarks:	Samples for histopathological analyses are best excised from the tissue after fixation.

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of two mid-transverse sections through both seminal vesicles and the adjacent coagulating glands. One longitudinal horizontal section is prepared from the prostate, including the dorsolateral, ventral lobe of the gland, and the urethra (Kittel et al., 2004). The male accessory sex glands of rodents and pigs cannot be directly compared. However, the number, location, and orientation of sections recommended in the Type-I-study sampling protocols allow for an adequate evaluation of porcine male accessory sex glands in routine studies.

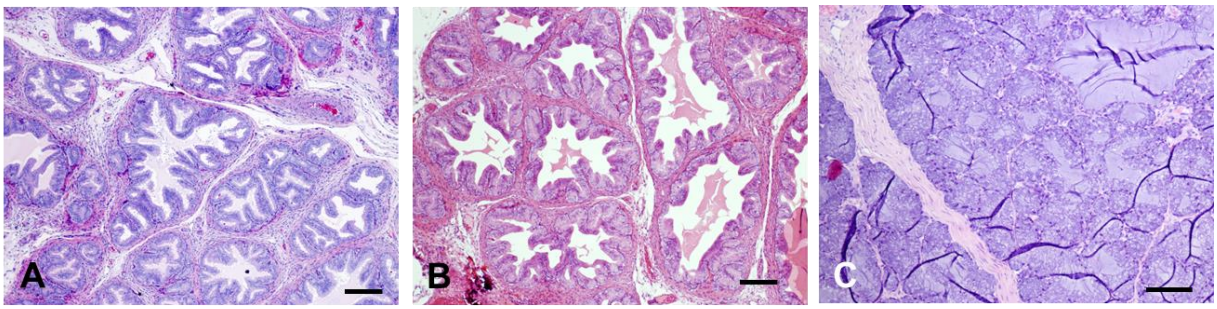


***Fig. S268. Histology of male accessory sex glands**. **A**:.Vesicular gland. **B**: Prostate gland. **C**: Bulbourethral gland. FF-PE. HE. Scale bars = 100 µm.*

2.11.1.2.2. Samples of male accessory sex glands in Type-II and Type-III studies

General systematic random sampling strategy

In each male accessory sex gland, the sampling positions are determined by systematic random sampling. The length along the longitudinal axis and the weight of each gland is determined after removal of the bulboglandularis muscle from the bulbourethral gland. Next, each gland is completely sectioned into parallel equidistant slabs orthogonal to the width axis of the organ. The vesicular and bulbourethral glands are cut in approximately 10 mm thick sections, and the body of the prostate gland is cut into approximately 5 mm thick sections. The first section is randomly positioned between 0 and 10 mm or 0 and 5 mm, respectively, from the margin of the glands (Fig. S269 and Fig. S270).

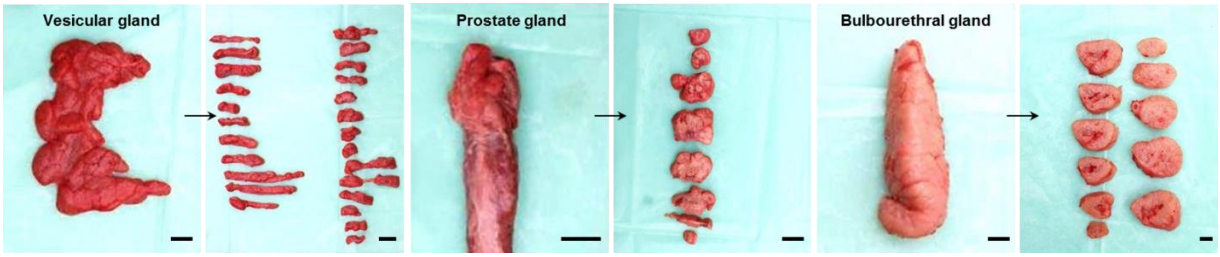


***Fig. S269. Freshly dissected and sliced male accessory sex glands for systematic random sampling in Type-II and Type-III studies.** Scale bars = 1 cm.*

The tissue slabs are placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 5 mm for the prostate gland and ~2 cm for the vesicular and bulbourethral glands, depending on the gland size and number of tissue slabs). The systematic random sampling of six tissue locations per gland is performed as described in section 1.4. and illustrated in Fig. S270. The sampled positions can be marked by placing pieces of confetti paper on the respective positions. An approximately 1.5x1.5x0.5-1-cm tissue piece is excised from each of the sampled locations, regardless of the structures contained in the tissue. The excised tissue specimens are subdivided for downstream analyses (Fig. S271, Table S39).

Table S39. The type and number of male accessory sex gland samples for histopathological examination and molecular analyses in Type-II and Type-III studies.

Sample type (gland)	Number of samples per location Type-II studies	Type-III studies
FF-PE (VG, PG, BUG)	1 per sampled location	1 per sampled location
MTC-PE (VG, BUG)	-	1 out of every 2nd location
CRYO (VG, PG, BUG)	1 out of every 3rd location	1 per sampled location
GA-EE (VG, PG, BUG)	1 per sampled location	1 per sampled location
FF-IUR-PRE* (VG,BUG)	-	1 per sampled location
Molecular analyses (-80°C) (VG, PG, BUG)	1 per sampled location	1 per sampled location

VG: Vesicular gland. PG: Prostate gland. BUG: Bulbourethral gland.: GMA/MMA.*

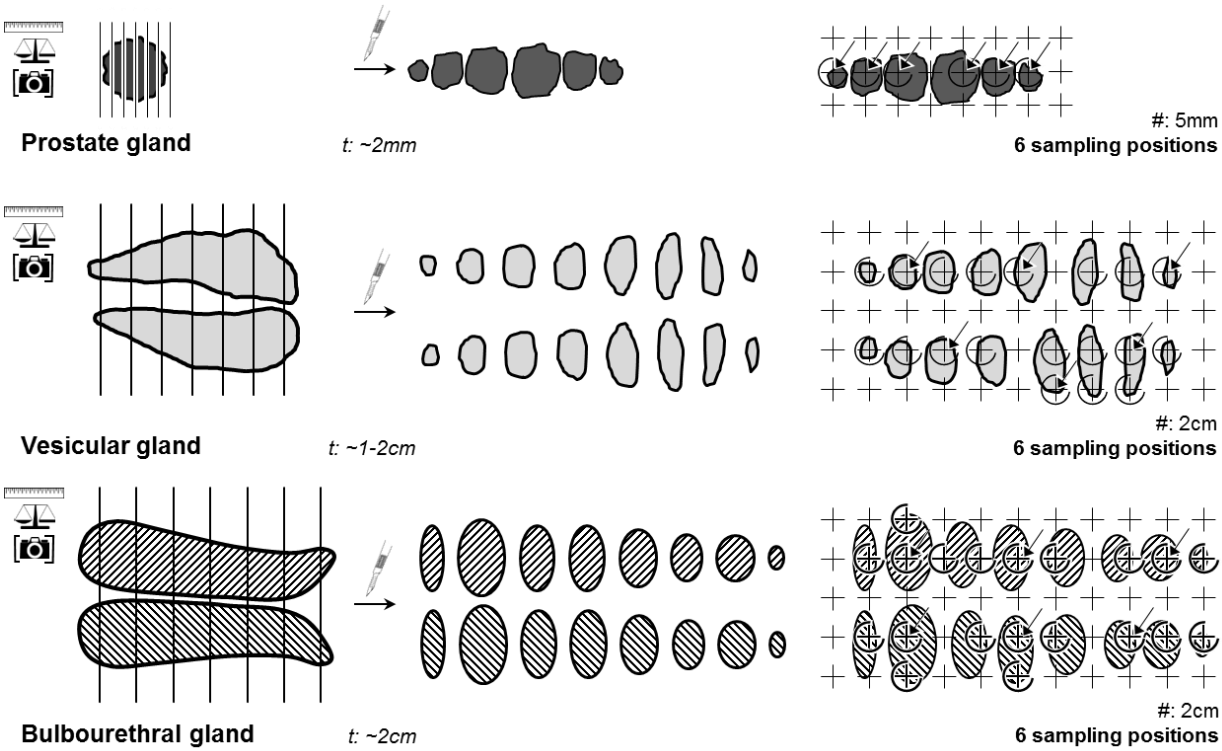


Fig. S270. *A schematic illustration of the systematic random sampling of male accessory sex glands in Type-II/III studies. #: The cross grid size and numbers of samples are indicated.*

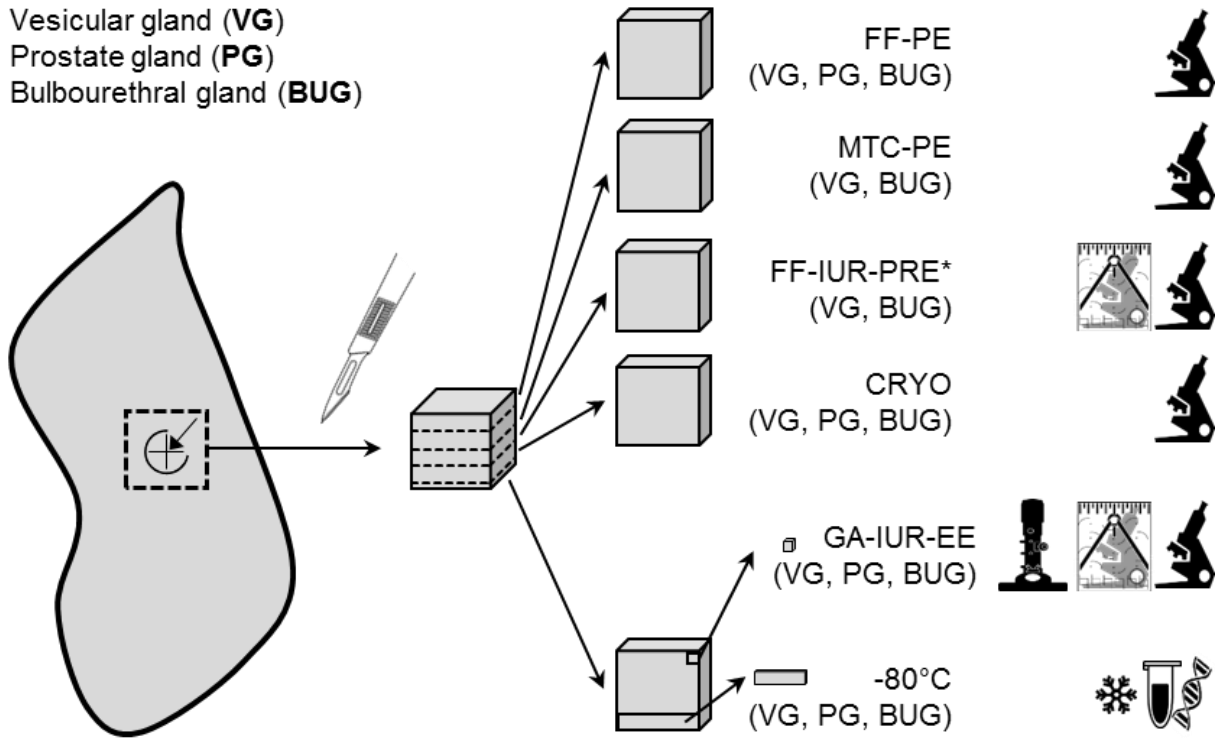


Fig. S271. *A schematic illustration of subsample preparation from one systematically randomly sampled location of male accessory sex gland tissue for downstream analyses in Type-II/III studies. *PR = GMA/MMA.*

Time and personnel requirements
At least 30 minutes must be scheduled for the execution of the complete Type-III-study sampling procedure by an experienced investigator. This estimate includes the time from the removal of the organs/tissues to the freezing/fixation of the sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time needed for further sample processing after fixation.

Related literature
(Creasy and Chapin, 2013, Lanning et al., 2002, Ljungvall et al., 2008)

2.11.1.3. Penis and prepuce

These sampling guidelines recommend the generation of routine (Type-I study) histological (FF-PE) samples of the penis and the prepuce. The generation of advanced analysis samples (study Type-II/III) is not recommended.

Relevant anatomical features/preparation
Male pigs have a fibroelastic penis. For gross examination, the penis is pulled out of the prepuce. The penis is removed from the ventral abdominal wall within its prepuce and cut off at the caudal surface of the pubic arch.

General examination parameters
The penis, prepuce and its dorsal preputial diverticulum (which releases a characteristic smell when incised) are macroscopically examined for pathological alterations. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

Histology samples

Location, number and orientation of sections:	One cross section of the penis, including the corpus cavernosum and urethra, is routinely taken for histopathological analysis. The sampling location is indicated in Fig. S266, section 2.11.1.2. An additional sample of the preputial diverticulum is taken (Fig. S273).
Fixation/Embedding:	FF-PE (Fig. S272 and Fig. S273).
Remarks:	Penis samples for histopathological analyses are best excised after fixation of the tissue.

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies

Separate sections of the penis and urethra are not commonly evaluated by histopathology in regulatory toxicity studies in rodents, although the urethra is usually present in sections taken from the prostate gland in males (Kittel et al., 2004). A cross-sectioned penile sample can be collected quickly and allows for the evaluation of interesting structures, such as the corpus cavernosum, numerous small caliber vessels and the urethra. However, a selective generation of specimens from a macroscopically unaltered penis for histopathological examination in routine toxicity studies involving pigs is not necessary. If animals of “newly developed” pig models are necropsied for an initial pathological examination, penis and prepuce samples should be collected. Therefore, the collection of penis and prepuce tissue samples for histopathological evaluation was included in the porcine Type-I-study sampling protocols.

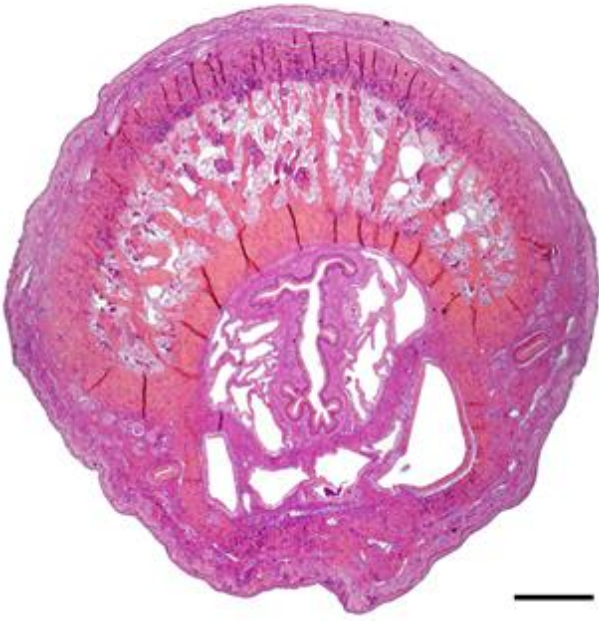


Fig. S272. Histological cross section of the penis with the urethra and corpus cavernosum. *FF-PE. HE. Scale bar = 1 mm.*

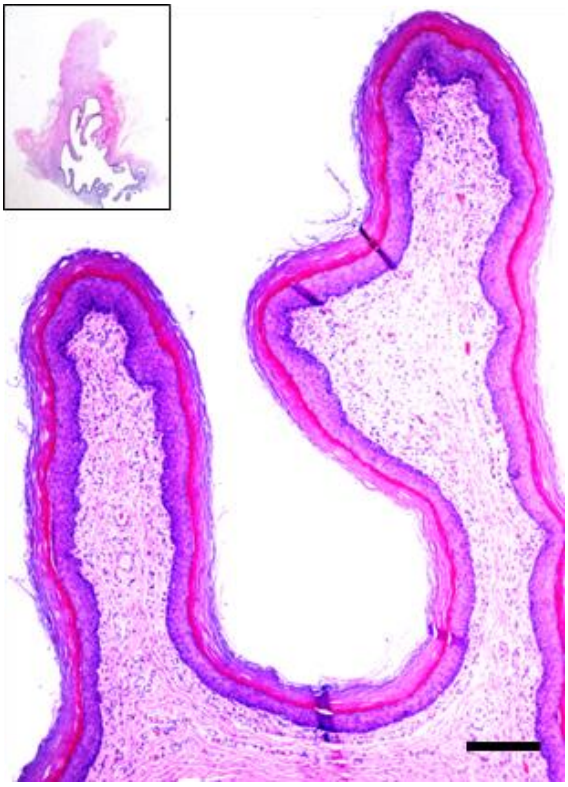


Fig. S273. Histology of the preputial diverticulum. *Top inset: Image of the histology slide. FF-PE. HE. Scale bar = 100 µm.*

Related literature

(Babinski et al., 2005, Creasy and Chapin, 2013, Foster, 2012c)

2.11.2. Female reproductive tract

This guide does not describe the sampling of the pregnant uterus or embryonic or fetal specimens.

Relevant anatomical features/preparation

After removal of the gastrointestinal tract, the female urogenital tract, including the kidneys, ureters, bladder, ovaries, Fallopian tubes, uterus, cervix, and vagina, is removed from the abdominal/pelvic cavity. The kidneys and ureters are separated from the genital tract. The genital tract is then dissected as shown in Fig. S274 and Fig. S275. The vagina, cervix and uterus are opened by a longitudinal incision if the diameter of the organs is large enough. If the diameter of the uterus is ≤1 cm, it is left unopened until the samples have been collected.

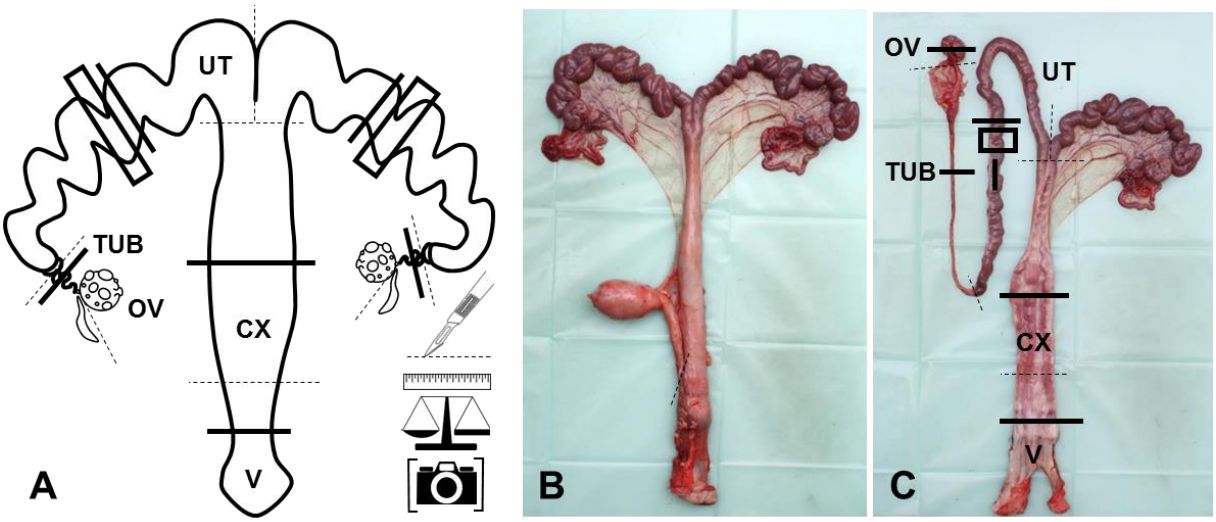


Fig. S274. *A schematic illustration (**A**) and images (**B**, **C**) of the female genital tract. The locations and orientations of the histology (black lines) and molecular analyses (black rectangles) samples for Type-I studies are indicated. Vagina (V), cervix (CX), uterus (UT), Fallopian tube (TUB), ovary (OV). The incision dissection lines are indicated by dashed lines. In Type-I studies, both ovaries, Fallopian tubes, the left and right uterine horn, body of the uterus, cervix and vagina are sampled (not indicated).*

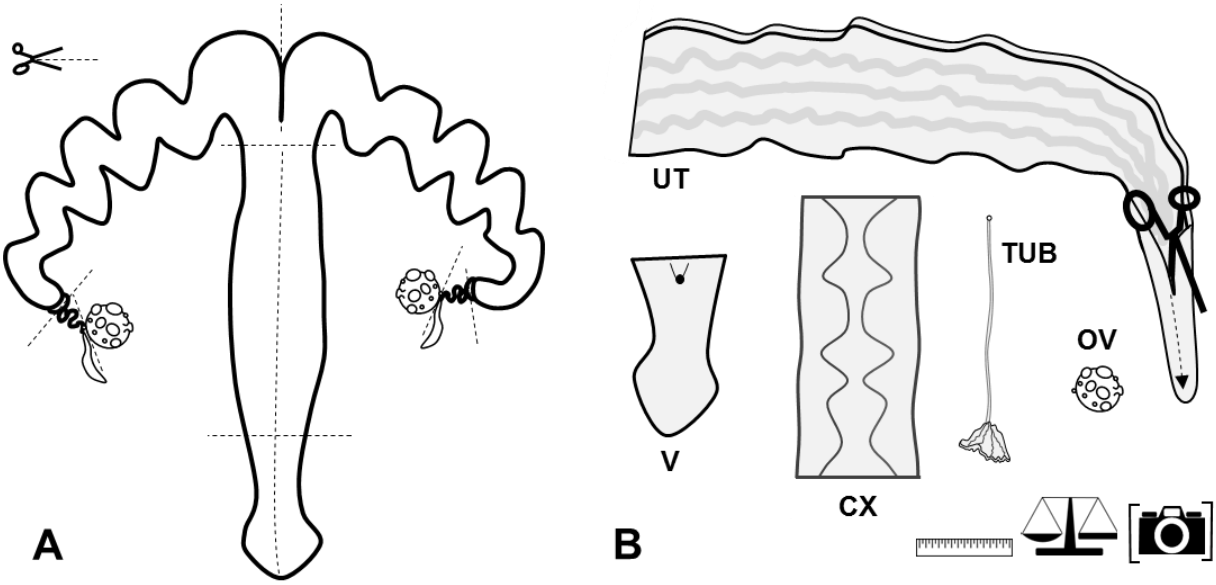


Fig. S275. *A schematic illustration of the dissection of the female reproductive tract. **A**: The dissection lines are indicated by dashed lines. **B**: The distinct parts of the genital tract are weighed, measured, and examined for alterations. Vagina (V), cervix (CX), uterus (UT), Fallopian tube (TUB), ovary (OV). The dissection lines are indicated by dashed lines.*

General examination parameters

The sizes (dimensions), lengths and weights of the right and left ovary and uterus are determined. In addition, the length of the Fallopian tubes is determined. If present, pathological alterations are described and photographed as necessary. Additional samples for histopathology, microbiology, virology, and molecular analyses are taken from the altered site(s) if required.

2.11.2.1. Female reproductive tract sampling in Type-I studies

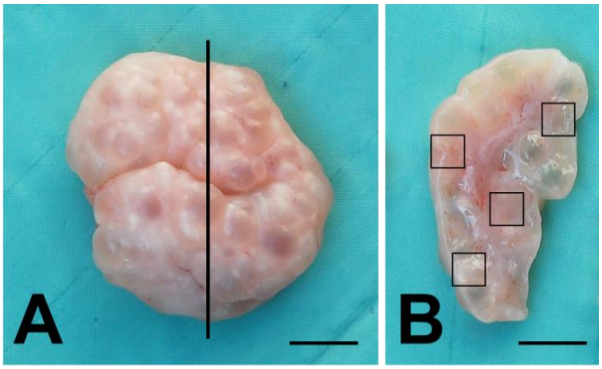


Fig. S276. A*: Juvenile ovary (fresh tissue).* ***B****: Slice of the cross-sectioned ovary. The locations and orientations of the histology (black line) and molecular analyses (black rectangles) samples for Type-I studies are indicated. Scale bars = 1 cm.*

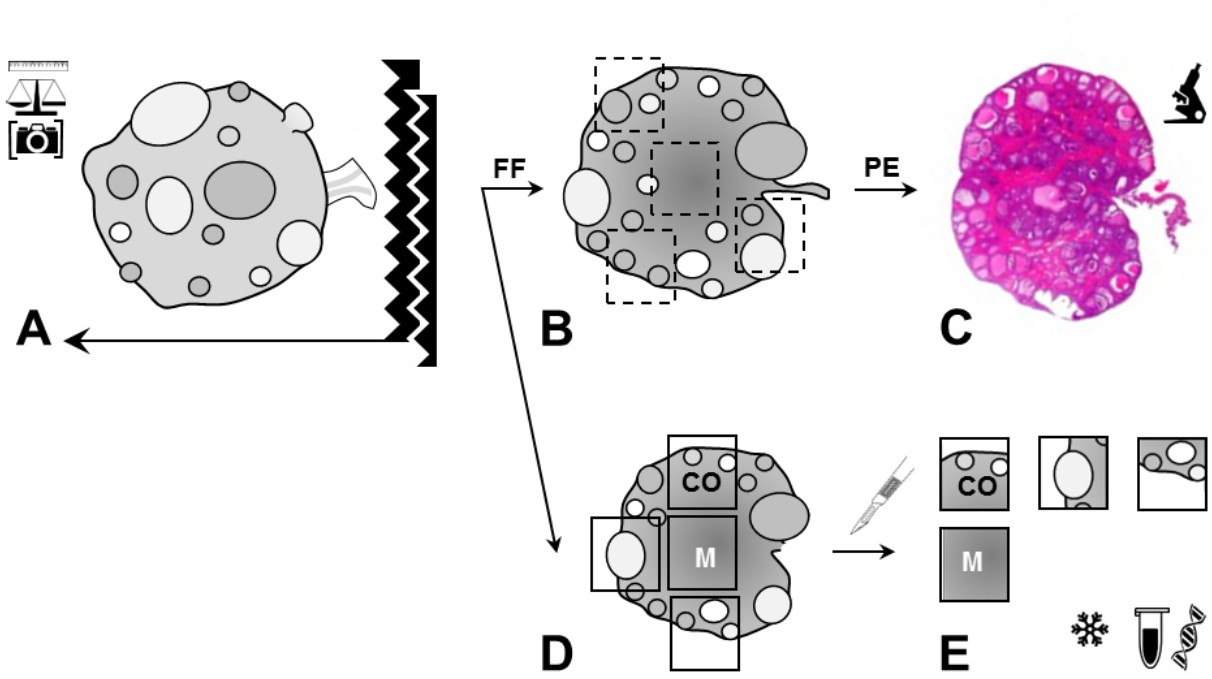


Fig. S277. *A schematic illustration of sample preparation from one ovary in Type-I studies (samples are generated from both ovaries).* ***A****: The ovary is weighed and measured. Sagittal slices are cut from the organ.* ***B****: One slice is fixed in a 4% formaldehyde solution and then processed for paraffin embedding. Depending on the size of the ovary, the entire slice can be embedded in paraffin or subsamples (dashed rectangles) are taken from the center and periphery of the ovary and then embedded in paraffin.* ***C****: Image of a HE stained histological slide with a sagittal section of an ovary.* ***D****: The samples for molecular analyses are excised from a fresh slice of ovary tissue.* ***E****: Samples are cut from the periphery (cortex, CO) and the center (medulla, M) of the ovary, labeled accordingly, and frozen at -80°C until further analysis.*

Samples for molecular analyses in Type-I studies

Location:	The sampling positions are indicated in Fig. S274-S277.
Number of samples:	Three cortical samples and one medullary sample are collected from the left and right ovary. One sample is collected from each Fallopian tube and uterine horn. One sample is collected from the cervix, and one sample is collected from the vagina.
Processing:	Samples are frozen on dry ice and stored at -80°C.
Downstream analyses:	DNA, RNA, and protein analyses (OMICS profiling).

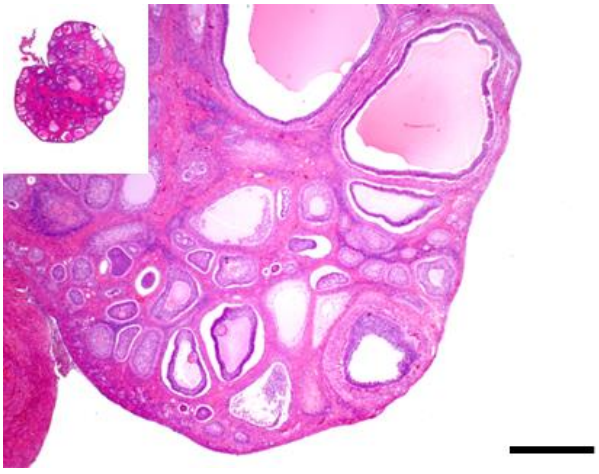
Histology samples

Location, number and orientation of sections:	One cross section of the right and left Fallopian tubes and one mid-sagittal section of each ovary (entire slice, or cortical/central subsamples, depending on the size of the ovary) are prepared. The section orientations are illustrated in Fig. S274-277. Additionally, one cross section and one longitudinal section of the right and left uterine horns are taken from the middle of the organ. In addition, one cross section of the cervix and one cross section of the vagina are collected (Fig. S274).
Fixation/Embedding:	FF-PE (Fig. S278-S280).

A comparison of the proposed Type-I-study sampling scheme with the established recommendations for histopathological examination in routine toxicity studies in rodents

For regulatory toxicity studies, the revised guides for organ sampling and trimming in rats and mice recommend the preparation of longitudinal sections through both ovaries. The uterine tubes may be sectioned together with the ovaries or separately embedded *in toto*. One mid-transverse section is prepared from each uterine horn, and one horizontal section is prepared from the uterine body with the cervix and vagina (Kittel et al., 2004).

Considering the size of the porcine female genital tract, a comparable sampling technique is not applicable in pigs. Therefore, the generation of several sections is recommended in Type-I-study sampling protocols to allow for an adequate evaluation of the porcine female reproductive tract in routine studies.



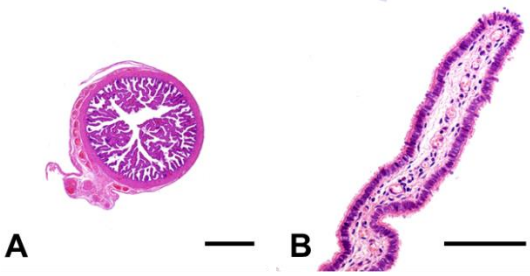


Fig. S278 (↑). *Histology of the Fallopian tube **A**: Cross section. **B**: Infundibulum of the Fallopian tube. Scale bars = 100 µm. FF-PE. HE.*

Fig. S279 (←). *Histology of the ovary (juvenile). Top inset: Image of a HE stained histological slide. Scale bar = 1 mm.*

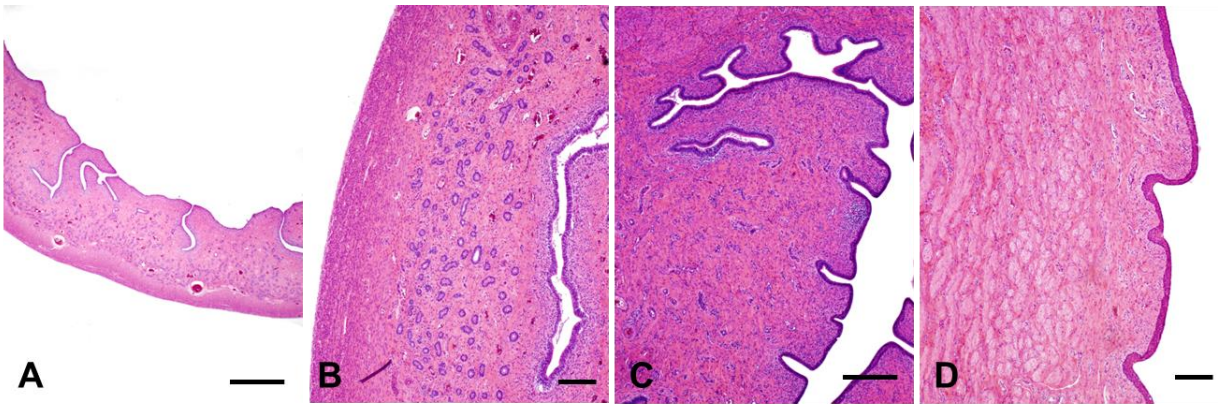


Fig. S280. *Histology of the (juvenile) uterus (**A**, longitudinal; **B**, cross section), cervix (**C**), and vagina (**D**). FF-PE. HE. Scale bars = 1 mm (A), 100 µm (B-D).*

2.11.2.2. Female reproductive tract samples in Type-II and Type-III studies

The generation of samples of the cervix and vagina is not recommended in Type-II/III studies.

2.11.2.2.1. Ovary samples in Type-II and Type-III studies

General systematic random sampling strategy: The type and number of samples for histopathological examination and molecular analyses of the ovaries in Type-II and Type-III studies

The weight and the dimensions of both ovaries are determined. One ovary is randomly selected, fixed completely in a formaldehyde solution (4%), sliced and embedded in paraffin for histopathological and quantitative stereological analyses (Kraugerud et al., 2012). The other ovary is processed for the generation of FF-PE, CRYO, MTC-PE, GA-IUR-EE and molecular analysis samples. The sampling positions and organ size-dependent sample numbers are determined by systematic random sampling. The ovary is cut into parallel equidistant slabs approximately 5-10 mm thick orthogonal to the longitudinal axis of the organ. The first section is randomly positioned between 0 and 10 mm from the margin of the organ (Fig. S281). The organ slabs are placed on the same side and counted. Next, the slabs are overlaid with a cross grid printed on a transparency (grid size: 5-20 mm, depending on the organ size and number and area of slabs). The systematic random sampling of eight (Type-II study) or twelve (Type-III study) tissue locations is performed as described in section 1.4. and shown in Fig. S281. An approximately 3x2x2-mm tissue piece is excised from each of the sampled locations. Every second sample is frozen at -80°C for molecular analyses. The remaining four (Type-II study) or six (Type-III study) specimens are GA-fixed and then processed for the generation of IUR-sectionable Epon-embedded specimens for downstream quantitative stereological analyses, semithin section histology and ultrastructural examination. For details, see Fig. S281 and Table S40.

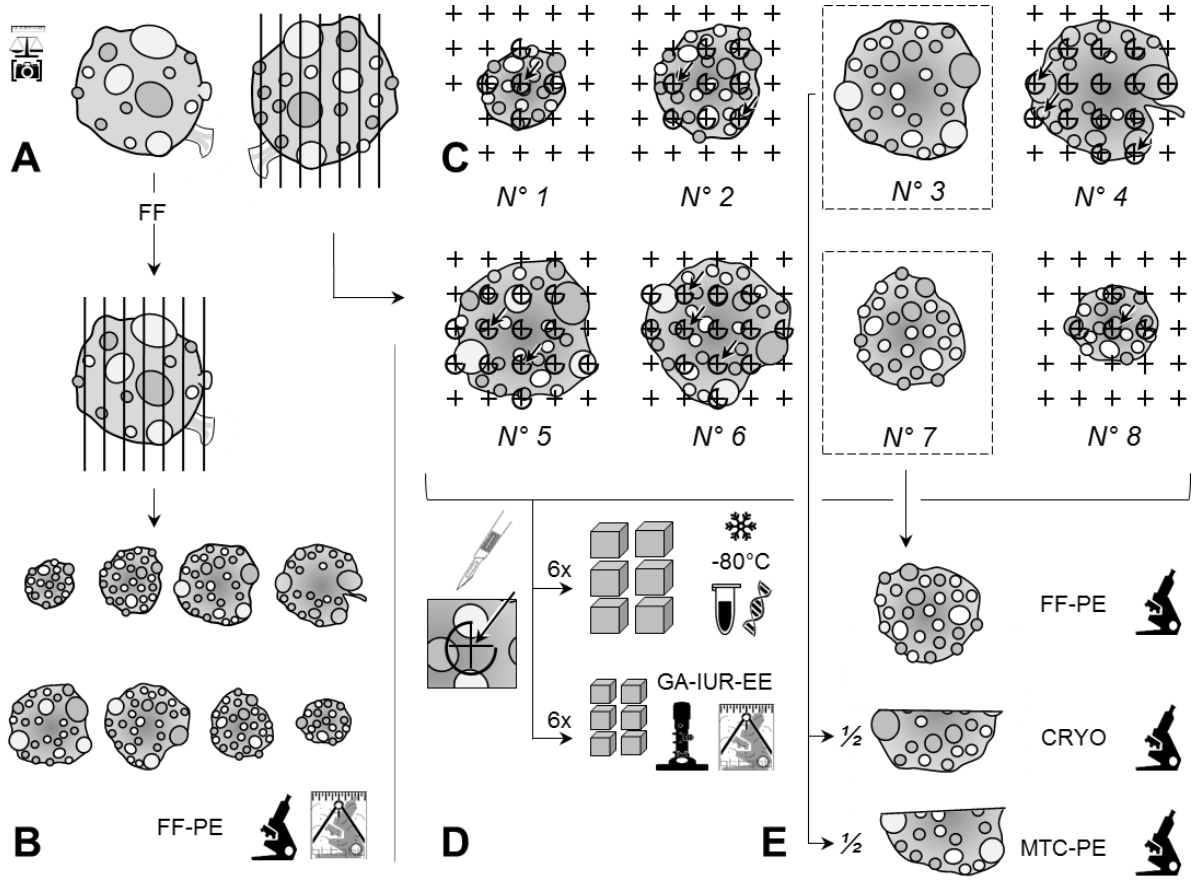


Fig. S281. *A schematic illustration of the sampling of the ovaries in Type-III studies.* ***A****: The ovaries are weighed and measured.* ***B****: One ovary is randomly selected, fixed in formalin* in toto*, completely sectioned into parallel slices (~5 mm) and embedded in paraffin.* ***C****: The second ovary is cut into parallel equidistant slabs (~5-10 mm thick), which are then placed on the same side. Two slabs are systematically randomly sampled. In the present example, there are eight slabs. For the systematic random selection of two slabs, every fourth slab is sampled. The first slab is randomly chosen between locations 1 and 4. In the present example, slabs N° 4 and N° 7 are sampled. One of these slabs is randomly selected and processed for FF-PE, and the other slab is halved (****E****). One half of this specimen is processed for cryo-sectioning and the other half is processed for MTC-PE (in Type-III studies, Table S40).* ***D****: The remaining tissue slabs are overlaid with a cross grid (grid size: 5-20 mm), printed on a transparency for the subsequent systematic random sampling of twelve tissue locations in Type-III studies (arrows). An approximately 3x2x2-mm tissue piece is excised from each of the sampled locations. Every second sample (= 6) is frozen at -80°C for molecular analyses. The remaining six specimens are GA-fixed and then processed for the generation of IUR-sectionable EE specimens (ORIENTATOR or ISECTOR) for downstream quantitative stereological analyses, semithin section histology, and ultrastructural examination.*

Table S40. The type and number of ovary samples in Type-II and Type-III studies.

Side	Sample type	Number of locations/samples Type-II studies	Type-III studies
First ovary		FF, complete sectioning (5 mm), PE	
Second ovary			
	FF-PE	-	*1 slab*
	MTC-PE	-	½ slab
	CRYO	*½ slab*	½ slab
Systematically sampled locations in the remaining tissue		8	12
		Number of samples	
GA-IUR-EE		1 out of every 2nd location	1 out of every 2nd location
Molecular analyses (-80°C)		1 out of every 2nd location	1 out of every 2nd location

2.11.2.2.2. Fallopian tube samples in Type-II and Type-III studies

General systematic random sampling strategy and the type and number of samples for histopathological examination and molecular analyses of the Fallopian tube in Type-II and Type-III studies

The weight and the dimensions of both Fallopian tubes are determined. The sampling positions are determined by systematic random sampling. The Fallopian tubes are laid on a planar surface and overlaid with a plastic transparency with printed equidistant (2-3 cm) parallel test lines. Out of the test lines that hit the tissue, three locations are systematically selected per side as shown in Fig. S282. From each of the sampled locations, an approximately 1.5-2-cm-long piece is excised and divided into subsamples for downstream analyses. For details, see Fig. S283 and Table S41.

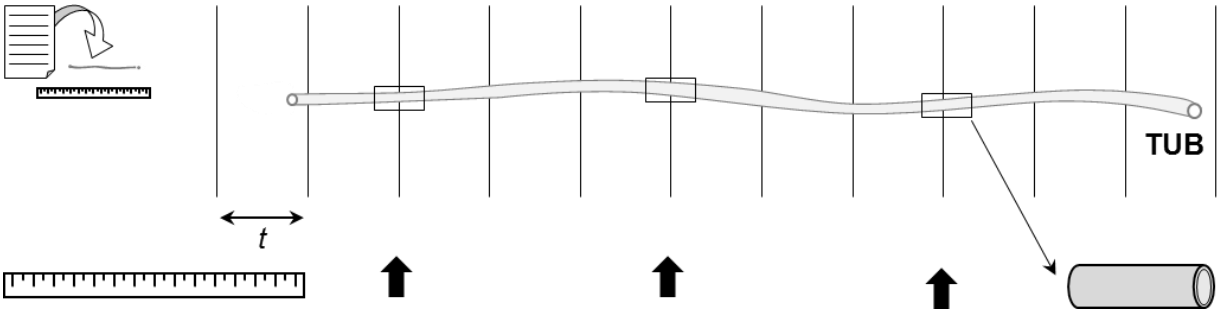


Fig. S282. *A schematic illustration of the systematic random sampling of the Fallopian tubes (tTUB) in Type-II/III studies (shown for one tube). t = 2-3 cm.*

Table S41. The type and number of Fallopian tube samples in Type-II and Type-III studies.

Sample type	N° of sampled locations per side/samples per location Type-II studies	Type-III studies
Sampled locations (per tube)	*3*	*3*
	Number of samples per location	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 per sampled location
CRYO	1 per side	1 per sampled location
GA-VUR-EE	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

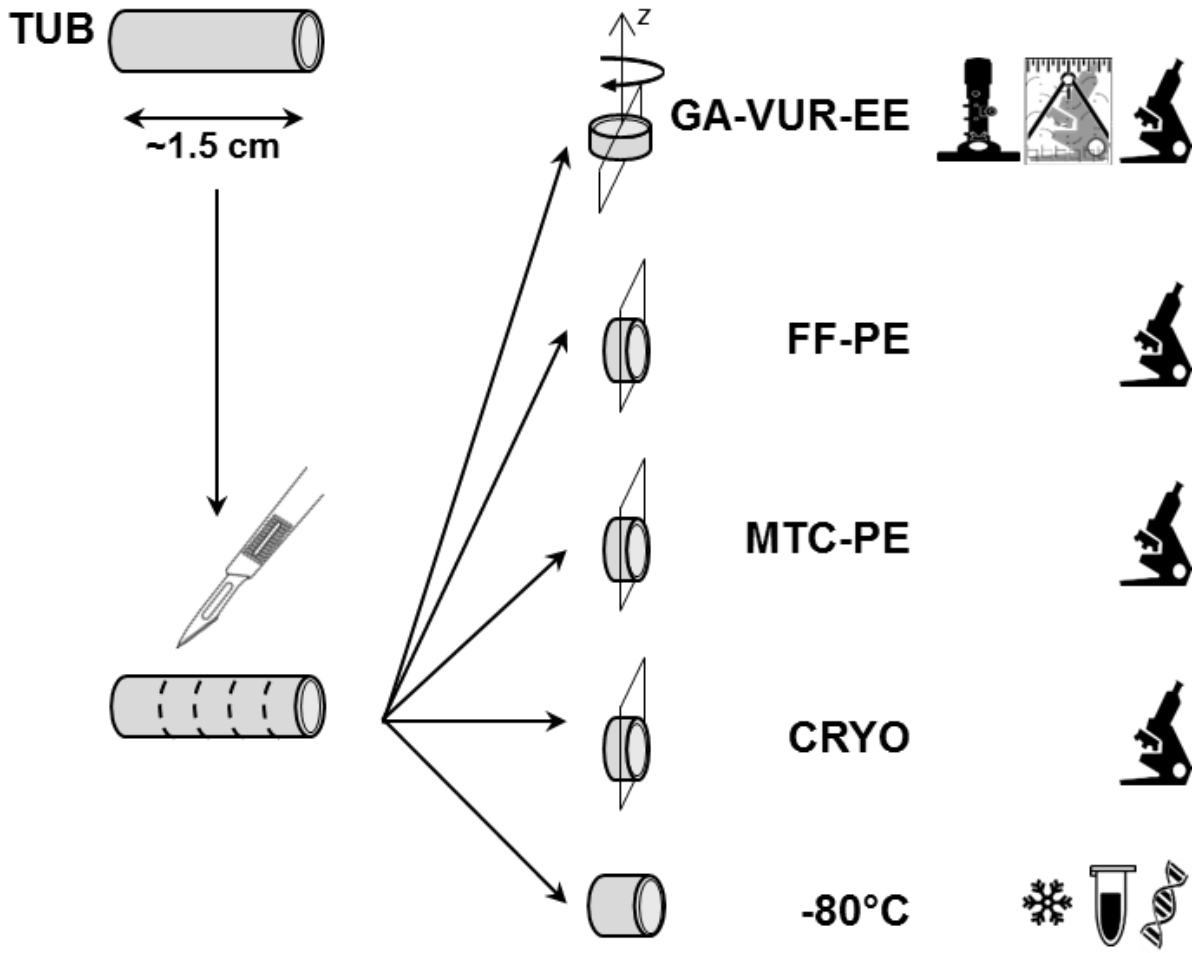


Fig. S283. *A schematic illustration of subsample preparation from one systematically randomly sampled location of the Fallopian tube for downstream analyses (Type-II/III studies) (TUB).*

2.11.2.2.3. Uterus samples in Type-II and Type-III studies

General systematic random sampling strategy and the type and number of samples for histopathological examination and molecular analyses of the uterus in Type-II and Type-III studies

If the uterus diameter is ≤ 1 cm, the organ is not cut open prior to sampling and the sampling is performed analogous to the sampling procedure described for the Fallopian tubes (section 2.11.2.2.2.). The weight and dimensions of the complete uterus is determined as shown in (Fig. S275). The uterus is opened (Fig. S275), and both uterine horns are placed on a planar surface with the mucosal side up. The sampling positions are determined by systematic random sampling. The tissue is overlaid with a cross grid printed on a transparency (grid size: 1-4 cm, depending on the organ size and tissue area). The systematic random sampling of tissue locations is performed as described in section 1.4. and shown in Fig. S284. The sampled positions can be marked by placing pieces of confetti paper on the respective positions. An approximately 1x1x2-cm tissue piece is excised from each of the sampled locations and subdivided for downstream analyses. To generate VUR-sectionable samples (Table S42), round specimens are punched out from the uterus with the mucosal side up using a hole punch and hammer as indicated in Fig. S285.

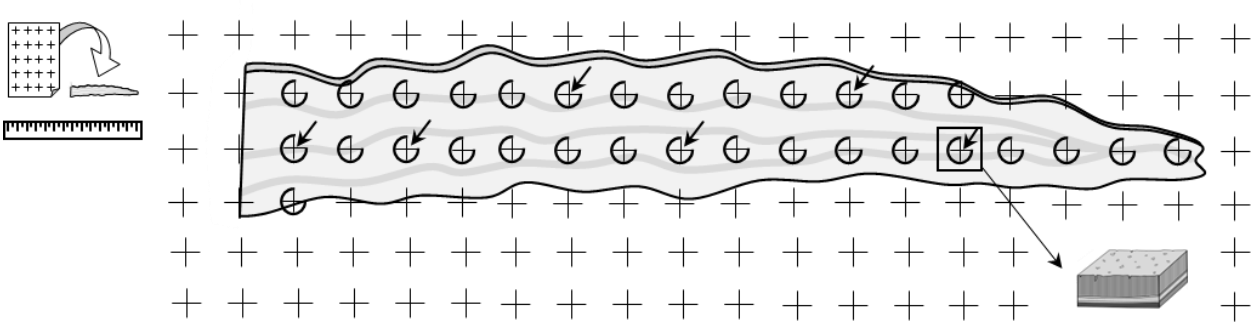


Fig. S284. *A schematic illustration of the systematic random sampling of the uterus in Type-II/III studies (shown for one uterine horn).*

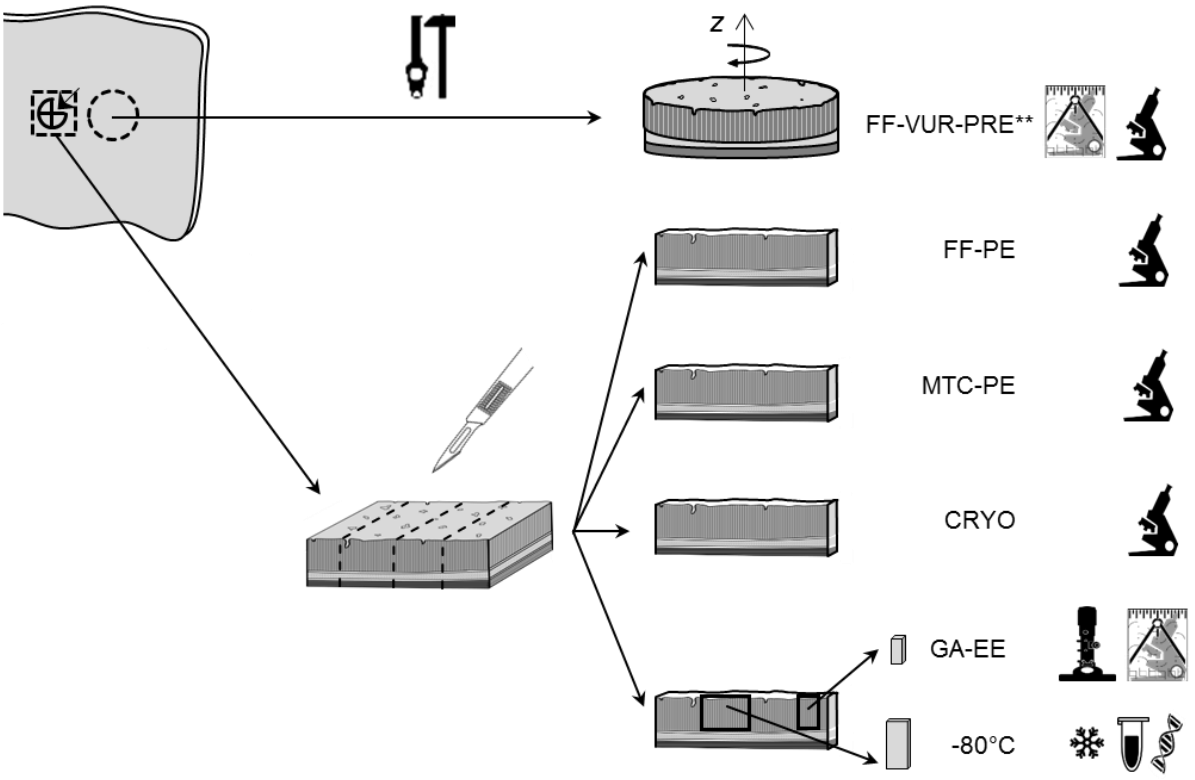


Fig. S285. *A schematic illustration of the preparation of subsamples from one systematically randomly sampled location of uterine tissue for downstream analyses (Type-II/III studies). **PR = GMA/MMA.*

Table S42. The type and number of uterus samples in Type-II and Type-III studies.

Sample type	Number of sampled locations per side and of samples per location Type-II studies	Type-III studies
Sampled locations per side (r/l)	*3*	*6*
	Number of samples per location	
FF-PE	1 per sampled location	1 per sampled location
MTC-PE	-	1 per sampled location
CRYO	1 out of every 2nd location	1 out of every 2nd location
GA- EE	1 per sampled location	1 per sampled location
FF-VUR-PRE	1 per sampled location	1 per sampled location
Molecular analyses (-80°C)	1 per sampled location	1 per sampled location

Time and personnel requirements

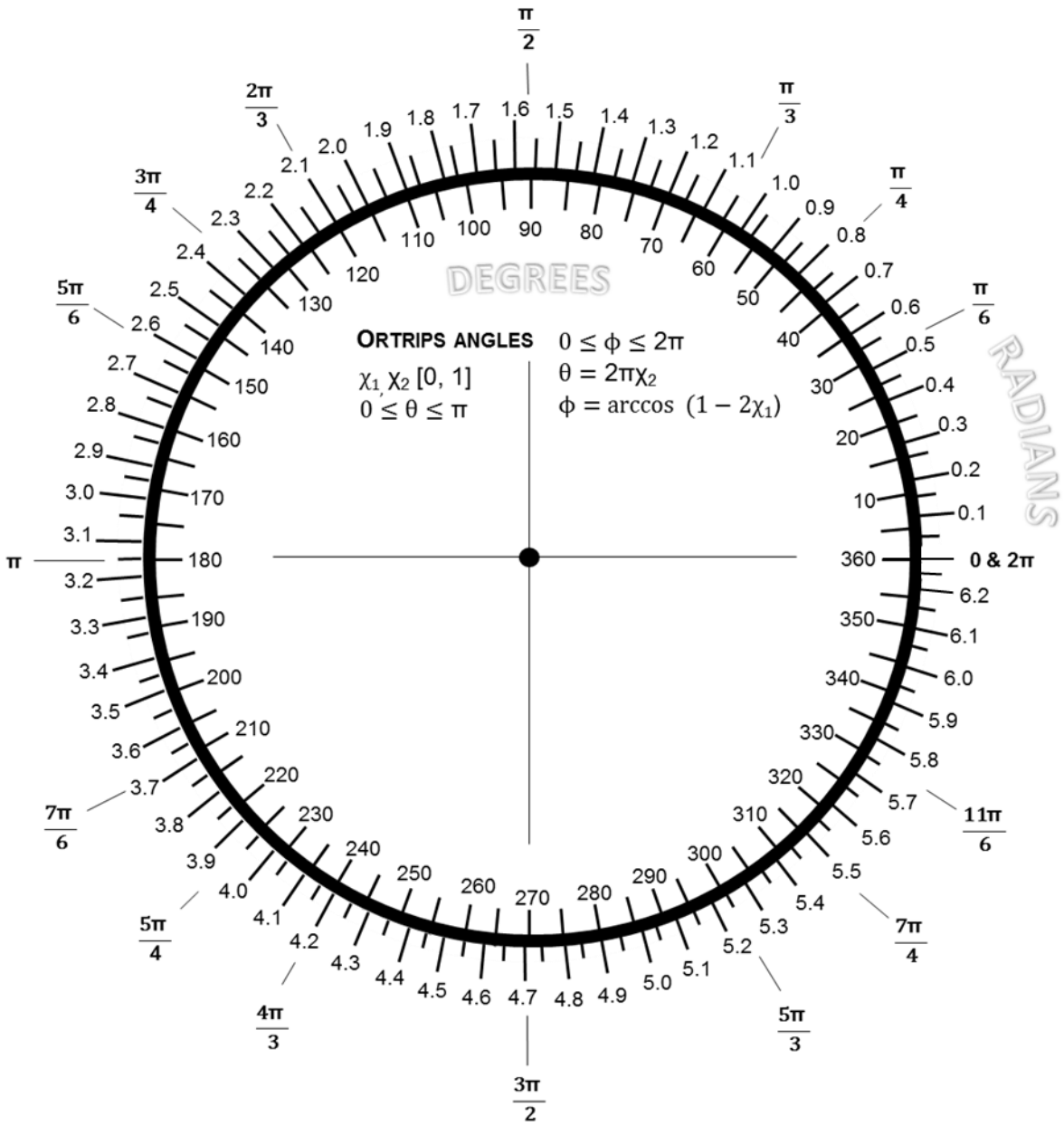
At least 35 minutes must be scheduled for the execution of the complete Type-III-study sampling procedures for sampling of the ovaries, Fallopian tubes and uterus by an experienced investigator and one assistant. This estimate includes the time from the removal of the organs to the freezing/fixation of sampled specimen; however, it does not include the time needed for the preparation and labeling of the necessary test tubes, sample vessels, embedding cassettes, fixative containers, and storage boxes or the time required for the further sample processing after fixation.

Related literature

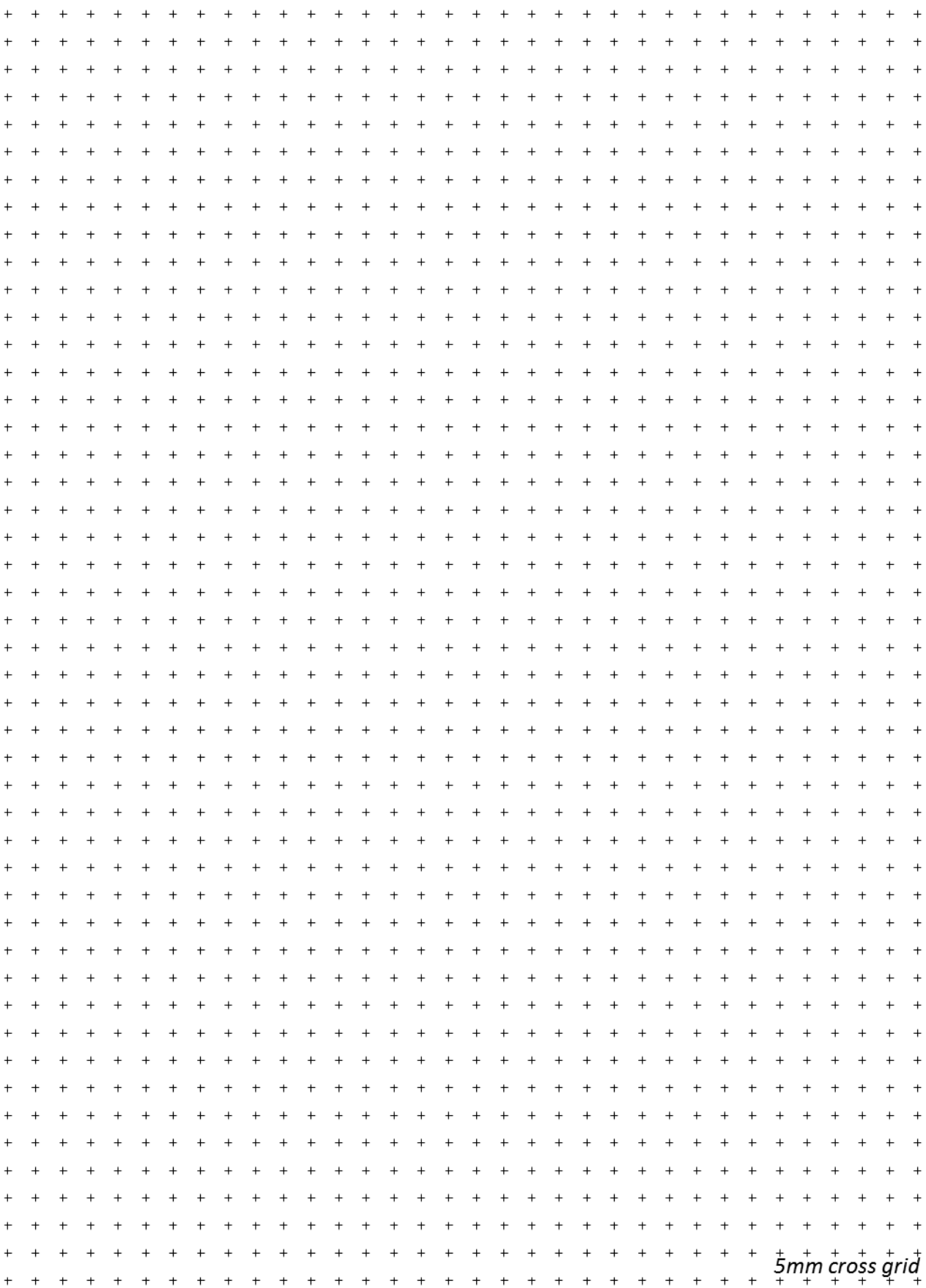
(Dixon et al., 2014, Foster, 2012b, Kittel et al., 2004, Kraugerud et al., 2012, Merkl et al., 2010, Romek and Karasinski, 2011, Rudmann and Foley, 2013)

3. Appendix

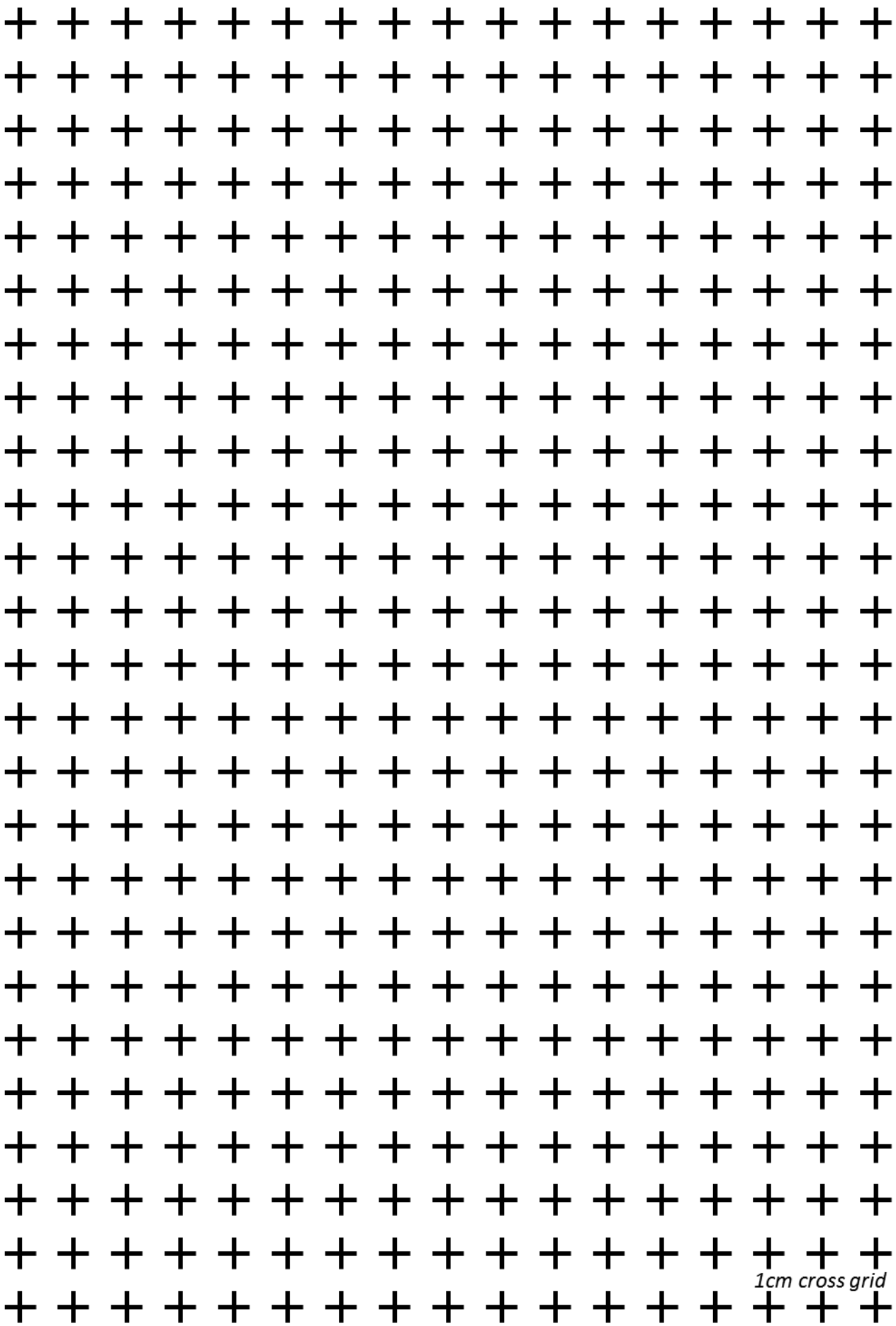
3.1. Copy of template of radian scaled circle for preparation of Orthogonal Triplet Probes (ORTRIPS)



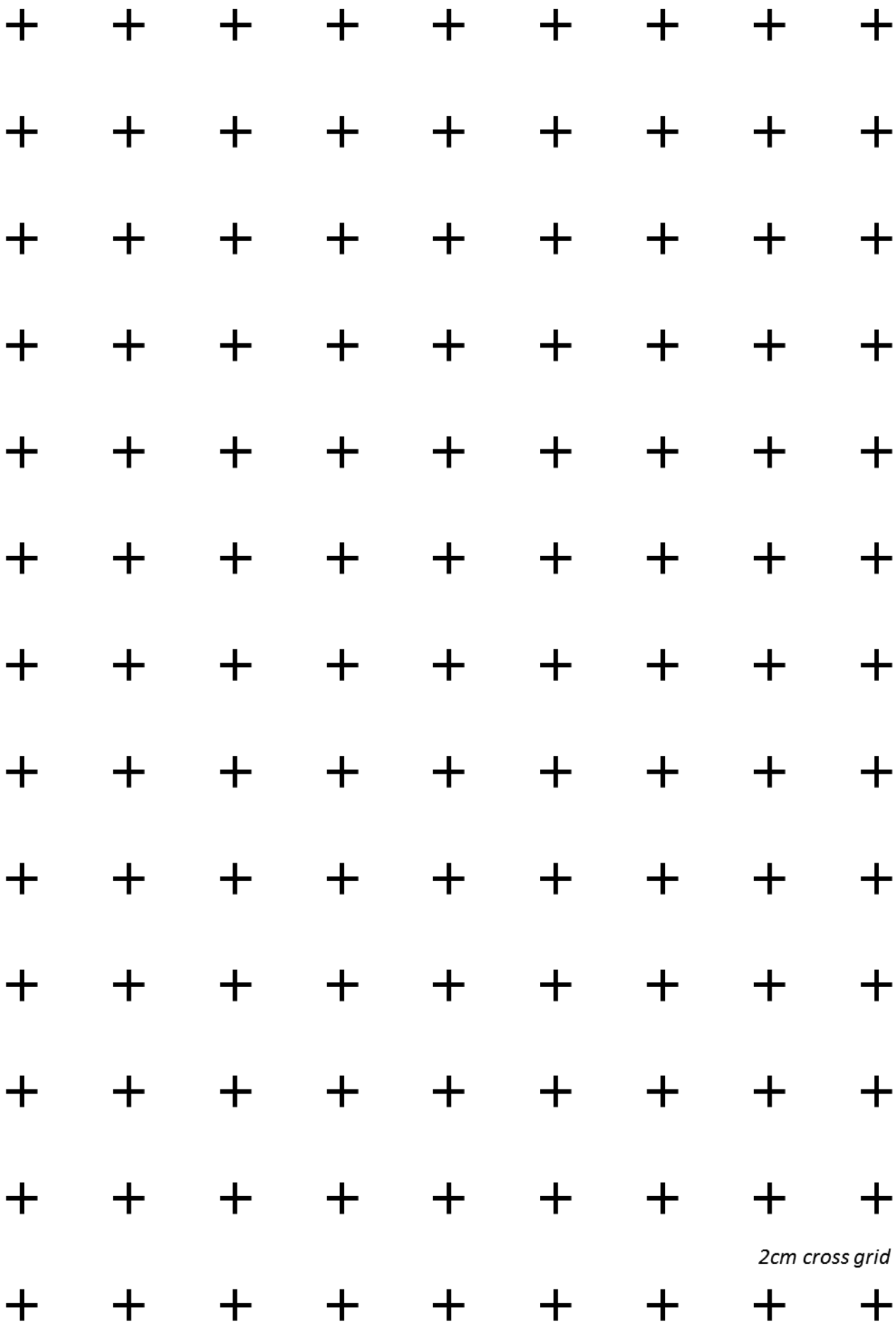
3.2. Copy of templates of cross grids for systematic random sampling



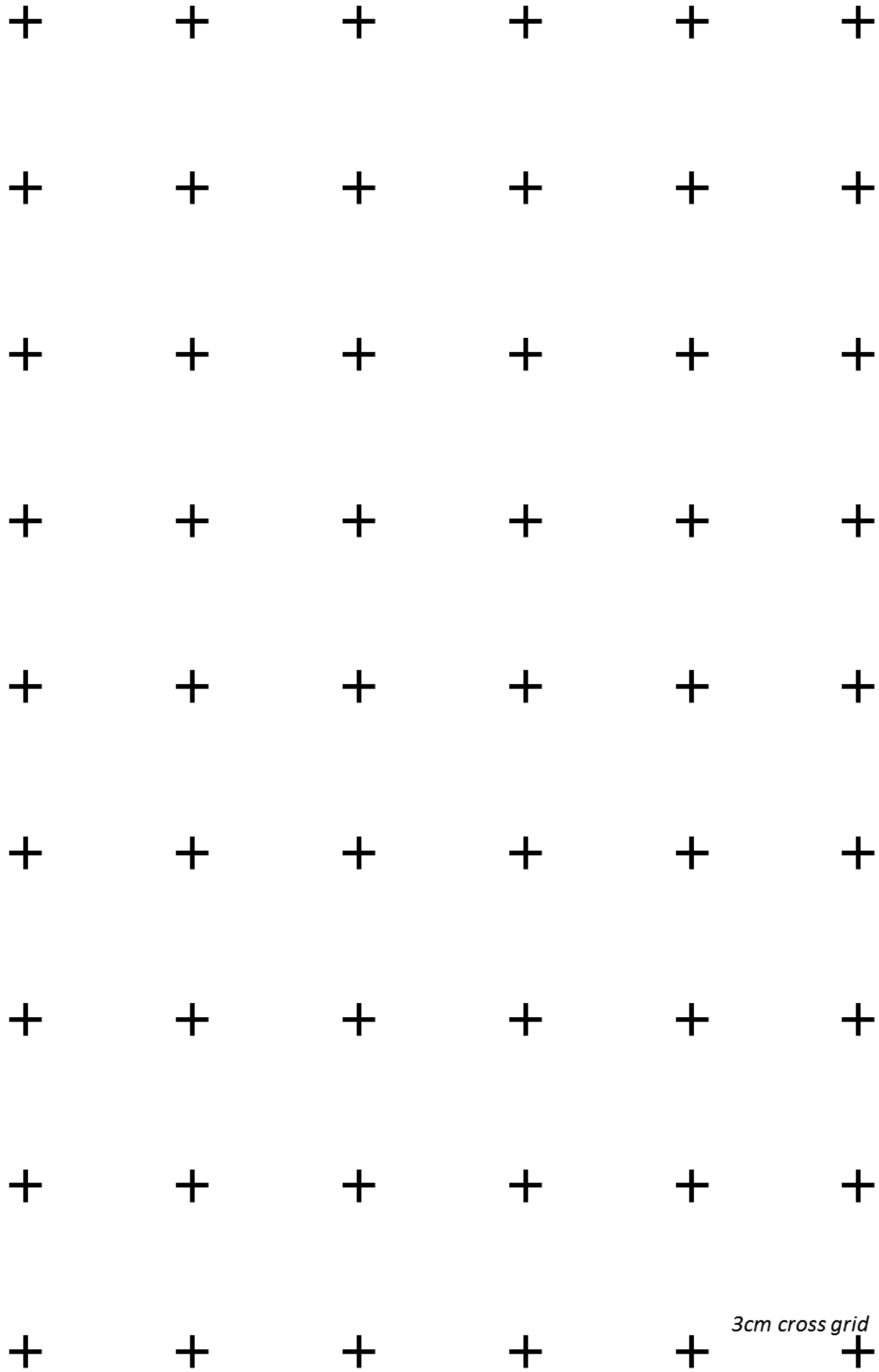
5mm cross grid



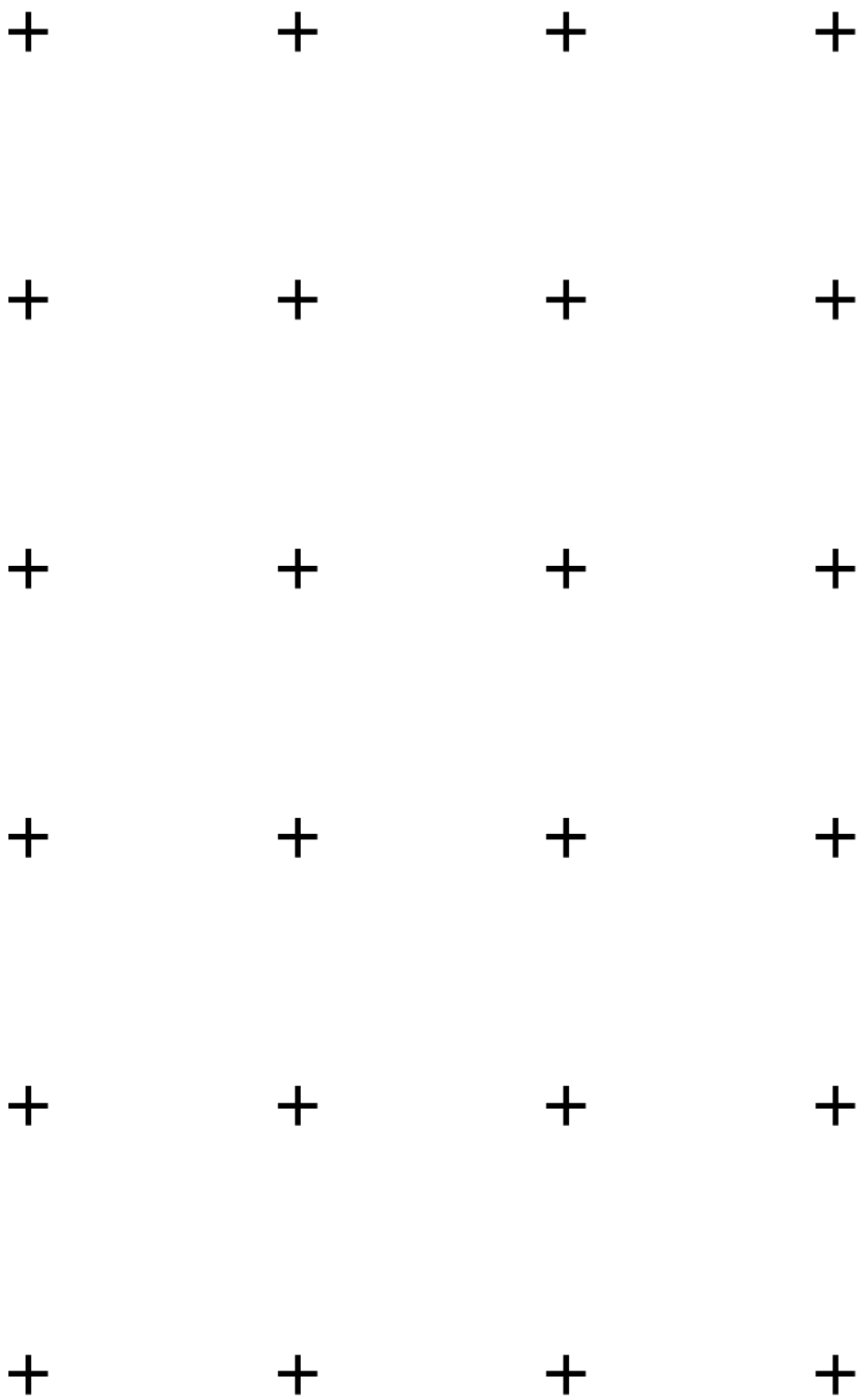
1cm cross grid



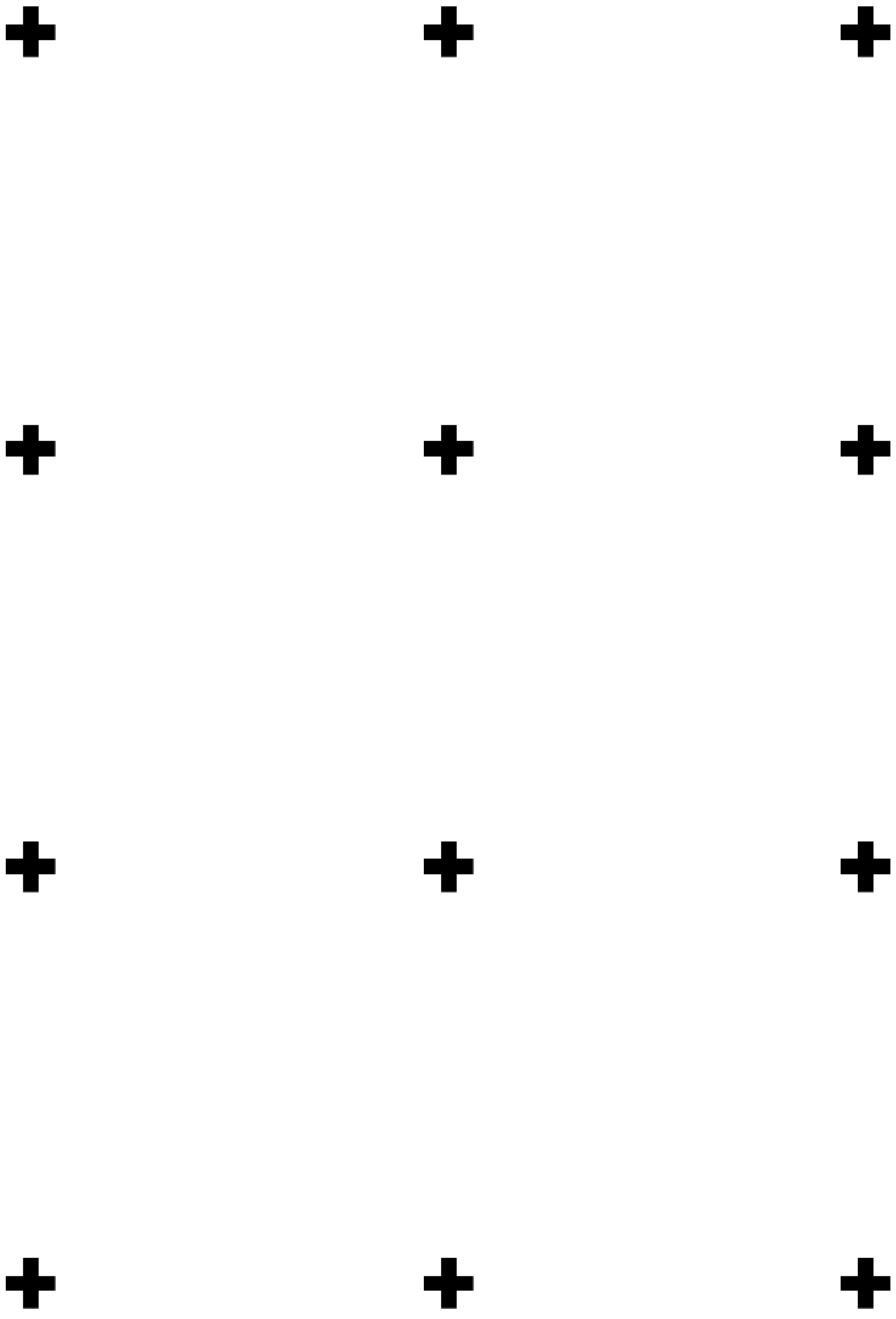
2cm cross grid



3cm cross grid



4cm cross grid



6cm cross grid

References

Abbott, A. (2015). Inside the first pig biobank. *Nature,* **519,** 397-398.

Al-Mashhadi, R.H., Bjorklund, M.M., Mortensen, M.B., Christoffersen, C., Larsen, T., Falk, E. and Bentzon, J.F. (2015). Diabetes with poor glycaemic control does not promote atherosclerosis in genetically modified hypercholesterolaemic minipigs. *Diabetologia*.

Almeida, F.F., Leal, M.C. and Franca, L.R. (2006). Testis morphometry, duration of spermatogenesis, and spermatogenic efficiency in the wild boar (Sus scrofa scrofa). *Biol Reprod,* **75,** 792-799.

Babinski, M.A., deBrito-Gitirana, L., Chagas, M.A., Abidu-Figueiredo, M., Costa, W.S. and Sampaio, F.J. (2005). Immunohistochemical analysis of smooth muscle cells and volumetric density of the elastic system fibers of wild boar (Sus scrofa) penis. *Anim Reprod Sci,* **86,** 317-328.

Baddeley, A.J., Gundersen, H.J. and Cruz-Orive, L.M. (1986). Estimation of surface area from vertical sections. *J Microsc,* **142,** 259-276.

Beck, K.D., Nguyen, H.X., Galvan, M.D., Salazar, D.L., Woodruff, T.M. and Anderson, A.J. (2010). Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. *Brain,* **133,** 433-447.

Berg, F., Gustafson, U. and Andersson, L. (2006). The uncoupling protein 1 gene (UCP1) is disrupted in the pig lineage: a genetic explanation for poor thermoregulation in piglets. *PLoS Genet,* **2,** e129.

Berridge, B.R., Van Vleet, J.F. and Herman, E. (2013). Cardiac, Vascular, and Skeletal Muscle Systems. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 1568-1666. Academic Press. INC., London.

Bertram, T.A., Ludlow, J.W., Basu, J. and Muthupalani, S. (2013). Digestive Tract. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 2277-2360. Academic Press. INC., London.

Bishop, S.P. (1999). Necropsy Techniques for the Heart and Great Vessels. In *Textbook of canine and feline cardiology* (Fox, P.*, et al.* eds.). Saunders, Philadelphia.

Blutke, A., Borjes, P., Herbach, N., Pfister, K., Hamel, D., Rehbein, S. and Wanke, R. (2015). Acaricide treatment prevents adrenocortical hyperplasia as a long-term stress reaction to psoroptic mange in cattle. *Vet Parasitol,* **207,** 125-133.

Blutke, A., Schneider, M.R., Renner-Muller, I., Herbach, N., Wanke, R. and Wolf, E. (2014). Genetic dissection of IGF1-dependent and -independent effects of permanent GH excess on postnatal growth and organ pathology of mice. *Mol Cell Endocrinol,* **394,** 88-98.

Bock, T., Pakkenberg, B. and Buschard, K. (2005). Genetic background determines the size and structure of the endocrine pancreas. *Diabetes,* **54,** 133-137.

Böhme, G. (1992). The Nervous System, the Endocrine Glands, and the Sensory Organs of the Domestic Mammals (Volume 4). In *The Anatomy of the Domestic Animals* (Nickel, R., Schummer, A., Seiferle, E. eds.), pp 28-61. Paul Parey, Berlin and Hamburg.

Bolliger, A.P. (2004). Cytologic evaluation of bone marrow in rats: indications, methods, and normal morphology. *Vet Clin Pathol,* **33,** 58-67.

Bolon, B., Butt, M.T., Garman, R.H. and Dorman, D.C. (2013a). Nervous System. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 2005-2095. Academic Press. INC., London.

Bolon, B., Garman, R.H., Pardo, I.D., Jensen, K., Sills, R.C., Roulois, A., Radovsky, A., Bradley, A., Andrews-Jones, L., Butt, M. and Gumprecht, L. (2013b). STP position paper: Recommended practices for sampling and processing the nervous system (brain, spinal cord, nerve, and eye) during nonclinical general toxicity studies. *Toxicologic Pathology,* **41,** 1028-1048.

Botti, M., Gazza, F., Ragionieri, L., Minelli, L.B. and Panu, R. (2011). Localization of the autonomic, somatic and sensory neurons innervating the cranial tibial muscle of the pig. *Ital J Anat Embryol,* **116,** 20-29.

Braber, S., Verheijden, K.A., Henricks, P.A., Kraneveld, A.D. and Folkerts, G. (2010). A comparison of fixation methods on lung morphology in a murine model of emphysema. *Am J Physiol Lung Cell Mol Physiol,* **299,** L843-851.

Brown, R.S., Edwards, J., Bartlett, J.W., Jones, C. and Dogan, A. (2002). Routine acid decalcification of bone marrow samples can preserve DNA for FISH and CGH studies in metastatic prostate cancer. *J Histochem Cytochem,* **50,** 113-115.
Carlson, C.S. and Weisbrode, S.E. (2012). Bones, Joints, Tendons, and Ligaments. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 920-971. Mosby Elsevier.
Cattely, R.C. and Cullen, J.M. (2013). Liver And Gall Bladder. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 1509-1567. Academic Press. INC., London.
Cheng, C., Sun, W.K., Liu, R., Wang, R.M., Chen, Y.H., Wang, Y., Li, J.L., Lu, X.B. and Gao, R. (2015). Comparison of gene expression of Toll-like receptors and antimicrobial peptides in immune organs and tissues between Yorkshire and Tibetan pigs. *Anim Genet,* **46,** 272-279.
Cohen, S.M. (2013). Lower Urinary Tract. In *Haschek and Rousseaux´s Handbook of t Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 1775-1794. Academic Press. INC., London.
Creasy, D., Bube, A., de Rijk, E., Kandori, H., Kuwahara, M., Masson, R., Nolte, T., Reams, R., Regan, K., Rehm, S., Rogerson, P. and Whitney, K. (2012). Proliferative and nonproliferative lesions of the rat and mouse male reproductive system. *Toxicologic Pathology,* **40,** 40s-121s.
Creasy, D.M. and Chapin, R.E. (2013). Male reproductive system. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 2493-2598. Academic Press. INC., London.
Cullen, J.M. and Brown, D.L. (2012). Hepatobiliary System and Exocrine Pancreas. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 405-457. Mosby Elsevier.
Culling, C.F.A.(1974). *Handbook of Histopathological and Histochemical Techniques,* Butterworths, London, UK.
Dalle Carbonare, L., Valenti, M.T., Bertoldo, F., Zanatta, M., Zenari, S., Realdi, G., Lo Cascio, V. and Giannini, S. (2005). Bone microarchitecture evaluated by histomorphometry. *Micron,* **36,** 609-616.
Dixon, D., Alison, R., Bach, U., Colman, K., Foley, G.L., Harleman, J.H., Haworth, R., Herbert, R., Heuser, A., Long, G., Mirsky, M., Regan, K., Van Esch, E., Westwood, F.R., Vidal, J. and Yoshida, M. (2014). Nonproliferative and proliferative lesions of the rat and mouse female reproductive system. *J Toxicol Pathol,* **27,** 1s-107s.
Docampo, M.J., Cabrera, J., Segales, J. and Bassols, A. (2014). Immunohistochemical investigation of extracellular matrix components in the lymphoid organs of healthy pigs and pigs with systemic disease caused by circovirus type 2. *J Comp Pathol,* **151,** 1-9.
Drogemuller, C., Becker, D., Kessler, B., Kemter, E., Tetens, J., Jurina, K., Jaderlund, K.H., Flagstad, A., Perloski, M., Lindblad-Toh, K. and Matiasek, K. (2010). A deletion in the N-myc downstream regulated gene 1 (NDRG1) gene in Greyhounds with polyneuropathy. *PLoS One,* **5,** e11258.
Duenwald-Kuehl, S., Kondratko, J., Lakes, R.S. and Vanderby, R., Jr. (2012). Damage mechanics of porcine flexor tendon: mechanical evaluation and modeling. *Ann Biomed Eng,* **40,** 1692-1707.
Feitosa, V.L., Reis, F.P., Esquisatto, M.A., Joazeiro, P.P., Vidal, B.C. and Pimentel, E.R. (2006). Comparative ultrastructural analysis of different regions of two digital flexor tendons of pigs. *Micron,* **37,** 518-525.
Foster, B.L. (2012a). Methods for studying tooth root cementum by light microscopy. *Int J Oral Sci,* **4,** 119-128.
Foster, R.A. (2012b). Female Reproductive System and Mammary Gland. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 1085-1126. Mosby Elsevier.
Foster, R.A. (2012c). Male Reproductive System. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 1127-1152. Mosby Elsevier.
Frazier, K.S., Seely, J.C., Hard, G.C., Betton, G., Burnett, R., Nakatsuji, S., Nishikawa, A., Durchfeld-Meyer, B. and Bube, A. (2012). Proliferative and nonproliferative lesions of the rat and mouse urinary system. *Toxicologic Pathology,* **40,** 14s-86s.
Fry, M.M. and McGavin, M.D. (2012). Bone Marrow, Blood Cells, and the Lymphatic System. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 698-770. Mosby Elsevier.
Garca, M., Ruiz-Ederra, J., Hernandez-Barbachano, H. and Vecino, E. (2005). Topography of pig retinal ganglion cells. *J Comp Neurol,* **486,** 361-372.

Gelberg, H.B. (2012). Alimentary System and the Peritoneum, Omentum, Mesentery, and Peritoneal Cavity. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 322-404. Mosby Elsevier.

Ghetie, V. and Pastea, E.(1958). *Atlas de Anatomica comparative Vol. II.,* Bucuresti: Editura Agro-Silvica des Stat.

Glorieux, S., Van den Broeck, W., van der Meulen, K.M., Van Reeth, K., Favoreel, H.W. and Nauwynck, H.J. (2007). In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract. *J Virol Methods,* **142,** 105-112.

Gorti, G.K., Birchall, M.A., Haverson, K., Macchiarini, P. and Bailey, M. (1999). A preclinical model for laryngeal transplantation: anatomy and mucosal immunology of the porcine larynx. *Transplantation,* **68,** 1638-1642.

Greaves, P., Chouinard, L., Ernst, H., Mecklenburg, L., Pruimboom-Brees, I.M., Rinke, M., Rittinghausen, S., Thibault, S., Von Erichsen, J. and Yoshida, T. (2013). Proliferative and non-proliferative lesions of the rat and mouse soft tissue, skeletal muscle and mesothelium. *J Toxicol Pathol,* **26,** 1s-26s.

Gundersen, H.J. (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *J Microsc,* **143,** 3-45.

Gundersen, H.J. and Jensen, E.B. (1987). The efficiency of systematic sampling in stereology and its prediction. *J Microsc,* **147,** 229-263.

Gundersen, H.J.G., Mirabile, R., Brown, D. and Boyce, R.W. (2013). Stereological Principles and Sampling Procedures for Toxicologic Pathologists. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 215-286. Academic Press. INC., London.

Gunson, D., Gropp, K.E. and Varela, A. (2013). Bone and Joints. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 2761-2858. Academic Press. INC., London.

Haley, P., Perry, R., Ennulat, D., Frame, S., Johnson, C., Lapointe, J.M., Nyska, A., Snyder, P., Walker, D. and Walter, G. (2005). STP position paper: best practice guideline for the routine pathology evaluation of the immune system. *Toxicologic Pathology,* **33,** 404-407; discussion 408.

Harkema, J.R., Nikula, K.J. and Haschek, W.M. (2013). Respiratory System. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 1935-2004. Academic Press. INC., London.

Hart, A.M. and Terenghi, G. (2004). Frozen-section fluorescence microscopy and stereology in the quantification of neuronal death within dorsal root ganglia. *J Mol Histol,* **35,** 565-580.

Hartlev, L.B., Nyengaard, J.R., Thomsen, J.S., Boel, L.W., Stengaard-Pedersen, K. and Hauge, E.M. (2013). Application of design-based stereology for estimation of absolute volume and surface area of the articular and calcified cartilage compartments of undecalcified human femoral heads. *J Microsc,* **251,** 133-143.

Hennig-Pauka, I., Koch, R., Hoeltig, D., Gerlach, G.F., Waldmann, K.H., Blecha, F., Brauer, C. and Gasse, H. (2012). PR-39, a porcine host defence peptide, is prominent in mucosa and lymphatic tissue of the respiratory tract in healthy pigs and pigs infected with Actinobacillus pleuropneumoniae. *BMC Res Notes,* **5,** 539.

Herbach, N., Bergmayr, M., Goke, B., Wolf, E. and Wanke, R. (2011). Postnatal development of numbers and mean sizes of pancreatic islets and beta-cells in healthy mice and GIPR(dn) transgenic diabetic mice. *PLoS One,* **6,** e22814.

Herbach, N., Goeke, B., Schneider, M., Hermanns, W., Wolf, E. and Wanke, R. (2005). Overexpression of a dominant negative GIP receptor in transgenic mice results in disturbed postnatal pancreatic islet and beta-cell development. *Regul Pept,* **125,** 103-117.

Herbach, N., Schairer, I., Blutke, A., Kautz, S., Siebert, A., Goke, B., Wolf, E. and Wanke, R. (2009). Diabetic kidney lesions of GIPRdn transgenic mice: podocyte hypertrophy and thickening of the GBM precede glomerular hypertrophy and glomerulosclerosis. *Am J Physiol Renal Physiol,* **296,** F819-829.

Hermanns, W., Liebig, K. and Schulz, L.C. (1981). Postembedding immunohistochemical demonstration of antigen in experimental polyarthritis using plastic embedded whole joints. *Histochemistry,* **73,** 439-446.
Hoeflich, A., Weber, M.M., Fisch, T., Nedbal, S., Fottner, C., Elmlinger, M.W., Wanke, R. and Wolf, E. (2002). Insulin-like growth factor binding protein 2 (IGFBP-2) separates hypertrophic and hyperplastic effects of growth hormone (GH)/IGF-I excess on adrenocortical cells in vivo. *FASEB J,* **16,** 1721-1731.
Howard, C.V. and Reed, M.G.(2005). *Unbiased Stereology,* QTP Publications, Coleraine, UK.
Hunziker, E.B. (2007). Cartilage histomorphometry. *Methods Mol Med,* **135,** 147-166.
Hyde, D.M., Tyler, N.K. and Plopper, C.G. (2007). Morphometry of the respiratory tract: avoiding the sampling, size, orientation, and reference traps. *Toxicologic Pathology,* **35,** 41-48.
Jackson, P.G.G. and Cockcroft, P.D.(2007). *Handbook of pig medicine,* SAUNDERS Eslevier Limited
Juhl, C.O., Vinter-Jensen, L., Poulsen, S.S., Orntoft, T.F. and Dajani, E.Z. (1995). Chronic treatment with epidermal growth factor causes esophageal epithelial hyperplasia in pigs and rats. *Dig Dis Sci,* **40,** 2717-2723.
Kaufmann, W., Bolon, B., Bradley, A., Butt, M., Czasch, S., Garman, R.H., George, C., Groters, S., Krinke, G., Little, P., McKay, J., Narama, I., Rao, D., Shibutani, M. and Sills, R. (2012). Proliferative and nonproliferative lesions of the rat and mouse central and peripheral nervous systems. *Toxicologic Pathology,* **40,** 87s-157s.
Khan, K.N.M., Hard, G.C. and Alden, C.L. (2013). Kidney. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M*., et al.* eds.), pp. 1667-1774. Academic Press. INC., London.
Kittel, B., Ruehl-Fehlert, C., Morawietz, G., Klapwijk, J., Elwell, M.R., Lenz, B., O'Sullivan, M.G., Roth, D.R., Wadsworth, P.F., Group, R. and Group, N. (2004). Revised guides for organ sampling and trimming in rats and mice--Part 2. A joint publication of the RITA and NACAD groups. *Exp Toxicol Pathol,* **55,** 413-431.
Klymiuk, N., Blutke, A., Graf, A., Krause, S., Burkhardt, K., Wuensch, A., Krebs, S., Kessler, B., Zakhartchenko, V., Kurome, M., Kemter, E., Nagashima, H., Schoser, B., Herbach, N., Blum, H., Wanke, R., Aartsma-Rus, A., Thirion, C., Lochmuller, H., Walter, M.C. and Wolf, E. (2013). Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle. *Hum Mol Genet,* **22,** 4368-4382.
Knust, J., Ochs, M., Gundersen, H.J. and Nyengaard, J.R. (2009). Stereological estimates of alveolar number and size and capillary length and surface area in mice lungs. *Anat Rec (Hoboken),* **292,** 113-122.
Kraugerud, M., Aleksandersen, M., Nyengaard, J.R., Ostby, G.C., Gutleb, A.C., Dahl, E., Berg, V., Farstad, W., Schweder, T., Skaare, J.U. and Ropstad, E. (2012). In utero and lactational exposure to PCB 118 and PCB 153 alter ovarian follicular dynamics and GnRH-induced luteinizing hormone secretion in female lambs. *Environ Toxicol,* **27,** 623-634.
Kristiansen, S.L. and Nyengaard, J.R. (2012). Digital stereology in neuropathology. *APMIS,* **120,** 327-340.
Kuper, C.F., Ernst, H., van Oostrum, L.C., Rittinghausen, S., Penninks, A.H., Ganderup, N.C. and Wolterbeek, A.P. (2012). Nasal passages of Gottingen minipigs from the neonatal period to young adult. *Toxicologic Pathology,* **40,** 656-666.
Kuper, C.F., Ruehl-Fehlert, C., Elmore, S.A. and Parker, G.A. (2013). Immune System. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M*., et al.* eds.), pp. 1795-1862. Academic Press. INC., London.
La Perle, K.M.D. (2012). Endocrine System. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 660-697. Mosby Elsevier.
Lanning, L.L., Creasy, D.M., Chapin, R.E., Mann, P.C., Barlow, N.J., Regan, K.S. and Goodman, D.G. (2002). Recommended approaches for the evaluation of testicular and epididymal toxicity. *Toxicologic Pathology,* **30,** 507-520.
Latendresse, J.R., Warbrittion, A.R., Jonassen, H. and Creasy, D.M. (2002). 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.

Lefaucheur, L. and Vigneron, P. (1986). Post-natal changes in some histochemical and enzymatic characteristics of three pig muscles. *Meat science,* **16,** 199-216.
Liebich, H.G. and König, H.E. (2008). Immune System and Lymphatic Organs (Organa Lymphopoietica): In *Veterinary anatomy of domestic mammals* (Liebich, H.G. & König, H.E. eds.), pp. 475-488. Schattauer GmbH, New York.
Liu, X.B. and Schumann, C.M. (2014). Optimization of electron microscopy for human brains with long-term fixation and fixed-frozen sections. *Acta Neuropathol Commun,* **2,** 42.
Ljungvall, K., Veeramachaneni, D.N., Hou, M., Hulten, F. and Magnusson, U. (2008). Morphology and morphometry of the reproductive organs in prepubertal and postpubertal male pigs exposed to di(2-ethylhexyl) phthalate before puberty: Precocious development of bulbourethral glands. *Theriogenology,* **70,** 984-991.
Lovell, J.M. and Harper, G.M. (2007). The morphology of the inner ear from the domestic pig (Sus scrofa). *J Microsc,* **228,** 345-357.
Madsen, L.W., Svensmark, B., Elvestad, K. and Jensen, H.E. (2001). Otitis interna is a frequent sequela to Streptococcus suis meningitis in pigs. *Vet Pathol,* **38,** 190-195.
Manojlovic-Stojanoski, M.N., Filipovic, B.R., Nestorovic, N.M., Sosic-Jurjevic, B.T., Ristic, N.M., Trifunovic, S.L. and Milosevic, V. (2014). Morpho-functional characteristics of rat fetal thyroid gland are affected by prenatal dexamethasone exposure. *Steroids,* **84,** 22-29.
Mao, H., Seo, S.J., Biswal, M.R., Li, H., Conners, M., Nandyala, A., Jones, K., Le, Y.Z. and Lewin, A.S. (2014). Mitochondrial oxidative stress in the retinal pigment epithelium leads to localized retinal degeneration. *Invest Ophthalmol Vis Sci,* **55,** 4613-4627.
Marcos, R., Monteiro, R.A. and Rocha, E. (2012). The use of design-based stereology to evaluate volumes and numbers in the liver: a review with practical guidelines. *J Anat,* **220,** 303-317.
Mattfeldt, T., Mall, G., Gharehbaghi, H. and Moller, P. (1990). Estimation of surface area and length with the orientator. *J Microsc,* **159,** 301-317.
Mattfeldt, T., Mobius, H.J. and Mall, G. (1985). Orthogonal triplet probes: an efficient method for unbiased estimation of length and surface of objects with unknown orientation in space. *J Microsc,* **139,** 279-289.
Mecklenburg, L., Kusewitt, D., Kolly, C., Treumann, S., Adams, E.T., Diegel, K., Yamate, J., Kaufmann, W., Muller, S., Danilenko, D. and Bradley, A. (2013). Proliferative and non-proliferative lesions of the rat and mouse integument. *J Toxicol Pathol,* **26,** 27s-57s.
Merkl, M., Ulbrich, S.E., Otzdorff, C., Herbach, N., Wanke, R., Wolf, E., Handler, J. and Bauersachs, S. (2010). Microarray analysis of equine endometrium at days 8 and 12 of pregnancy. *Biol Reprod,* **83,** 874-886.
Meurens, F., Summerfield, A., Nauwynck, H., Saif, L. and Gerdts, V. (2012). The pig: a model for human infectious diseases. *Trends Microbiol,* **20,** 50-57.
Miller, L.M., Van Vleet, J.F. and Gal, A. (2012). Cardiovascular System and Lymphatic Vessels. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 539-588. Mosby Elsevier.
Morawietz, G., Ruehl-Fehlert, C., Kittel, B., Bube, A., Keane, K., Halm, S., Heuser, A., Hellmann, J., Group, R. and Group, N. (2004). Revised guides for organ sampling and trimming in rats and mice--Part 3. A joint publication of the RITA and NACAD groups. *Exp Toxicol Pathol,* **55,** 433-449.
Muhlfeld, C., Nyengaard, J.R. and Mayhew, T.M. (2010). A review of state-of-the-art stereology for better quantitative 3D morphology in cardiac research. *Cardiovasc Pathol,* **19,** 65-82.
Munkeby, B.H., Smith, H.J., Winther-Larssen, E.H., Bjornerud, A. and Bjerkas, I. (2006). Magnetic resonance imaging of the Harderian gland in piglets. *J Anat,* **209,** 699-705.
Navarro, R., Juhas, S., Keshavarzi, S., Juhasova, J., Motlik, J., Johe, K., Marsala, S., Scadeng, M., Lazar, P., Tomori, Z., Schulteis, G., Beattie, M., Ciacci, J.D. and Marsala, M. (2012). Chronic spinal compression model in minipigs: a systematic behavioral, qualitative, and quantitative neuropathological study. *J Neurotrauma,* **29,** 499-513.
Newman, S.J. (2012). The Urinary System. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 589-659. Mosby Elsevier.

Nielsen, K.K., Andersen, C.B. and Kromann-Andersen, B. (1995). A comparison between the effects of paraffin and plastic embedding of the normal and obstructed minipig detrusor muscle using the optical dissector. *J Urol,* **154,** 2170-2173.
Noorafshan, A., Hoseini, L., Karbalay-Doust, S. and Nadimi, E. (2012). A simple stereological method for estimating the number and the volume of the pancreatic beta cells. *JOP,* **13,** 427-432.
Nyengaard, J.R. (1999). Stereologic methods and their application in kidney research. *J Am Soc Nephrol,* **10,** 1100-1123.
Nyengaard, J.R. and Alwasel, S.H. (2014). Practical stereology of the stomach and intestine. *Ann Anat,* **196,** 41-47.
Nyengaard, J.R. and Gundersen, H.J. (2006a). Direct and efficient stereological estimation of total cell quantities using electron microscopy. *J Microsc,* **222,** 182-187.
Nyengaard, J.R. and Gundersen, H.J.G. (1992). The isector: a simple and direct method for generating isotropic, uniform random sections from small specimens. *Journal of Microscopy,* **165,** 427-431.
Nyengaard, J.R. and Gundersen, H.J.G. (2006b). Sampling for stereology in lungs. *Eur Respir Rev,* **15,** 107-114.
Ochs, M. and Muhlfeld, C. (2013). Quantitative microscopy of the lung: a problem-based approach. Part 1: basic principles of lung stereology. *Am J Physiol Lung Cell Mol Physiol,* **305,** L15-22.
Ouchi, N., Parker, J.L., Lugus, J.J. and Walsh, K. (2011). Adipokines in inflammation and metabolic disease. *Nat Rev Immunol,* **11,** 85-97.
Pardo, I.D., Garman, R.H., Weber, K., Bobrowski, W.F., Hardisty, J.F. and Morton, D. (2012). Technical guide for nervous system sampling of the cynomolgus monkey for general toxicity studies. *Toxicologic Pathology,* **40,** 624-636.
Parzefall, B., Schmahl, W., Blutke, A., Baiker, K. and Matiasek, K. (2009). A rapid approach to ultrastructural evaluation and DNA analysis of the vestibular labyrinth and ganglion in dogs and cats. *J Neurosci Methods,* **177,** 217-224.
Perkins, J.A., Shcherbatyy, V. and Liu, Z.J. (2008). Morphologic and histologic outcomes of tongue reduction surgery in an animal model. *Otolaryngol Head Neck Surg,* **139,** 291-297.
Pikkarainen, M., Martikainen, P. and Alafuzoff, I. (2010). The effect of prolonged fixation time on immunohistochemical staining of common neurodegenerative disease markers. *J Neuropathol Exp Neurol,* **69,** 40-52.
Ramaiah, L., Bounous, D.I. and Elmore, S.A. (2013). Hematopoietic System. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 1863-1934. Academic Press. INC., London.
Reagan, W.J., Irizarry-Rovira, A., Poitout-Belissent, F., Bolliger, A.P., Ramaiah, S.K., Travlos, G., Walker, D., Bounous, D., Walter, G. and Bone Marrow Working Group of, A.S. (2011). Best practices for evaluation of bone marrow in nonclinical toxicity studies. *Vet Clin Pathol,* **40,** 119-134.
Renne, R., Brix, A., Harkema, J., Herbert, R., Kittel, B., Lewis, D., March, T., Nagano, K., Pino, M., Rittinghausen, S., Rosenbruch, M., Tellier, P. and Wohrmann, T. (2009). Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. *Toxicologic Pathology,* **37,** 5s-73s.
Renner, S., Braun-Reichhart, C., Blutke, A., Herbach, N., Emrich, D., Streckel, E., Wunsch, A., Kessler, B., Kurome, M., Bahr, A., Klymiuk, N., Krebs, S., Puk, O., Nagashima, H., Graw, J., Blum, H., Wanke, R. and Wolf, E. (2013). Permanent neonatal diabetes in INS(C94Y) transgenic pigs. *Diabetes,* **62,** 1505-1511.
Renner, S., Fehlings, C., Herbach, N., Hofmann, A., von Waldthausen, D.C., Kessler, B., Ulrichs, K., Chodnevskaja, I., Moskalenko, V., Amselgruber, W., Goke, B., Pfeifer, A., Wanke, R. and Wolf, E. (2010). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. *Diabetes,* **59,** 1228-1238.
Romek, M. and Karasinski, J. (2011). Quantification of connexin43 gap junctions in porcine myometrium by confocal microscopy and stereology. *Reprod Domest Anim,* **46,** 29-38.
Rosol, T.J., DeLellis, R.A., Harvey, P.W. and Sutcliffe, C. (2013). Endocrine System. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 2392-2492. Academic Press. INC., London.

Rudmann, D., Cardiff, R., Chouinard, L., Goodman, D., Kuttler, K., Marxfeld, H., Molinolo, A., Treumann, S. and Yoshizawa, K. (2012). Proliferative and nonproliferative lesions of the rat and mouse mammary, Zymbal's, preputial, and clitoral glands. *Toxicologic Pathology,* **40,** 7s-39s.
Rudmann, D.G. and Foley, G.L. (2013). Female Reproductive System. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 2599-2664. Academic Press. INC., London.
Ruehl-Fehlert, C., Kittel, B., Morawietz, G., Deslex, P., Keenan, C., Mahrt, C.R., Nolte, T., Robinson, M., Stuart, B.P., Deschl, U., Group, R. and Group, N. (2003). Revised guides for organ sampling and trimming in rats and mice--part 1. *Exp Toxicol Pathol,* **55,** 91-106.
Scherle, W. (1970). A simple method for volumetry of organs in quantitative stereology. *Mikroskopie,* **26,** 57-60.
Schmitz, C. and Hof, P.R. (2005). Design-based stereology in neuroscience. *Neuroscience,* **130,** 813-831.
Schneider, J.P. and Ochs, M. (2014). Alterations of mouse lung tissue dimensions during processing for morphometry: a comparison of methods. *Am J Physiol Lung Cell Mol Physiol,* **306,** L341-350.
Soshin, T., Takai, H., Kato, C., Fujii, E., Matsuo, S., Ito, T. and Suzuki, M. (2010). A method for sampling and tissue preparation of the parathyroid glands in miniature pigs for toxicity studies. *J Toxicol Sci,* **35,** 235-238.
Summerfield, A., Meurens, F. and Ricklin, M.E. (2015). The immunology of the porcine skin and its value as a model for human skin. *Mol Immunol,* **66,** 14-21.
Thoolen, B., Maronpot, R.R., Harada, T., Nyska, A., Rousseaux, C., Nolte, T., Malarkey, D.E., Kaufmann, W., Kuttler, K., Deschl, U., Nakae, D., Gregson, R., Vinlove, M.P., Brix, A.E., Singh, B., Belpoggi, F. and Ward, J.M. (2010). Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicologic Pathology,* **38,** 5s-81s.
Trayhurn, P., Temple, N.J. and Van Aerde, J. (1989). Evidence from immunoblotting studies on uncoupling protein that brown adipose tissue is not present in the domestic pig. *Can J Physiol Pharmacol,* **67,** 1480-1485.
Tschanz, S., Schneider, J.P. and Knudsen, L. (2014). Design-based stereology: Planning, volumetry and sampling are crucial steps for a successful study. *Ann Anat,* **196,** 3-11.
Turner, N.J., Pezzone, D. and Badylak, S.F. (2015). Regional variations in the histology of porcine skin. *Tissue Eng Part C Methods,* **21,** 373-384.
Turner, R.T., Maran, A., Lotinun, S., Hefferan, T., Evans, G.L., Zhang, M. and Sibonga, J.D. (2001). Animal models for osteoporosis. *Rev Endocr Metab Disord,* **2,** 117-127.
Uhl, P.B., Amann, B., Hauck, S.M. and Deeg, C.A. (2015). Novel localization of peripherin 2, the photoreceptor-specific retinal degeneration slow protein, in retinal pigment epithelium. *Int J Mol Sci,* **16,** 2678-2692.
Uhl, P.B., Szober, C.M., Amann, B., Alge-Priglinger, C., Ueffing, M., Hauck, S.M. and Deeg, C.A. (2014). In situ cell surface proteomics reveals differentially expressed membrane proteins in retinal pigment epithelial cells during autoimmune uveitis. *J Proteomics,* **109,** 50-62.
Valentine, B.A. and McGavin, M.D. (2012). Skeletal Muscle. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 871-919. Mosby Elsevier.
Vilahur, G., Padro, T. and Badimon, L. (2011). Atherosclerosis and thrombosis: insights from large animal models. *J Biomed Biotechnol,* **2011,** 907575.
Vovk, T., Bogataj, M. and Mrhar, A. (2009). Antioxidant levels in the pig urinary bladder: distribution within the bladder wall and in the urothelium derived from different bladder regions. *Biol Pharm Bull,* **32,** 801-806.
Wallig, M.A. and Sullivan, J.M. (2013). Exocrine Pancreas. In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 2361-2391. Academic Press. INC., London.
Wang, S., Liu, Y., Fang, D. and Shi, S. (2007). The miniature pig: a useful large animal model for dental and orofacial research. *Oral Dis,* **13,** 530-537.
Wang, T., Jiang, A., Guo, Y., Tan, Y., Tang, G., Mai, M., Liu, H., Xiao, J., Li, M. and Li, X. (2013). Deep sequencing of the transcriptome reveals inflammatory features of porcine visceral adipose tissue. *Int J Biol Sci,* **9,** 550-556.

Wanke, R., Milz, S., Rieger, N., Ogiolda, L., Renner-Muller, I., Brem, G., Hermanns, W. and Wolf, E. (1999). Overgrowth of skin in growth hormone transgenic mice depends on the presence of male gonads. *J Invest Dermatol,* **113,** 967-971.
Wanke, R., Wolf, E., Brem, G. and Hermanns, W. (2001). [Role of podocyte damage in the pathogenesis of glomerulosclerosis and tubulointerstitial lesions: findings in the growth hormone transgenic mouse model of progressive nephropathy]. *Verh Dtsch Ges Pathol,* **85,** 250-256.
Weibel, E.R., Hsia, C.C. and Ochs, M. (2007). How much is there really? Why stereology is essential in lung morphometry. *J Appl Physiol (1985),* **102,** 459-467.
Yamamoto, A., Tanaka, H., Ishida, T. and Horiike, K. (2010). D-aspartate oxidase localisation in pituitary and pineal glands of the female pig. *J Neuroendocrinol,* **22,** 1165-1172.
Zachary, J.F. (2012). Nervous System. In *Pathologic basis of veterinary disease* (McGavin, M.D. & Zachary, J.F. eds.), pp. 771-870. Mosby Elsevier.

4 Diskussion

4.1 Grundlagen und Ziele der vorliegenden Arbeit

Ziel der vorliegenden Arbeit ist es, standardisierte Sektions- und Probennahmeprotokolle für porzine Tiermodelle in der biomedizinischen Forschung zu etablieren. Die erstellten Probennahmepläne wurden am Institut für Tierpathologie der Ludwig-Maximilians-Universität München, basierend auf den langjährigen Erfahrungen bei Sektionen sowie in der pathomorphologischen Charakterisierung verschiedener Schweinemodelle und im Zusammenhang mit der Etablierung einer Gewebe- und Organproben-Biobank eines gentechnisch modifizierten diabetischen Schweinemodells erarbeitet (Abbott, 2015, Aigner *et al.*, 2010, Kemter *et al.*, 2012, Klymiuk *et al.*, 2013, Klymiuk *et al.*, 2012a, Klymiuk *et al.*, 2012b, Klymiuk *et al.*, 2012c, Renner *et al.*, 2013, Renner *et al.*, 2010, Renner *et al.*, 2012, Streckel *et al.*, 2015, Wünsch *et al.*, 2014). Hierbei wurde besonderer Wert auf die Berücksichtigung von in der Toxikopathologie und der pharmazeutischen Industrie gebräuchlichen Standards gelegt.

Die Notwendigkeit solcher standardisierter Sektions- und Probennahmeanleitungen liegt in der immer weiter zunehmenden Nutzung porziner Tiermodelle für unterschiedliche Forschungsziele und der damit verbundenen immer größer werdenden Bedeutung der adäquaten Probengewinnung und Nutzung dieser Schweine. Aufgrund ihrer Staffelung in Typ-I bis Typ-III Studien werden die vorliegenden Probennahmeprotokolle für Schweinemodelle unterschiedlichen Anwendungen und Studienzielen gerecht und können individuell an das experimentelle Design der jeweiligen Studien angepasst werden.

Bereits existierende Vorgaben verschiedener internationaler und nationaler Organisationen und Einrichtungen, wie beispielsweise der European Medicines

Agency (EMA), der United States Environmental Protection Agency (EPA), der Food and Drug Administration (FDA), der International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) und der Society of Toxicologic Pathology (STP) für die Probennahme und die Routineprozessierung von Organen und Gewebe bei Nagetiermodellen und Nicht-Nagetiermodellen nennen meist lediglich die zu untersuchenden Organe und Gewebe. Diese Richtlinien spezifizieren aber nicht, welcher Anteil oder welches Kompartiment eines Organs untersucht werden soll, beziehungsweise aus welchen Lokalisationen die erforderlichen Proben mithilfe welcher Probennahmeverfahren gewonnen werden sollen (Gundersen *et al.*, 2013, Ruehl-Fehlert *et al.*, 2003). Im Gegensatz dazu werden in den von der RITA (Registry of Industrial Toxicology Animal-data) und NACAD (North American Controll Animal Database) vorgeschlagenen “Revised guides for organ sampling and trimming in rats and mice--Part 1-3.“ für in toxikopathologischen Studien verwendete Nagetiermodelle definierte Entnahmelokalisationen für Proben aus diversen Organen und Geweben definiert (Kittel *et al.*, 2004, Morawietz *et al.*, 2004, Ruehl-Fehlert *et al.*, 2003). Bei den Nagermodellen haben die Etablierung und breite Akzeptanz dieser spezifizierten Probennahmeprotokolle maßgeblich zur Standardisierung und Reproduzierbarkeit bei der Probennahme und damit zur Verbesserung der Qualität des generierten Probenmaterials beigetragen (Ruehl-Fehlert *et al.*, 2003).

Die in der vorliegenden Arbeit entwickelten Sektions- und Probennahmeprotokolle für porzine Tiermodelle sollen dazu dienen, die Probennahme beim Schwein zu standardisieren und repräsentatives Probenmaterial dieser Tiere für verschiedene Untersuchungsansätze zu gewinnen. Um die Durchführbarkeit eines breit gefächerten Spektrums von verschiedenen Analyseverfahren zu ermöglichen, wird im Gegensatz zu bestehenden Vorgaben, in den Probennahmeplänen für Schweinemodelle routinemäßig Probenmaterial einer weitaus größeren und

umfassenderen Bandbreite, sowohl im Zusammenhang mit der Organ- und Gewebeauswahl, als auch bei der Probenanzahl und der weiteren Prozessierung der Proben, gewonnen. Dies ermöglicht die Etablierung und Anwendung der Probennahmeprotokolle für porzine Tiermodelle in einer Vielzahl unterschiedlicher wissenschaftlicher Gebiete. Da die bestehenden Probennahmepläne für murine Tiermodelle in der Toxikopathologie durch die grundlegenden anatomischen Unterschiede nicht auf das Schwein als Tiermodell übertragen werden können und auch die Gewinnung und Nutzung des vorhandenen Tier- und Probenmaterials im Hinblick auf das Untersuchungsspektrum erheblich erweitert werden sollte, wurden Probennahmepläne für die einzelnen Organe und Gewebe des Schweins konzipiert und detailliert ausgearbeitet. In diesem Zusammenhang wurden für die Entwicklung der Probennahmeprotokolle für Schweine zusätzlich zu den oben erwähnten Nagerprotokollen weitere bereits bestehende Probennahmeanweisungen berücksichtigt, um auch die Vergleichbarkeit mit bereits bestehenden Forschungsergebnissen zu gewährleisten und die Erfahrungen daraus mit einfließen zu lassen. Im Weiteren werden zu allen Organen und Geweben, die in anderen Studien und Richtlinien (Übersicht siehe Tabelle 1 der Publikation) sowohl bei Nagetiermodellen als auch bei Nicht-Nagetiermodellen beprobt werden, in den vorliegenden Protokollen zusätzlich schweinespezifische Organe beprobt. Auch Organe, welche beim Schwein als Prädilektionsstelle bestimmter Krankheiten gelten, wie beispielsweise die Papilla ilealis bei einer Infektion mit *Brachyspira hyodysenteriae,* werden bei der jeweiligen Probennahme berücksichtigt (Jackson and Cockcroft, 2007).

Die entwickelten Probennahmeprotokolle für mehr als fünfzig Organe und Gewebe sollen somit die Basis für eine adäquate Probennahme darstellen und können sowohl für spezifische Fragestellungen einzelner Studien, als auch für die Etablierung von

umfangreichen Gewebe- und Biobanken angewendet werden. Dem Benutzer soll mit den entwickelten Probennahmeprotokollen eine Grundlage an die Hand gegeben werden, die dazu beiträgt, den routinemäßigen Ablauf und die Organisation der Probennahme bei der Sektion sowie bei der weiteren Probenprozessierung zu vereinfachen, die Qualität des so generierten Probenmaterials sicherzustellen sowie die Effizienz und Reproduzierbarkeit der gesamten Probennahme zu steigern. Um die Sektion und die Probennahme für den Sekanten, vor allem auch in Bezug auf den Zeit- und Arbeitsaufwand, möglichst einfach, übersichtlich und standardisiert zu gestalten, wurden zusätzlich zu den dargestellten und erläuterten anatomischen Hintergrundinformationen detaillierte Sektionsanleitungen zu den jeweiligen Organen und Geweben erstellt.

Die im Zuge einer Sektion basierend auf den vorliegenden Probennahmeprotokollen gewonnenen Proben eignen sich sowohl für quantitative und qualitative morphologische Untersuchungen, eingeschlossen Gefrierschnitt-, Kunststoff- und Paraffinhistologie, Elektronenmikroskopie, Immunhistochemie und In-situ-Hybridisierung, als auch für molekulare Untersuchungen von DNA, RNA, Proteinen sowie Metaboliten und Elektrolyten. Vor allem im Hinblick auf den Umfang des Probenmaterials, welcher an die unterschiedlichen Studiendesigns und Forschungsziele individuell angepasst werden kann, und die Bandbreite verschiedener damit durchführbarer Analyse- und Untersuchungsverfahren, werden die Unterschiede zu den bereits bestehenden Probennahmeprotokollen bei Nagern oder anderen Tiermodellen deutlich.

4.2 Methodik und Aufbau der Probennahmepläne

Um die erstellten Probennahmeprotokolle für das Schwein übersichtlich darzustellen, wurde, neben der Aufteilung anhand der einzelnen Organsysteme und Gewebe, eine Staffelung in drei unterschiedlich umfangreiche und ausgestaltete Studientypen (I-III) vorgenommen. Diese Untergliederung in drei verschiedene, unterschiedlich ausführliche Studientypen ermöglicht dabei, die vorliegenden Protokolle in vielen wissenschaftlichen Gebieten für unterschiedliche Studienziele und Versuchsaufbauten zu nutzen und individuell an deren Anforderungen anzupassen. Zur besseren Veranschaulichung der Probennahmeprotokolle und um die Umsetzung in die Praxis so einfach wie möglich zu gestalten, werden detaillierte Empfehlungen zur Probennahme gegeben und mit Schemazeichnungen sowie mit fotografischen Abbildungen der einzelnen Organe und Gewebe und der verschiedenen Versuchsaufbauten während den verschiedenen Sektionsschritten und mit aus der Probennahme resultierenden histologischen Schnitten dargestellt. Für jedes einzelne Organ oder Gewebe werden dabei schweinespezifische anatomische Besonderheiten und praktische Empfehlungen zur Sektion herausgearbeitet. Die Schnittrichtungen und die Orientierung der Gewebeproben für die Histopathologie, aber auch Verfahren zur weiteren Prozessierung der einzelnen Proben werden anhand von unterschiedlichen Symbolen verdeutlicht und sowohl in den Schemazeichnungen, als auch in den fotografischen Abbildungen dargestellt. Vergleichbare Sektionsprotokolle, vor allem im Hinblick auf die Ausführlichkeit und Anschaulichkeit der hier vorliegenden Arbeit, wurden für das Schwein bisher noch nicht erstellt und sollen so eine hilfreiche Grundlage für die Probennahme in der Praxis beim porzinen Tiermodell darstellen.

Um die Repräsentativität, die Reproduzierbarkeit und die Effizienz der Probennahmeverfahren zu gewährleisten, werden Empfehlungen zur Anzahl der

Probenahmelokalisationen, beruhend auf statistischen sowie auf organgrößenspezifischen Parametern, aber auch zur Größe der einzelnen Proben, je nach Verwendungszweck, und die weitere Prozessierung des Probenmaterials (Fixation, Einbettung usw.) dargelegt.

Um Proben schnell, standardisiert und mit möglichst geringem Arbeitsaufwand für Standardscreening und Routineuntersuchungen von morphologischen Alterationen und molekulare Analysen, für Einsatzgebiete in toxikopathologischen Routinestudien zu generieren, werden in Typ-I Protokollen genau definierte Probenahmelokalisationen festgelegt. Die Probennahme beinhaltet in diesem Fall zusätzlich eine festgelegte Anzahl an Proben für jedes Organ oder Gewebe, mit einheitlicher Probengröße und festgelegter Orientierung. Dies kann auch dazu genutzt werden, in Ergänzung zu festgelegten spezifischen Untersuchungszielen zusätzliches standardisiertes Probenmaterial für weiterführende Analyseverfahren zu gewinnen, beziehungsweise um das vorliegende Tiermodell näher zu charakterisieren. Sollten sich makroskopische pathologische Veränderungen an den Organen oder Geweben finden, werden (selbstverständlich) zusätzlich Proben für histopathologische, mikrobiologische und molekularbiologische Untersuchungen aus den veränderten Stellen gewonnen. Am Ende des Abschnitts für Typ-I Studien des jeweiligen Organs findet sich ein detaillierter Vergleich des vorliegenden Probennahmeprotokolls beim Schwein mit den jeweils dazu etablierten Richtlinien für histopathologische Routinestudien in der Toxikopathologie (STP, NACAD/RITA) bei Nagern, um die Unterschiede und Gemeinsamkeiten unter anderem bezüglich der Probenlokalisation und –orientierung sowie Probenanzahl und Probenprozessierung darzustellen.

Ziel der Typ-II und Typ-III Studienprotokolle ist es, umfangreiches Probenmaterial zu gewinnen, welches für weiterführende und vertiefende Studien mit einer großen

Bandbreite an Untersuchungsarten und Verwendungsmöglichkeiten geeignet ist. Um die Generierung von repräsentativem und reproduzierbarem, für quantitative und qualitative Untersuchungen geeignetem Probenmaterial für diverse Untersuchungsverfahren zu gewährleisten, werden in den Probennahmeprotokollen systematisch zufällige Probennahmetechniken angewendet (Gundersen *et al.*, 2013, Howard and Reed, 2005, Nyengaard, 1999). Diese ausführlichen Studientypen können beispielsweise auch im Rahmen der Erstellung einer Biobank verwendet oder als Möglichkeit zur Generierung von Rückstellproben genutzt werden, welche für eventuell erst im Laufe der weiteren Untersuchungen auftretende Fragestellungen benötigt werden. Im Rahmen von Typ-II und Typ-III Studien wird bei allen Organen eine volumengewichtete systematisch zufällige Probennahme durchgeführt und ausführlich beschrieben. Für eine zielgerichtete, schnelle und arbeitseffektive Probennahme werden aus jeder so bestimmten Probenahmelokalisation im weiteren Verlauf mehrere Einzelproben für unterschiedliche Prozessierungsverfahren gewonnen. Um dem komplexeren Aufbau einiger Organe, wie beispielsweise Herz, Niere oder Gehirn, gerecht zu werden, werden auch in Typ-II und Typ-III Studien routinemäßig gezielt Proben aus definierten Lokalisationen dieser Organe gewonnen. Für weiterführende speziellere Studien können aber auch bei diesen Organen aus definierten Bereichen systematisch zufällige Proben gewonnen werden (Beispiel siehe Supplemente Abschnitt 2.8.1.1, Gehirn).

Bei einigen Organen wurden aufgrund der nur sehr seltenen Erfassung und Untersuchung in Routinestudien oder dem nicht Vorhandensein vergleichbarer Strukturen beim Menschen, keine weiterführenden Typ-II und Typ-III Probennahmeprotokolle erstellt (Beispiel: Hardersche Drüse). Sollten diese Organe und Gewebe Bestandteil intensiverer Studien sein, wird der Anwender auf weiterführende spezielle Literatur verwiesen. Mit den Zeit- und Arbeitsaufwand

einsparenden unterschiedlichen Probennahmeverfahren wird eine robuste Grundlage für eine effiziente Probennahme mit der Gewinnung von qualitativ hochwertigem Probenmaterial erstellt. Das im Rahmen dieser Probennahmeverfahren generierte und für das jeweilige Organ oder Gewebe repräsentative Probenmaterial, kann somit die Vergleichbarkeit und Reproduzierbarkeit in einer oder zwischen verschiedenen Studien sicherstellen.

Die Protokolle können für Schweine zwischen 10 und 400 Kilogramm angewendet, modifiziert und kombiniert, und somit an die jeweiligen Anforderungen unterschiedlicher Studien angepasst werden. Hierbei sollte vor Anwendung der dargestellten Sektionsanleitungen genau geprüft werden, welche Proben im Rahmen des vorliegenden Versuchsaufbaus benötigt werden. Bei spezifischen Fragestellungen, die einzelne Organe und deren spezielle Untersuchungen betreffen, sind neben den dargelegten Probennahmetechniken, weiterführende Untersuchungen und Proben, entsprechend der jeweiligen Fragestellung zu generieren. In diesem Fall können die vorliegenden Probennahmetechniken nur als richtungsweisende Grundlage angesehen werden. Bei der Adaptierung der Probennahmeprotokolle an die jeweilige Studie, sollten das Ziel der Studie und die Reihenfolge sowie der Umfang des zu generierenden Probenmaterials im Vorhinein festgelegt und angepasst werden, damit eine strukturierte und zielgerichtete Durchführung der Sektion ermöglicht wird.

Um dem erheblichen Zeit-, Arbeits- und Personalaufwand gerecht zu werden, sollte zu Beginn der Sektionen ein genauer Zeitplan und eine Sektionsanleitung mit Schritt für Schritt dargestellten Arbeitsanweisungen für jedes einzelne Organ oder Gewebe erstellt werden. Diese Anleitung sollte die zeitliche Abfolge der einzelnen Schritte der Sektion im Hinblick auf die Reihenfolge der Entnahme der Organe und Gewebe, sowie die genaue Abfolge der Probennahme für jedes einzelne Organ oder Gewebe

darstellen. Dabei sollen sowohl Anzahl, Lokalisation, Orientierung, Größe der Proben und die weitere Probenprozessierung, sowie gleich zu Beginn zu bestimmende Parameter wie etwa Maße und Gewicht des Organs aufgeführt und beschrieben werden. Hierfür empfiehlt sich die Erstellung von Checklisten mit allen geforderten Parametern und Proben, sowie dem für die Sektion und Probennahme benötigtem Material (verschiedene Fixantien, Probenröhrchen, Kapseln, die jeweilige Beschriftung und weitere spezielle Materialien). Diese Checklisten gewährleisten eine reibungslose Abfolge der Sektion und Probennahme und garantieren die Vollständigkeit der Probennahme und des gewonnenen Probenmaterials.

Vor allem in umfangreichen Biobank-Projekten (Abbott, 2015) mit mehreren Sekanten und Helfern muss die Organisation mit Vor- und Nachbereitung der Proben einen wichtigen Stellenwert einnehmen. Um den Anforderungen der jeweiligen Studie gerecht werden zu können, wird am Ende des jeweiligen Organabschnitts eine ungefähre Einschätzung des Zeit- und Arbeitsaufwands für Typ-II und Typ-III Studien, anhand der zugrundeliegenden Erfahrungen mit porzinen Tiermodellen am Institut für Tierpathologie der Ludwig-Maximilians-Universität München, angegeben. Zusätzlich werden für jedes Organ relevante, weiterführende Literaturquellen angegeben. Der mit dieser großen Bandbreite des Probenmaterials verbundene erhöhte Aufwand, der bei der Sektion der Tiere, sowie auch bei der Nachbearbeitung und Aufarbeitung des Probenmaterials entsteht, aber auch die logistischen Leistung der Probenlagerung und der damit verbundene erhöhte Zeit-, Arbeits- und Kostenfaktor, müssen im Einzelfall abgewogen werden.

4.3 Optimale Nutzung des von einem Tier generierbaren Probenmaterials für unterschiedliche Analyseverfahren

Ein weiterer Grund für die Erstellung standardisierter Probennahmeprotokolle für porzine Tiermodelle stellt die optimale Nutzung des vorhandenen Tierkollektivs und Probenmaterials dar. Die im Vergleich zu Nagermodellen deutlich mehr Zeit beanspruchende Generierung und Etablierung von gentechnisch modifizierten Schweinemodellen verbunden mit höheren Haltungskosten und erhöhten Arbeitsaufwand, die beispielsweise bei der Aufzucht von Tiermodellen mit prolongierten Krankheitsverläufen entstehen, machen das vorhandene Probenmaterial besonders wertvoll und fordern eine vorausschauende und optimierte Probennahme (Aigner *et al.*, 2010). Im Hinblick auf das Auftreten von weiteren, nicht im Rahmen der Versuchsplanung vorhersehbaren Fragestellungen im Verlauf einer Studie, und dem damit verbundenen zusätzlich benötigtem Probenmaterial, welches unter Umständen dann nicht mehr neu verfügbar oder generierbar ist, kann bereits gewonnenes und asserviertes Probenmaterial genutzt werden. In diesem Zusammenhang können die Probennahmepläne in der biomedizinischen Forschung auch zur Umsetzung des 3R-Konzeptes im Hinblick auf die Nutzung von porzinen Tiermodellen in der Wissenschaft beitragen (Replacement – Reduction – Refinement) (Tornqvist *et al.*, 2014). Da das Schwein als Modellorganismus humaner Erkrankungen einen wichtigen Platz in der Grundlagenforschung sowie auch in der Entwicklung und Validierung neuer Therapieansätze und Medikamente einnimmt, kann das vorhandene Tier- und Probenmaterial durch die Anwendung der vorliegenden Probennahmepläne in Verbindung mit einer Optimierung der jeweiligen Studienziele umfangreich und effektiv genutzt und eingesetzt werden. Auf Grundlage einer vorausschauenden Forschungsarbeit mit dem Ziel, möglichst viele

Informationen aus den vorhandenen Tiermodellen zu gewinnen, kann ein Beitrag zur Reduktion der Versuchstierzahlen geleistet werden.

4.4 Entwicklung und praktische Erprobung der Probennahmeprotokolle: Etablierung der „Munich-MIDY-Pig-Biobank“

Die vorliegenden Sektions- und Probennahmeprotokolle wurden im Zusammenhang mit dem Aufbau einer Biobank mit umfangreichem Probenmaterial eines transgenen Schweinemodells entwickelt und dabei bereits in der Praxis angewendet und evaluiert.

Ziel der Zusammenarbeit von Pathologen, Molekularbiologen, Reproduktionsmedizinern, Genetikern und Physiologen der Ludwig-Maximilians-Universität München (LMU) sowie der Eidgenössischen Technischen Hochschule Zürich (ETH) (Institut für Tierpathologie (AG Wanke), LMU; Institut für Molekulare Tierzucht und Biotechnologie (AG Wolf), LMU; Genzentrum (AG Wolf), LMU; Institut für Tierphysiologie (AG Deeg), LMU; Institute of Agricultural Sciences (AG Bauersachs), ETH) war es, das für die Diabetesforschung wertvolle und seltene Probenmaterial von langzeitdiabetischen INS^{C94Y}-Schweinen (> 2 Jahre) optimal auszunutzen und Material für verschiedene Untersuchungen auf molekularer, biochemischer sowie morphologischer Ebene zu gewinnen (Abbott, 2015, Renner *et al.*, 2013). Der Aufbau der Biobank mit Probenmaterial von fünf diabetischen transgenen Sauen und fünf nicht diabetischen Kontrolltieren stellt die effektive Nutzung sowie langfristige Asservierung des wertvollen Probenmaterials sicher. Die vor diesem Hintergrund erstellten Sektions- und Probennahmeprotokolle stellen die dabei notwendige Grundlage für den Aufbau einer solchen Gewebe- und Organprobensammlung dar und wurden in diesem Zusammenhang zum ersten Mal ausführlich in der Praxis angewendet, evaluiert und anhand der dabei gewonnenen

praktischen Erfahrungen weiter optimiert. Durch den in den Probennahmeprotokollen dargelegten Einsatz von unterschiedlichen Probennahmetechniken sowie verschiedenen Fixations- und Konservierungsmethoden, konnte für jedes Organ und Gewebe eine große Bandbreite an Probenmaterial für unterschiedlichste Analysen gewonnen werden. Die erstellten Probennahmeprotokolle wurden an das zur Verfügung stehende porzine Tiermodell und die damit verbundenen Anforderungen im Laufe der Sektionsvorbereitungen adaptiert und mithilfe einer detaillierten Arbeitsanweisung für jedes Organ und das zugehörige Sektionsteam konnte eine strukturierte, umfangreiche Sektion durchgeführt werden. Die Etablierung einer solchen Organ- und Gewebebank von gut charakterisiertem Probenmaterial für eine große Bandbreite an verschiedenen Analysen erlaubt es effizienten und vollen Nutzen aus diesem hier zur Verfügung stehenden Tiermodell zu ziehen und ermöglicht gleichzeitig auch eine erhebliche Einsparung an Zeit- und Kostenaufwand sowie Tiermaterial. Insgesamt wurden bei diesem Biobank-Projekt etwa 2000 Proben verschiedener Körperflüssigkeiten (Urin, Serum, Plasma, Liquor, Synovia) sowie über 12000 Proben von circa fünfzig verschiedenen Organen für qualitative und quantitative morphologische Untersuchungen auf licht- und elektronenmikroskopischer Ebene (Plastik- und Paraffinhistologie, Kryostatschnitte) gewonnen und circa 8000 Proben bei minus 80°Grad Celsius für DNA-, RNA-, Protein- und metabolomische Untersuchungen tiefgefroren. Nicht zu vernachlässigen sind beim Aufbau einer solchen Biobank die logistischen und arbeitsintensiven Leistungen sowohl in der Vor- und Nachbereitung der Sektion, bei der Sektion selbst, bei der circa zwanzig Sekanten und Helfer gleichzeitig tätig sind, sowie die weitere umfangreiche Probenprozessierung im Anschluss an die Sektion und die damit verbundene Probenlagerung und Probenlogistik. Die Munich-MIDY-Pig-Biobank konnte mittlerweile durch zwei weitere transgene diabetische Eber erweitert werden

und soll auch in Zukunft mit umfangreichem Probenmaterial der wissenschaftlichen Fachwelt zur Verfügung stehen und weiter ausgebaut werden (Abbott, 2015).

Die Erstellung der Sektions- und Probennahmepläne verbunden mit der praktischen Erprobung und Evaluation im Zusammenhang mit dem Aufbau der Munich-MIDY-Pig-Biobank erlaubt den Wissenschaftlern eine zielgerichtete, effektive und langfristig nutzbare Probensammlung des vorliegenden porzinen Diabetesmodells zu schaffen und gleichzeitig anderen Wissenschaftlern mit den erstellten Sektions- und Probennahmeplänen eine Grundlage an die Hand zu geben, welche für unterschiedliche porzine Tiermodelle in vielen Bereichen der translationalen Medizin, im Zusammenhang mit der Probengenerierung und -asservierung eingesetzt werden kann.

4.5 Ausblick

Die in der vorliegenden Arbeit etablierten Sektions- und Probennahmeprotokolle für das Schwein als Tiermodell in der biomedizinischen Forschung stellen eine neue robuste Basis zur Gewinnung von standardisierten, repräsentativen und reproduzierbaren Probenmaterial für ein breites Untersuchungs- und Analysenspektrum dar. Um der auch in Zukunft weiter zunehmenden Bedeutung porziner Tiermodelle in der Erforschung humaner Erkrankungen und der damit verbundenen Entwicklung und Validierung von Medikamenten und Therapien gerecht zu werden und um den optimalen Nutzen aus verfügbarem Tier- und Probenmaterial zu ziehen, stellt die Etablierung dieser Probennahmeprotokolle eine wichtige Grundlage für die zukünftige Nutzung und den Einsatz porziner Tiermodelle in der translationalen Medizin und den damit verbundenen Forschungsgebieten dar.

5 Zusammenfassung

Porzine Tiermodelle haben in der biomedizinischen Forschung in den letzten Jahren aufgrund der dem Menschen sehr ähnlichen Physiologie und Anatomie des Schweines und Fortschritten auf dem Gebiet der Gentechnik, erheblich an Bedeutung gewonnen. Schweinemodelle werden in verschiedenen Bereichen der translationalen Medizin genutzt. Hierzu zählen die Grundlagenforschung, die Erfassung von Erbkrankheiten des Menschen anhand gentechnisch veränderter „maßgeschneiderter“ porziner Modelle, die Erforschung von Pathogenitätsmechanismen, die toxikopathologische Evaluation neuer Wirkstoffe und die Entwicklung und Erprobung neuer therapeutischer Ansätze. Für diverse Nagetierspezies existieren etablierte Protokolle zur standardisierten Gewinnung von Organ- und Gewebeproben für toxikopathologische Untersuchungen. Für Schweinemodelle hingegen waren derartige Probennahmepläne bislang nicht verfügbar. In der vorliegenden Arbeit wurden standardisierte Probennahmeprotokolle für mehr als fünfzig verschiedene porzine Organe und Gewebe entwickelt und praktisch erprobt. Die Protokolle ermöglichen die reproduzierbare, standardisierte Generierung von repräsentativem Probenmaterial. Die Probennahmepläne beinhalten detaillierte Angaben zur Entnahmestelle, der Anzahl der zu entnehmenden Proben, ihrer Orientierung und weiteren Prozessierung für unterschiedliche qualitative und quantitative morphologische Analyseverfahren sowie molekularbiologische und biochemische Untersuchungen. Neben relevanten anatomischen Besonderheiten des Schweines und Hinweisen zur Sektionstechnik werden die Probennahmeprotokolle für sämtliche Organe und Gewebe durch detaillierte Schemazeichnungen sowie makroskopische und histologische Abbildungen veranschaulicht und durch den zu veranschlagenden Zeit-, Kosten- und

Personalaufwand ergänzt. In Anpassung an unterschiedliche Studienziele werden für die einzelnen Organe und Gewebe unterschiedliche Probennahmeprotokolle (Typ-I – Typ-III) vorgestellt. Diese können frei kombiniert und an das experimentelle Design einer jeweiligen Studie angepasst werden. Typ-I Probennahmeprotokolle sind für Übersichtsuntersuchungen morphologischer Alterationen und molekulare Analysen, beispielweise in toxikopathologischen Studien, vorgesehen. Hierbei erfolgt die Entnahme von Proben für histopathologische Routineuntersuchungen und molekularbiologische Analysen aus definierten Lokalisationen mit festgelegter Orientierung. Typ-II und Typ-III Probennahmeprotokolle wurden zur Generierung von Proben für detaillierte Untersuchungen einzelner Organe und Gewebe mit unterschiedlichen Analyseverfahren (Typ-II), beziehungsweise für die Erstellung von Gewebe-Biobanken (Typ-III) entwickelt. Zur reproduzierbaren Generierung repräsentativer Proben für ein breites Spektrum an quantitativen und qualitativen Untersuchungen werden die Entnahmelokalisationen der Proben in Typ-II und Typ-III Studien durch systematisch zufällige Verfahren bestimmt. Die Gewinnung von für verschiedene Analyseverfahren geeigneten Proben ermöglicht die Durchführung initial nicht geplanter Untersuchungen zur Beantwortung von sich erst im Verlauf einer Studie ergebenden Fragestellungen. Dies kann zur Verringerung der in einer Studie benötigten Tierzahl beitragen.

Die im Februar 2016 im Fachjournal *Toxicologic Pathology* veröffentlichten Probennahmeprotokolle leisten einen Beitrag zur standardisierten Gewinnung von qualitativ hochwertigen, repräsentativen Probenmaterials als Voraussetzung für die Vergleichbarkeit von Ergebnissen unterschiedlicher Studien porziner Tiermodelle in der biomedizinischen Forschung.

6 Summary

During the past decade(s), porcine animal models have gained a steadily growing popularity in biomedical research. This is due to the similar physiology and anatomy of pigs and humans, and the technical advances in genetic modification of the porcine genome. Porcine models are used in diverse areas of translational medicine, including basic research, generation of “tailored”, genetically modified pig models of human diseases, investigations of pathophysiological processes, surgery, transplantation research, toxicity testing of pharmacological substances, as well as development of new therapeutic strategies. For various rodent species used as experimental animal models, standardized sampling guidelines for reproducible collection of organ and tissue specimen exist. For porcine models, however, such guidelines have not been published so far.

In the present work, standardized sampling guidelines for more than 50 porcine organs and tissues were developed to facilitate the reproducible generation of representative specimen. For each organ and tissue, the sampling guidelines indicate the relevant anatomic features, and provide precise advices on the appropriate necropsy techniques and the estimated time and personnel expenses for sampling. Illustrated by detailed schematic drawings, macroscopic pictures and microscopic images of histological slides, the protocols specify the sampling locations and give recommendations on the adequate number of samples, their orientation, and the subsequent processing of the specimen for different qualitative and quantitative morphological investigations, as well as for molecular and biochemical analyses. According to the aims and purposes of different studies, different types of sampling protocols are provided (type I-III), which can be individually combined and adapted to the experimental design of a specific study.

Type-I sampling protocols are designed for studies in which a broad set of organs/tissues is examined in an overview fashion, using routine histopathological techniques and/or standard molecular analyses, as e.g., in general toxicity studies. Here, the samples are collected from deliberately defined locations and in predefined orientations.

Type-II and type-III sampling protocols were developed for advanced, detailed studies of distinct organs and tissues, using a wide range of different analytical methods (type-II), respectively for biobank projects (type-III), where particularly large numbers of different types of samples from a wide range of different organs and tissues are required. To enable the reproducible generation of representative specimen, the sampling locations in type-II and type-III studies are determined by efficient, systematic random sampling strategies. From each of the sampled locations, sub-samples are taken and processed for a broad spectrum of different analysis methods, including analyses not necessarily specified or planned at the time point of sampling. A suchlike ‘‘forward-looking’’ sampling strategy allows to perform additional experiments without a repeated generation of new samples, and might thus contribute to reduce the number of animals in a study.

The sampling protocols presented in this study were published in “*Toxicologic Pathology*” in February 2016. Their broad application will ensure the efficient generation of representative, high-quality samples of porcine organs and tissues, and contribute to the reproducibility of results and the intra-/interstudy comparability of research projects involving pigs as animal models.

7 Literaturverzeichnis

Abbott, A. (2015). Inside the first pig biobank. *Nature,* **519,** 397-398.

Adams, E.T. and Crabbs, T.A. (2013). Basic approaches in anatomic toxicologic pathology In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 149-173. Academic Press. INC., London.

Aigner, B., Renner, S., Kessler, B., Klymiuk, N., Kurome, M., Wünsch, A. and Wolf, E. (2010). Transgenic pigs as models for translational biomedical research. *J Mol Med (Berl),* **88,** 653-664.

American Diabetes Association. (2014). Diagnosis and classification of diabetes mellitus. *Diabetes Care,* **37 Suppl 1,** S81-90.

Baddeley, A.J., Gundersen, H.J. and Cruz-Orive, L.M. (1986). Estimation of surface area from vertical sections. *J Microsc,* **142,** 259-276.

Blutke, A., Block, C., Berendt, F., Herbach, N., Kemter, E., Amann, K., Frohlich, T., Arnold, G.J. and Wanke, R. (2011). Differential glomerular proteome analysis of two murine nephropathy models at onset of albuminuria. *Proteomics Clin Appl,* **5,** 375-381.

Boyce, R.W., Dorph-Petersen, K.A., Lyck, L. and Gundersen, H.J. (2010). Design-based stereology: introduction to basic concepts and practical approaches for estimation of cell number. *Toxicol Pathol,* **38,** 1011-1025.

Bregman, C.L., Adler, R.R., Morton, D.G., Regan, K.S. and Yano, B.L. (2003). Recommended tissue list for histopathologic examination in repeat-dose toxicity and carcinogenicity studies: a proposal of the Society of Toxicologic Pathology (STP). *Toxicol Pathol,* **31,** 252-253.

Brem, G., Brenig, B. and Goodman, H.M. (1985). Production of trangenic mice, rabbits and pigs by microinjection into pronuclei. *Zuchthygiene,* **20,** 251-252.

Bromberg, J.S. and LeRoith, D. (2006). Diabetes cure--is the glass half full? *N Engl J Med,* **355,** 1372-1374.

Cheon, D.J. and Orsulic, S. (2011). Mouse models of cancer. *Annu Rev Pathol,* **6,** 95-119.

Cooper, D.K., Ekser, B., Ramsoondar, J., Phelps, C. and Ayares, D. (2016). The role of genetically engineered pigs in xenotransplantation research. *J Pathol,* **238,** 288-299.

Davis, M.A., Eldridge, S. and Louden, C. (2013). Biomarkers: discovery, qualification and application In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 317-352. Academic Press. INC., London.

Fairclough, R.J., Wood, M.J. and Davies, K.E. (2013). Therapy for Duchenne muscular dystrophy: renewed optimism from genetic approaches. *Nat Rev Genet,* **14,** 373-378.

Flisikowska, T., Kind, A. and Schnieke, A. (2013). The new pig on the block: modelling cancer in pigs. *Transgenic Res,* **22,** 673-680.

Flisikowska, T., Kind, A. and Schnieke, A. (2014). Genetically modified pigs to model human diseases. *J Appl Genet,* **55,** 53-64.

Glerup, P., Grand, N. and Skydsgaard, M. (2013). The use of minipigs in non-clinical research In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 461-475. Academic Press. INC., London.

Groenen, M.A., Archibald, A.L., Uenishi, H., Tuggle, C.K., Takeuchi, Y., Rothschild, M.F., Rogel-Gaillard, C., Park, C., Milan, D., Megens, H.J., Li, S., Larkin, D.M., Kim, H., Frantz, L.A., Caccamo, M., Ahn, H., Aken, B.L., Anselmo, A., Anthon, C., Auvil, L., Badaoui, B., Beattie, C.W., Bendixen, C., Berman, D., Blecha, F., Blomberg, J., Bolund, L., Bosse, M., Botti, S., Bujie, Z., Bystrom, M., Capitanu,

B., Carvalho-Silva, D., Chardon, P., Chen, C., Cheng, R., Choi, S.H., Chow, W., Clark, R.C., Clee, C., Crooijmans, R.P., Dawson, H.D., Dehais, P., De Sapio, F., Dibbits, B., Drou, N., Du, Z.Q., Eversole, K., Fadista, J., Fairley, S., Faraut, T., Faulkner, G.J., Fowler, K.E., Fredholm, M., Fritz, E., Gilbert, J.G., Giuffra, E., Gorodkin, J., Griffin, D.K., Harrow, J.L., Hayward, A., Howe, K., Hu, Z.L., Humphray, S.J., Hunt, T., Hornshoj, H., Jeon, J.T., Jern, P., Jones, M., Jurka, J., Kanamori, H., Kapetanovic, R., Kim, J., Kim, J.H., Kim, K.W., Kim, T.H., Larson, G., Lee, K., Lee, K.T., Leggett, R., Lewin, H.A., Li, Y., Liu, W., Loveland, J.E., Lu, Y., Lunney, J.K., Ma, J., Madsen, O., Mann, K., Matthews, L., McLaren, S., Morozumi, T., Murtaugh, M.P., Narayan, J., Nguyen, D.T., Ni, P., Oh, S.J., Onteru, S., Panitz, F., Park, E.W*., et al.* (2012). Analyses of pig genomes provide insight into porcine demography and evolution. *Nature,* **491,** 393-398.

Gun, G. and Kues, W.A. (2014). Current progress of genetically engineered pig models for biomedical research. *Biores Open Access,* **3,** 255-264.

Gundersen, H.J. and Jensen, E.B. (1987). The efficiency of systematic sampling in stereology and its prediction. *J Microsc,* **147,** 229-263.

Gundersen, H.J.G., Mirabile, R., Brown, D. and Boyce, R.W. (2013). Stereological principles and sampling procedures for toxicologic pathologists In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M*., et al.* eds.), pp. 215-286. Academic Press. INC., London.

Hammer, R.E., Pursel, V.G., Rexroad, C.E., Jr., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. and Brinster, R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature,* **315,** 680-683.

Hoeflich, A., Weber, M.M., Fisch, T., Nedbal, S., Fottner, C., Elmlinger, M.W., Wanke, R. and Wolf, E. (2002). Insulin-like growth factor binding protein 2 (IGFBP-2) separates hypertrophic and hyperplastic effects of growth hormone (GH)/IGF-I excess on adrenocortical cells in vivo. *FASEB J,* **16,** 1721-1731.

Holm, I.E., Alstrup, A.K. and Luo, Y. (2016). Genetically modified pig models for neurodegenerative disorders. *J Pathol,* **238,** 267-287.

Howard, C.V. and Reed, M.G. (2005). *Unbiased Stereology,* QTP Publications, Coleraine, UK.

Jackson, P.G.G. and Cockcroft, P.D. (2007). *Handbook of Pig Medicine,* SAUNDERS Elsevier Limited, Edinburgh, UK.

Jeppesen, G. and Skydsgaard, M. (2015). Spontaneous background pathology in Gottingen minipigs. *Toxicol Pathol,* **43,** 257-266.

Jirka, S.M., Tanganyika-de Winter, C.L., Boertje-van der Meulen, J.W., van Putten, M., Hiller, M., Vermue, R., de Visser, P.C. and Aartsma-Rus, A. (2015). Evaluation of 2'-Deoxy-2'-fluoro Antisense Oligonucleotides for Exon Skipping in Duchenne Muscular Dystrophy. *Mol Ther Nucleic Acids,* **4,** e265.

Kemter, E., Lieke, T., Kessler, B., Kurome, M., Wünsch, A., Summerfield, A., Ayares, D., Nagashima, H., Baars, W., Schwinzer, R. and Wolf, E. (2012). Human TNF-related apoptosis-inducing ligand-expressing dendritic cells from transgenic pigs attenuate human xenogeneic T cell responses. *Xenotransplantation,* **19,** 40-51.

Kemter, E. and Wolf, E. (2015). Pigs pave a way to de novo formation of functional human kidneys. *Proc Natl Acad Sci U S A,* **112,** 12905-12906.

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

Klymiuk, N., Aigner, B., Brem, G. and Wolf, E. (2010). Genetic modification of pigs as organ donors for xenotransplantation. *Mol Reprod Dev,* **77,** 209-221.

Klymiuk, N., Blutke, A., Graf, A., Krause, S., Burkhardt, K., Wünsch, A., Krebs, S., Kessler, B., Zakhartchenko, V., Kurome, M., Kemter, E., Nagashima, H., Schoser, B., Herbach, N., Blum, H., Wanke, R., Aartsma-Rus, A., Thirion, C., Lochmuller, H., Walter, M.C. and Wolf, E. (2013). Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle. *Hum Mol Genet,* **22,** 4368-4382.

Klymiuk, N., Bocker, W., Schonitzer, V., Bahr, A., Radic, T., Frohlich, T., Wünsch, A., Kessler, B., Kurome, M., Schilling, E., Herbach, N., Wanke, R., Nagashima, H., Mutschler, W., Arnold, G.J., Schwinzer, R., Schieker, M. and Wolf, E. (2012a). First inducible transgene expression in porcine large animal models. *FASEB J,* **26,** 1086-1099.

Klymiuk, N., Mundhenk, L., Kraehe, K., Wünsch, A., Plog, S., Emrich, D., Langenmayer, M.C., Stehr, M., Holzinger, A., Kroner, C., Richter, A., Kessler, B., Kurome, M., Eddicks, M., Nagashima, H., Heinritzi, K., Gruber, A.D. and Wolf, E. (2012b). Sequential targeting of CFTR by BAC vectors generates a novel pig model of cystic fibrosis. *J Mol Med (Berl),* **90,** 597-608.

Klymiuk, N., Seeliger, F., Bohlooly, Y.M., Blutke, A., Rudmann, D.G. and Wolf, E. (2015). Tailored Pig Models for Preclinical Efficacy and Safety Testing of Targeted Therapies. *Toxicol Pathol*.

Klymiuk, N., van Buerck, L., Bahr, A., Offers, M., Kessler, B., Wünsch, A., Kurome, M., Thormann, M., Lochner, K., Nagashima, H., Herbach, N., Wanke, R., Seissler, J. and Wolf, E. (2012c). Xenografted islet cell clusters from INSLEA29Y transgenic pigs rescue diabetes and prevent immune rejection in humanized mice. *Diabetes,* **61,** 1527-1532.

Kurome, M., Kessler, B., Wünsch, A., Nagashima, H. and Wolf, E. (2015). Nuclear transfer and transgenesis in the pig. *Methods Mol Biol,* **1222,** 37-59.

Kurome, M., Ueda, H., Tomii, R., Naruse, K. and Nagashima, H. (2006). Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. *Transgenic Res,* **15,** 229-240.

Liu, M., Hodish, I., Haataja, L., Lara-Lemus, R., Rajpal, G., Wright, J. and Arvan, P. (2010). Proinsulin misfolding and diabetes: mutant INS gene-induced diabetes of youth. *Trends Endocrinol Metab,* **21,** 652-659.

Lunney, J.K. (2007). Advances in swine biomedical model genomics. *Int J Biol Sci,* **3,** 179-184.

Luo, Y., Lin, L., Bolund, L., Jensen, T.G. and Sorensen, C.B. (2012). Genetically modified pigs for biomedical research. *J Inherit Metab Dis,* **35,** 695-713.

Madsen, K.M. (1999). The art of counting. *J Am Soc Nephrol,* **10,** 1124-1125.

Mattfeldt, T. (1990). *Stereologische Methoden in der Pathologie.* Thieme, Stuttgart, New York.

Mattfeldt, T., Mall, G., Gharehbaghi, H. and Moller, P. (1990). Estimation of surface area and length with the orientator. *J Microsc,* **159,** 301-317.

Mattfeldt, T., Mobius, H.J. and Mall, G. (1985). Orthogonal triplet probes: an efficient method for unbiased estimation of length and surface of objects with unknown orientation in space. *J Microsc,* **139,** 279-289.

McDorman, K.S., Chan, C., Rojko, J., Satterwhite, C.M. and Morrison, J.P. (2013). Special techniques in toxicologic pathology In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 175-214. Academic Press. INC., London.

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

Müller, S., Wanke, R. and Distl, O. (1995). Segregation of pigment cell anomalies in Munich miniature swine (MMS) Troll crossed with German Landrace. *Dtsch Tierärztl Wochenschr,* **102,** 391-394.

Murakami, T., Hitomi, S., Ohtsuka, A., Taguchi, T. and Fujita, T. (1997). Pancreatic insulo-acinar portal systems in humans, rats, and some other mammals: scanning electron microscopy of vascular casts. *Microsc Res Tech,* **37,** 478-488.

Nyengaard, J.R. (1999). Stereologic methods and their application in kidney research. *J Am Soc Nephrol,* **10,** 1100-1123.

Nyengaard, J.R. and Gundersen, H.J.G. (1992). The isector: a simple and direct method for generating isotropic, uniform random sections from small specimens. *Journal of Microscopy,* **165,** 427-431.

Nyengaard, J.R. and Gundersen, H.J.G. (2006). Sampling for stereology in lungs. *Eur Respir Rev,* **15,** 107-114.

Ochs, M. and Mühlfeld, C. (2013). Quantitative microscopy of the lung: a problem-based approach. Part 1: basic principles of lung stereology. *Am J Physiol Lung Cell Mol Physiol,* **305,** L15-22.

Ouchi, N., Parker, J.L., Lugus, J.J. and Walsh, K. (2011). Adipokines in inflammation and metabolic disease. *Nat Rev Immunol,* **11,** 85-97.

Prather, R.S., Lorson, M., Ross, J.W., Whyte, J.J. and Walters, E. (2013). Genetically engineered pig models for human diseases. *Annu Rev Anim Biosci,* **1,** 203-219.

Renner, S., Blutke, A., Streckel, E., Wanke, R. and Wolf, E. (2016). Incretin actions and consequences of incretin-based therapies: lessons from complementary animal models. *J Pathol,* **238,** 345-358.

Renner, S., Braun-Reichhart, C., Blutke, A., Herbach, N., Emrich, D., Streckel, E., Wünsch, A., Kessler, B., Kurome, M., Bahr, A., Klymiuk, N., Krebs, S., Puk, O., Nagashima, H., Graw, J., Blum, H., Wanke, R. and Wolf, E. (2013). Permanent neonatal diabetes in INS(C94Y) transgenic pigs. *Diabetes,* **62,** 1505-1511.

Renner, S., Fehlings, C., Herbach, N., Hofmann, A., von Waldthausen, D.C., Kessler, B., Ulrichs, K., Chodnevskaja, I., Moskalenko, V., Amselgruber, W., Goke, B., Pfeifer, A., Wanke, R. and Wolf, E. (2010). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. *Diabetes,* **59,** 1228-1238.

Renner, S., Romisch-Margl, W., Prehn, C., Krebs, S., Adamski, J., Goke, B., Blum, H., Suhre, K., Roscher, A.A. and Wolf, E. (2012). Changing metabolic signatures of amino acids and lipids during the prediabetic period in a pig model with impaired incretin function and reduced beta-cell mass. *Diabetes,* **61,** 2166-2175.

Rousseaux, C.G. and Gad, S.C. (2013). Statistical assessment of toxicologic pathology studies In *Haschek and Rousseaux´s Handbook of Toxicologic Pathology* (Haschek, W.M.*, et al.* eds.), pp. 894-988. Academic Press. INC., London.

Ruehl-Fehlert, C., Kittel, B., Morawietz, G., Deslex, P., Keenan, C., Mahrt, C.R., Nolte, T., Robinson, M., Stuart, B.P., Deschl, U., Group, R. and Group, N. (2003). Revised guides for organ sampling and trimming in rats and mice--part 1. *Exp Toxicol Pathol,* **55,** 91-106.

Schneider, J.P. and Ochs, M. (2014). Alterations of mouse lung tissue dimensions during processing for morphometry: a comparison of methods. *Am J Physiol Lung Cell Mol Physiol,* **306,** L341-350.

Smith, A.C. and Swindle, M.M. (2006). Preparation of swine for the laboratory. *ILAR J,* **47,** 358-363.

Steiner, D.J., Kim, A., Miller, K. and Hara, M. (2010). Pancreatic islet plasticity: interspecies comparison of islet architecture and composition. *Islets,* **2,** 135-145.

Streckel, E., Braun-Reichhart, C., Herbach, N., Dahlhoff, M., Kessler, B., Blutke, A., Bahr, A., Ubel, N., Eddicks, M., Ritzmann, M., Krebs, S., Goke, B., Blum, H., Wanke, R., Wolf, E. and Renner, S. (2015a). Effects of the glucagon-like peptide-1 receptor agonist liraglutide in juvenile transgenic pigs modeling a pre-diabetic condition. *J Transl Med,* **13,** 73.

Swindle, M.M., Makin, A., Herron, A.J., Clubb, F.J., Jr. and Frazier, K.S. (2012). Swine as models in biomedical research and toxicology testing. *Vet Pathol,* **49,** 344-356.

Swindle, M.M., Smith, A.C., Laber-Laird, K. and Dungan, L. (1994). Swine in biomedical research: Management and models. *ILAR J,* **36,** 1-5.

Tornqvist, E., Annas, A., Granath, B., Jalkesten, E., Cotgreave, I. and Oberg, M. (2014). Strategic focus on 3R principles reveals major reductions in the use of animals in pharmaceutical toxicity testing. *PLoS One,* **9,** e101638.

Turner, N.J., Pezzone, D. and Badylak, S.F. (2015). Regional variations in the histology of porcine skin. *Tissue Eng Part C Methods,* **21,** 373-384.

van Buerck, L., Schuster, M., Rathkolb, B., Sabrautzki, S., Hrabe de Angelis, M., Wolf, E., Aigner, B., Wanke, R. and Herbach, N. (2012). Enhanced oxidative stress and endocrine pancreas alterations are linked to a novel glucokinase missense mutation in ENU-derived Munich Gck(D217V) mutants. *Mol Cell Endocrinol,* **362,** 139-148.

Wanke, R. (2013). Pigmentzellanomalien beim Münchner Miniaturschwein Troll: Ein porzines Melanommodell In *Das Melanom - der "schwarze Tod" der Neuzeit* (Brem, G. & Stingl, G. eds.). Österreichische Akademie der Wissenschaften, Wien.

Weibel, E.R. (1979). *Stereological methods. I. Practical methods for biological morphometry.* Academic Press, London.

Whitelaw, C.B., Sheets, T.P., Lillico, S.G. and Telugu, B.P. (2016). Engineering large animal models of human disease. *J Pathol,* **238,** 247-256.

Whyte, J.J. and Prather, R.S. (2011). Genetic modifications of pigs for medicine and agriculture. *Mol Reprod Dev,* **78,** 879-891.

Wolf, E., Braun-Reichhart, C., Streckel, E. and Renner, S. (2014). Genetically engineered pig models for diabetes research. *Transgenic Res,* **23,** 27-38.

Wünsch, A., Baehr, A., Bongoni, A.K., Kemter, E., Blutke, A., Baars, W., Haertle, S., Zakhartchenko, V., Kurome, M., Kessler, B., Faber, C., Abicht, J.M., Reichart, B., Wanke, R., Schwinzer, R., Nagashima, H., Rieben, R., Ayares, D., Wolf, E. and Klymiuk, N. (2014). Regulatory sequences of the porcine THBD gene facilitate endothelial-specific expression of bioactive human thrombomodulin in single- and multitransgenic pigs. *Transplantation,* **97,** 138-147.

8 Beiträge auf wissenschaftlichen Kongressen

Teile der vorliegenden Arbeit in Vorträgen wurden auf wissenschaftlichen Kongressen präsentiert und als Abstracts veröffentlicht.

Vorträge auf wissenschaftlichen Tagungen (*vortragende Person)

Hofmann B*. Aktueller Stand der APAM Biobank. Pig Biobank Meeting Mini-Symposium. München, Deutschland, 2. Oktober 2014.

Albl B, Haesner S, Braun-Reichhart C, Streckel E, Renner S, Seeliger F, Wanke R, Wolf E, Blutke A*. Standardized Tissue Sampling in Porcine Biomedical Models. Minipig Workshop. Freising, Deutschland, 23. und 24. September 2015.

Abstracts in Tagungsbänden

Blutke A, Braun-Reichhart C, Albl B, Streckel E, Haesner S, Renner S, Wanke R, Wolf E. The Munich MIDY-PIG Biobank: a unique resource for translational diabetes research. Opening Conference of COST Action BM1308 “Sharing Advances on Large Animal Models – SALAAM”. München, Deutschland, 15. bis 17. Dezember 2014. Abstract im Tagungsband Seite 32.

Blutke A, Albl B, Haesner S, Braun-Reichhart C, Wolf E, Wanke R. Etablierung einer Gewebe-Biobank eines porcinen Diabetes mellitus Modelles. 58. Jahrestagung der Fachgruppe Pathologie der Deutschen Veterinärmedizinischen Gesellschaft. Fulda, Deutschland, 7. und 8. März 2015. Abstract im Tagungsband Seite 16.

Abstracts in „Refereed Journals”

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