The Royal College of Pathologists
Pathology: the science behind the cure

Tissue pathways for non-neoplastic neuropathology specimens

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Foreword

The tissue pathways published by The Royal College of Pathologists (RCPath) are guidelines which enable pathologists to deal with routine surgical specimens in a consistent manner and to a high standard. This ensures that accurate diagnostic and prognostic information is available to clinicians for optimal patient care and ensures appropriate management for specific clinical circumstances. This guideline has been developed to cover most common circumstances. However, we recognise that guidelines cannot anticipate every pathological specimen type and clinical scenario. Occasional variation from the practice recommended in this guideline may therefore be required to report a specimen in a way that maximises benefit to the patient.

The guidelines themselves constitute the tools for implementation and dissemination of good practice.

The stakeholders consulted for this document were:

- The British Neuropathological Society
- Representatives of the British Myology Society.

As with many areas of diagnostic cellular pathology, evidence for these recommendations is not based on systematic, controlled studies. Evidence was evaluated using modified SIGN guidance. The evidence is predominantly classified as: “Good practice point (GPP): Recommended best practice based on the clinical experience of the authors of the writing group” (see Appendix A).

No major organisational changes or cost implications have been identified that would hinder the implementation of the tissue pathway.

The information used to develop this tissue pathway was collected from electronic searches of the medical literature, previous recommendations of the RCPath and local guidelines in the UK. Published evidence was evaluated using modified SIGN guidance. Consensus of evidence in the tissue pathways was achieved by expert review.

A formal revision cycle for all tissue pathways takes place on a five-yearly basis. However, each year, the College will ask the author(s) of the tissue pathways, in conjunction with the relevant subspecialty adviser to the College, to consider whether or not the document needs to be updated or revised. A full consultation process will be undertaken if major revisions are required. If minor revisions are required, an abridged consultation process will be undertaken whereby a short note of the proposed changes will be placed on the College website for two weeks for members’ attention. If members do not object to the changes, the short notice of change will be incorporated into the pathways and the full revised version (incorporating the changes) will replace the existing version on the publications page of the College. All changes will be documented in the data control section of the relevant pathway.

The pathway has been reviewed by the Clinical Effectiveness department and Working Group on Cancer Services and placed on the College website for abridged consultation with the membership from 12 October to 26 October 2017. All comments received from the Working Group and membership will be addressed by the authors to the satisfaction of the Tissue Pathway Coordinator and the Director of Publishing and Engagement.

This pathway was developed without external funding to the writing group. The College requires the authors of tissue pathways to provide a list of potential conflicts of interest; these are monitored by the Clinical Effectiveness department and are available on request. The authors of this document have declared that there are no conflicts of interest.

1 Introduction

This document covers tissue pathways for specific areas within diagnostic neuropathology. The guidance in the previous editions of the *Tissue pathways for non-neoplastic neuropathology specimens* has been piloted in laboratory practice, either by the authors or in the laboratories of those responding to the consultation.

As with many areas of diagnostic cellular pathology, evidence for these recommendations is not based on systematic, controlled studies. Such evidence, classified as good practice point (GPP; see Appendix A), is typically expert opinion without explicit critical appraisal, or based on physiology, bench research or 'first principles'. In view of this, references cited in support of the evidence are mainly drawn from textbooks.

There are few studies that document specificity and sensitivity for the range of diagnoses being considered in this section of diagnostic neuropathology. Users of these guidelines are recommended to consult PubMed to see if any original publications exist in cases where knowing the specificity and sensitivity of a biopsy technique would influence a clinical decision to undertake a procedure.

Neoplastic neuropathology cases are covered in The Royal College of Pathologists' *Dataset for tumours of the central nervous system, including the pituitary gland (4th edition, 2016).*[1]

1.1 Target users and health benefits of this guideline

The target primary users of the tissue pathway are trainee and consultant cellular pathologists and, on their behalf, the suppliers of IT products to laboratories. The secondary users are surgeons and oncologists, cancer registries and the National Cancer Intelligence Network.

1.2 Staffing and workload

Central nervous system (CNS) biopsies should be reported by neuropathologists or histopathologists with a special interest in neuropathology who participate in the UK national neuropathology external quality assessment (EQA) scheme.

Muscle biopsies should be processed by accredited departments that participate in the national muscle technical EQA scheme.

Ideally, two or more neuropathologists in a unit should have the requisite skills, knowledge and externally validated diagnostic competence in order to provide continuity of service for periods of leave. In centres that have only one neuropathologist, arrangements with other units should be agreed to allow continuity of service, making use of telepathology if available and appropriate.

The minimum and maximum number of neurosurgical biopsies for maintenance of diagnostic accuracy, without risking errors, currently lacks an evidence base. All procedures should be carried out in adequately equipped and staffed centres by teams with experience in removing, handing and processing these samples.

Any on-call service offered to support neurosurgery should be staffed by neuropathologists who contribute to the routine neuropathology service and hold EQA evidence of competence.

1.3 Laboratory and workplace facilities

Routine light microscopy, immunohistochemistry, special histochemistry and access to electron microscopy (EM) are all required for a comprehensive service encompassing nerve,

muscle and CNS (brain, spinal cord and meningeal) biopsies. There should be facilities to review and discuss radiological findings with local specialists in this area. In order to reduce the risk of erroneous diagnoses, the reporting pathologists should have sessional time to attend regular multidisciplinary meetings, during which the pathological findings can be correlated with radiological and clinical findings.

There should be facilities for storage and archiving of fresh-frozen material at -80°C, or in liquid nitrogen, and facilities and resources to enable the transport of samples on dry ice using courier services for specialised investigation.

Processing of unfixed 'high risk' specimens (e.g. cytospin, intraoperative smear and frozen sections, or routine frozen sections such as muscle biopsies) and any decisions on whether to provide a service dealing with such material should be based on local risk assessments, the availability of appropriately trained staff and any necessary laboratory containment facilities.

1.4 Specimen submission: general considerations

Biopsies should be accompanied by adequate clinical, laboratory and radiological information to focus the differential diagnosis and choice of special investigations. All National Specialised Commissioning Team (NSCT; formerly NCG or NSCAG) referrals must be accompanied by a current NSCT form (completed by the referring clinician), up-to-date clinic letters, muscle biopsy reports and all relevant available information pertaining to the case.

Further details and forms are available to download from the websites of the NSCT centres:

- www.gosh.nhs.uk/research-and-innovation/nihr-great-ormond-street-brc/brc-news/dubowitz-neuromuscular-centre-confirmed-centre-paediatric-clinical-and-research-excellence (accessed and valid in July 2017)
- www.ncl.ac.uk/igm/services/ngs/#purpose (accessed and valid in July 2017).

Peripheral nerve and muscle biopsy should include:

- information about the biopsy site and laterality
- details of onset, distribution and progression of the disorder
- involvement of other organs
- results of nerve conduction studies and electromyography or MRI of brain, spinal cord and adjacent nerve roots or muscle (if performed)
- drug history
- occupational exposure(s)
- past and current personal and family medical history
- plasma creatinine kinase (CK) level (essential)
- plasma and cerebrospinal fluid (CSF) lactate levels (muscle biopsies), other biochemical investigations and results of brain, spinal cord and adjacent nerve roots or muscle MRI (if performed and as relevant).

Where applicable, specimens and their request sheet should be labelled with a 'Risk of infection' sticker.

Specimens should be delivered to the laboratory in the appropriate state for the intended investigations, for example:

- CSF for cytology: within 1 hour of being taken

- fresh brain: within 15 minutes of being taken
- nerve (see section 3)
- muscle biopsies must ideally be transported immediately to the lab fresh at room temperature, either wrapped in gauze lightly moistened in saline or wrapped in cling film to prevent drying out. For histopathological studies, this is acceptable for samples with total transit time less than two hours from the time of collection. Samples with transit time exceeding two hours must be frozen on site and transported on dry ice. Samples for EM should be fixed in glutaraldehyde within 15 minutes of being taken. Samples collected for mitochondrial respiratory chain enzyme analysis must be frozen immediately (at the bedside or in theatre) or no later than 15 minutes to avoid loss of enzyme activity
- skin and chorionic villus samples (CVS) for culturing should be transported to the lab in sterile standard culture medium at room temperature within 24 hours
- ideally, skin samples for histopathological studies should be immediately transported to the lab fresh
- CVS samples for histopathology should be transported in sterile standard culture medium at room temperature within 24 hours.

1.5 Specimen dissection: general considerations

Handling should be appropriate to the category of risk indicated and in line with local guidelines and health and safety regulations (see sections 1.3 and 1.4). In practice, intraoperative frozen sectioning of known or suspected Hazard Group 3 pathogens, such as *Mycobacterium tuberculosis*, should be avoided. Given that a proportion of biopsied lesions from the CNS turn out to be undiagnosed infective conditions, intraoperative diagnostic material should be handled as a potential category 3 pathogen risk until proven otherwise.

2 Skeletal muscle[2,3]

2.1 Specimen submission and dissection

- Formalin-fixed specimens: these will usually constitute the minority in a specialist neurosciences centre. All specimens in which there is a risk of infection should be formalin fixed. In all other situations, the recommended practice is to examine snap-frozen tissue with the optional addition of a formalin-fixed specimen if there is sufficient material. After measurement, submit formalin-fixed muscle for paraffin processing with embedding in both transverse and longitudinal orientations. Barring high-risk samples and large open biopsies where it may be impractical to freeze down the entire specimen volume, the routine formalin fixation of biopsies is discouraged as muscle pathology can be focal and formalin fixation can limit histopathological testing, thereby hindering a diagnosis.
- The majority of specimens in a clinical neurosciences setting will be delivered either:
  - in a fresh state: subdivide for cryostat sectioning, molecular genetic, biochemical and EM studies. Fresh samples for cryostat sectioning must be orientated transversely (preferably using a dissecting microscope).
  - on dry ice, having been snap-frozen (with or without an accompanying piece of tissue in EM fixative): separate pieces (or subdivide) for cryostat sectioning, molecular genetic and biochemical studies. A minimum specimen diameter of 3–4 mm (size of an orange pip) is recommended for genetic and biochemical studies.

For both, note the interval between removal of the tissue from the patient and freezing. This will affect the interpretation of histochemical and biochemical (respiratory chain) data.

2.2 Embedding options

Water-based mountant (e.g. 'optimal cutting temperature') with sample orientated transversely. Resin for EM, as appropriate, with at least some samples longitudinally orientated.

2.3 Sectioning

Frozen tissue: cryostat at 8–12 µm (according to stain). Resin sections: ultra-microtome set at ~0.6–1 µm for semi-thin sections and ~50–90 nm for ultra-thin sections.

2.4 Typical routine staining (which may be adapted according to the clinical circumstances of a case)

Cryostat sections: routinely for haematoxylin and eosin (H&E); Gömöri trichrome (Engel and Cunningham); cytochrome oxidase (COX); succinate dehydrogenase (SDH); combined COX/SDH; NADH-TR; acid phosphatase; lipid stains (e.g. Oil Red O or Sudan Black); periodic acid-Schiff (PAS)/PAS-diastase (DPAS); acid phosphatase; ATPase at pH 9.5, 4.6 and 4.3; myosin heavy chain immunohistochemistry (slow, fast, fetal [neonatal] and developmental); and MHC class I. Myosin immunolabelling has the advantage of identifying fibres expressing more than one isoform and determining if a biopsy is normal or abnormal. (Depending on the preference of the diagnostic service, immunohistochemistry may be used instead of histochemical stains for fibre typing.)

Note: A diagnostic service may prefer to perform a subset of these routine stains first and, based on an initial histological analysis and consideration of clinical features in a case, decide on which additional routine investigations to perform alongside those considered in the next section (below). For resource planning, it is anticipated that most cases will require application of this panel to determine if a sample is normal or abnormal, and to differentiate between various possible diagnoses. All tests should be interpreted in the light of experience with positive and negative age-appropriate biopsies, which may include age-matched normal and/or disease controls and with a control sample stained at the same time.

2.5 Further investigations depending on initial results and phenotype

According to clinical indications or pathological findings, supplementary investigations may include:

- histochemistry for phosphorylase, phosphofructokinase, myoadenylate deaminase, aldolase, menadione NBT (without substrate)
- immunohistochemistry for dystrophy-associated proteins (e.g. dystrophin using antibodies to epitopes against N-terminal, rod and C-terminal domains; utrophin; NNOS; alpha-, beta-, gamma- and delta-sarcoglycan; caveolin-3; emerin; dysferlin; laminin α2 [80 and 300 kDa], laminin α5, laminin β1; and other appropriate dystrophin axis associated proteins); beta-spectrin and laminin γ1 immunohistochemistry for assessing sarcolemmal integrity
- Congo red, ubiquitin and p62 if inclusion body myositis or protein aggregation myopathy are suspected or for exclusion, or, if rimmed vacuoles are present, EM may also be undertaken
- immunohistochemistry for desmin, myotilin, alpha-B-crystallin, FHL-1, BAG-3, filamin A/C if a myofibrillar myopathy is suspected
- immunohistochemistry for MHC class I (HLA-ABC or β2-microglobulin); complement membrane attack complex (complement C5b-9 components); CD3; CD4; CD8; CD79a; CD68

- EM: indications including mitochondrial cytopathy, nemaline myopathy, cores, inclusion body myositis and clarification of features observed with light microscopy – fixative containing 2.5–6% glutaraldehyde in 0.1M cacodylate or phosphate buffer
- respiratory chain enzyme analysis for suspected mitochondrial cytopathy
- molecular genetic studies: indications including mitochondrial cytopathy
- western blot analysis: indications including various muscular dystrophies with raised creatine kinase
- specific single-enzyme studies: indications including glycogen storage diseases
- referral to NSCT services, as follows:
  - Dubowitz Neuromuscular Centre, London: for suspected congenital myopathy or congenital muscular dystrophy. Repertoire of tests includes immunoanalysis of dystroglycans, laminins and collagen VI in muscle, skin and CVS; fluorescent activated cell sorting quantification in cultured fibroblasts.
  - Newcastle: if CK raised and phenotype suggests limb girdle muscle dystrophy, or a myofibrillar myopathy is suspected
  - Oxford: if a myasthenic syndrome is suspected
  - Queen Square, London: if an ion channel disorder is suspected
  - NSCT mitochondrial service (Queen Square London, Newcastle, Oxford): if mitochondrial disorder is suspected.

2.6 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- biopsy dimensions and time of stations in handling, especially time to freeze
- description of the microscopic findings
- interpretation of the findings, with indication of limitations imposed by available clinical information, amounting to specific or differential diagnosis
- comparison with any previous biopsy
- any recommendation for re-biopsy if inadequate for diagnosis
- any recommendation for supra-regional referral (see section 2.5)
- any required pre-referral form
- suggested diagnosis
- conclusion
- SNOMED codes.

[Level of evidence GPP – the recommendations in this section are based on the expert opinions of the authors.]

3 Peripheral nerve

The specimen should be obtained from an affected nerve. Most neuropathies show distal accentuation. The sural nerve is purely sensory in more than 90% of patients and contains

only a few motor fibres in the remaining.[4] Moreover, it is easily accessible to surgery and therefore most frequently chosen for biopsy. It usually contains between five and ten nerve fascicles. In a large autopsy study, 3,300–8,000 myelinated and 10,500–45,500 unmyelinated nerve fibres were found in subjects without history of disease or ingestion of drugs known to affect peripheral nerve.[5] The nerve segment should be excised and handled with much care in order to minimise mechanical injury to the sample. Squeezing or stretching the nerve should be strictly avoided and excessive removal of fat or connective tissue should not be attempted. Nerve fibres are very sensitive to mechanical injury.

3.1 Specimen submission and dissection

The recommended length of the biopsied nerve segment is 4 cm. Removal of a shorter segment will be less useful diagnostically and can hamper a proper analysis, but will leave an identical sensory deficit.

Formalin and glutaraldehyde fixation of nerve segments is essential, freezing an unfixed segment is optional.

Submissions received from non-specialist centres can be in fixative, with one part in formalin and a separate piece in EM fixative (containing 2.5–6% glutaraldehyde in 0.1M cacodylate or phosphate buffer). Material fixed in glutaraldehyde containing fixative will be processed for semi-thin resin sections and potentially for EM and teased fibres. Formalin-fixed material will be processed into paraffin wax for histochemical and immunohistochemical studies.

The great majority of specimens derived from clinical neuroscience centres will be delivered fresh. However, the fresh tissue has to be fixed in both formalin and 3% glutaraldehyde containing fixative immediately (at most a 5 minute delay). Therefore, the tissue sample should not be transported in the fresh state (unfixed) when the courier time would exceed 5 minutes.

Subdivide into four segments (in order of priority):

- formalin fixation (or equivalent fixatives) and paraffin embedding; with one piece oriented longitudinally and the remainder transversally
- glutaraldehyde fixation and resin embedding; the glutaraldehyde-fixed specimen is cut transversally (longitudinal optionally in addition)
- glutaraldehyde fixation for teased fibre preparation
- optional: snap-freezing.

3.2 Embedding options

Paraffin processing for routine stains and immunohistochemistry; resin for EM. Optional preparation of frozen sections.

3.3 Sectioning

Microtome section at approximately 3–4 µm nominal thickness, at multiple levels. Ultra-microtome set at approximately ~0.6 µm for semi-thin sections and ~50–90 nm for ultra-thin sections.

3.4 Routine staining

Microtome sections: routinely for H&E; and optional for elastic van Gieson (EVG; or other suitable trichrome), Congo red and Perls'. Immunohistochemistry for neurofilament protein (e.g. NF200), myelin basic protein (e.g. SMI94), CD68 (macrophages), CD3 (T cells) and

CD20 (B cells). It is recommended to use immunostainings instead of tinctorial stains for axons and myelin sheath, as they are more specific and give a better signal to noise ratio.[6]

It is highly recommended to prepare semi-thin sections from glutaraldehyde-fixed material (stained for example with Toluidine blue or Methyl Blue Aniline–Basic Fuchsin) as standard, as it allows superior morphological assessment of a large range of pathological features.

3.5 Further investigation

Depending on clinical indications or pathological findings, the following stains are optional:

- histochemistry for fibrin
- epithelial membrane antigen for assessment of the perineurium
- immunohistochemistry for further immune cell subsets e.g. CD79a (B cells), CD8, CD4, (T cells), CD138 (plasma cells)
- EM
- teased fibres
- Wade-Fite (leprosy).

3.6 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- biopsy dimensions
- description of the histological findings
- morphometric data if relevant (e.g. density of myelinated fibres as well as other morphometric data)
- interpretation of the findings, with indication of limitations imposed by available clinical information, amounting to specific or differential diagnosis
- comparison with any previous biopsy
- any recommendation for re-biopsy if inadequate for diagnosis
- any recommendation for supra-regional referral
- any required pre-referral form
- conclusion
- SNOMED codes.

[Level of evidence B – the recommendations in this section are based on a body of evidence demonstrating consistency of results and comprising mainly high-quality systematic reviews of case studies with a very low risk of confounding or bias (see References below).]

4 Central nervous system (brain, spinal cord, meninges) incisional or excisional biopsy for non-neoplastic lesion[3]

4.1 Specimen submission and dissection

Usually, specimens will be received either in formalin or fresh, determined mainly but not exclusively by a requirement for intraoperative diagnosis. Specimens should be resected as a single piece, measuring at least 1x1 cm and should contain leptomeninges, cortex and subcortical white matter. Even if the clinical and radiological impression suggests a diffuse white matter process, it is strongly advised to include cortical grey matter in order to maximise the diagnostic options.

4.2 Immediate handling and intraoperative diagnosis

The biopsy of non-neoplastic lesions requires a different intraoperative approach than those of neoplastic or presumed neoplastic lesions. Generally, intraoperative diagnosis should not be requested for diagnostic biopsies of non-neoplastic lesions, unless the result can change the intraoperative approach. Intraoperative assessments give only very limited information and should be avoided to preserve the maximum possible amount of diagnostic material.

Check for infection risk label and also assess risk from clinical information provided.

If the request for intraoperative diagnosis is firm, consider smear preparation and/or frozen section. Frozen sections will have relatively greater value if the tissue texture precludes making an effective smear. Also, the block of frozen tissue can be fixed and processed through paraffin afterwards.

Issue a verbal report, making written record of the exact wording, the recipient and time the report was communicated.

If infection is possible, check that the surgeon has submitted separate material to microbiology. Do so oneself if this has not been done, the operative field has been closed and material is still unfixed.

Set aside tissue for freezing (further morphological investigations on frozen tissue, molecular genetics and tissue bank), EM and adjacent tissue paraffin processing, all according to diagnostic needs and terms of consent.

4.3 Embedding options

Paraffin processing for routine stains and immunohistochemistry; resin for EM. Treatment with formic acid is necessary on a proportion of the sample if prion disease is in the differential diagnosis (the other half/portion should be left in formalin until prion disease is either confirmed or excluded).

4.4 Sectioning

Microtome section at 4 µm, at multiple levels. Ultra-microtome set at ~0.6 µm for semi-thin sections and ~50–90 nm for ultra-thin sections.

4.5 Routine staining

Microtome sections: routinely for H&E.

4.6 Further investigation

Depending on clinical indications or pathological findings, the following stains are optional:

- if vascular malformation or evacuated blood clot, typically apply Congo red, Perls', EVG or equivalent. Aβ immunohistochemistry for amyloid angiopathy is strongly recommended as it is more sensitive than Congo red.
- if non-neoplastic space occupying lesion:
  - if looks infective/inflammatory: Zieh-Neelsen, Gram, Grocott, DPAS; add Toxoplasma immunohistochemistry, as appropriate
  - consider viral aetiology: herpes simplex virus, cytomegalovirus, measles, Epstein-Barr virus and human immunodeficiency virus. As the immunohistochemical investigation of biopsies with suspected viral aetiology is limited, it is strongly recommended to discuss submission of material (fresh/frozen) for viral sequencing, using next-generation sequencing (NGS) approaches. NGS can cover a wide range of viral pathogens, including a precise definition of the viral strain, if necessary.
  - if there is evidence of demyelination: myelin (myelin basic protein e.g. immunohistochemistry: SMI94; histochemistry: Luxol fast blue/cresyl violet) and axonal (neurofilament protein) stain; CD68; glial fibrillary acidic protein (GFAP). If progressive multifocal leuko-encephalopathy (PML) possible, perform immunohistochemistry or in situ hybridisation for polyoma virus (SV40).
  - EM if material available in appropriate fixative (glutaraldehyde) and DNA/RNA extraction for viral, bacterial (e.g. *Mycobacterium tuberculosis*) and fungal PCR. (Note that also possible from paraffin-embedded material.)
  - preserve a representative sample for freezing, to allow molecular assays for an extended range of pathogens (see assays for viral aetiology above).

4.7 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- biopsy dimensions
- description of the histological findings
- interpretation of the findings amounting to specific or differential diagnosis
- comparison with any previous biopsy
- any recommendation for re-biopsy if inadequate for diagnosis
- any recommendation for supra-regional referral (e.g. fungal, bacterial or viral reference lab)
- conclusion
- SNOMED codes.

[Level of evidence GPP – the recommendations in this section are based on the expert opinions of the authors.]

5 Brain biopsy for dementia

5.1 General comments[7–10]

Brain biopsy for dementia is typically considered when clinical or laboratory features suggest a reversible process such as vasculitis or infection in the differential diagnosis. Possible diagnoses include Creutzfeldt-Jakob disease (CJD), other neurodegenerative dementias, inflammatory disease, and cerebrovascular disease including cerebral amyloid angiopathy, multiple sclerosis, neoplasm, leukodystrophy, storage disorders and undetermined encephalopathies. Diagnostic yield ranges from 22 to 84%.[5]

5.2 Specimen submission and dissection

Specimens must be sent to the laboratory unfixed. Microbiological (bacteriological/ mycological) and virological samples should be submitted separately.

5.3 Immediate handling

- Check for infection risk labels and also assess risk from clinical information provided. Handle in appropriate containment if CJD is a possibility. Even if no infection risk label is present, it is recommended to consider such biopsies as high risk and manage/divide the biopsy accordingly (see below regarding prion disease).
- Assess for adequacy: cerebral full thickness including leptomeninges, and grey and white matter.
- Set aside tissue for freezing (molecular genetics; prion biochemistry), EM and adjacent tissue paraffin processing, all according to diagnostic needs.
- If prion disease is in the clinical differential diagnosis, divide specimen into three equal portions: two-thirds to be immediately fixed in formalin and one third to be frozen and preserved for immunoblotting, which will enable strain typing in a specialist centre, such as the National Creutzfeldt-Jakob Disease Surveillance Unit in Edinburgh. If the specimen is small, consider fixing the entire specimen in formalin in order to maximise morphological diagnosis. After overnight formalin fixation, subject part of the tissue to one hour immersion in 98% formic acid and subsequent formalin post fixation to reduce (potential) prion infectivity. Next, process this tissue through paraffin and use it for immunohistochemical study with a wide range of markers including prion protein. Some markers may not work as the antigens may be denatured by formic acid treatment. If prion disease is confirmed, the second tissue fragment will have to be treated with formic acid and processed with paraffin for archiving. If prion disease was excluded, the second formalin-fixed tissue may be used for further tests to explore other differential diagnoses. The frozen tissue can be used for further tests such as NGS for viral nucleic acid sequences.

5.4 Embedding options

Paraffin processing for routine stains and immunohistochemistry; resin for EM.

5.5 Sectioning

Microtome section at 4 µm, at multiple levels. Ultra-microtome set at ~0.6 µm for semi-thin sections and ~50–90 nm for ultra-thin sections.

5.6 Routine staining

Microtome sections: routinely for H&E.

5.7 Further investigation

According to clinical indications or pathological findings, supplementary investigations may include:

- special histochemical stains:
  - Luxol fast blue/cresyl violet (neuronal architecture)
  - PAS (granular deposits in the vessels)
  - Martius scarlet blue (fibrinoid necrosis of vessels)
  - Congo red (amyloid in the vessels and parenchyma)
- immunohistochemistry:
  - GFAP
  - Tau (e.g. antibody AT8)
  - neurofilament protein cocktail (70 and 200 kDa)
  - phosphorylated neurofilament
  - Aβ peptide
  - prion protein
  - ubiquitin
  - p62, alpha-synuclein, TDP-43 and FUS protein
- staining for CD20 (B-cell marker), CD3 (T-cell marker) and CD68 (microglia/macrophage/ monocyte marker)
- polyoma virus antigen immunohistochemistry or in situ hybridisation if PML is suspected.

5.8 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- clinical information received with the biopsy
- biopsy dimensions
- description of the histological findings
- interpretation of the findings amounting to specific or differential diagnosis
- comparison with any previous biopsy
- any recommendation for re-biopsy if inadequate for diagnosis
- any recommendation for supra-regional referral (e.g. fungal or viral reference lab)
- conclusion
- SNOMED codes.

5.9 Supra-regional referral

Contact the National Creutzfeldt-Jakob Disease Surveillance Unit in Edinburgh if prion disease is diagnosed or suspected.

[Level of evidence B – see section 3.6 and references below.]

6 Examination of tissue resections for the treatment of refractory epilepsy due to non-neoplastic lesion[11]

6.1 Specimen collection

Specimens will typically be received by the laboratory either fresh or in formalin. Intraoperative smears may be required in cases where an underlying tumour is in the differential diagnosis. The specimens may be received in several parts. Common types of specimen include temporal lobectomy, hippocampectomy or cortical resection. Close correlation with diagnostic imaging and pre-operative clinical pathological consultation can aid tissue sampling and diagnostic interpretation, in particular regarding history of invasive electroencephalogram prior to resective surgery.

6.2 Intraoperative diagnosis and immediate handling

Check for infection risk label and also assess risk from clinical information provided. Representative tissue is set aside for freezing (molecular genetics, tissue bank) and EM according to diagnostic needs, terms of consent and local project protocols. Larger specimens will require orientation and coloured ink can be used to mark resection boundaries or specific gyri. Larger specimens may be weighed. Photography of the macroscopic specimen may also be considered. The bulk of the specimen is fixed in formalin. Large specimens, including temporal lobectomies, will require overnight fixation before slicing and sampling tissue for processing. Hippocampal specimens should be orientated and cut coronally. Ideally, the entire specimen should be embedded with selected representative slices in larger specimens. The International League of Epilepsy has published guidelines regarding optimal sampling and protocols in epilepsy resections.[11]

6.3 Embedding options

Paraffin processing for routine stains and immunohistochemistry; resin for EM.

6.4 Sectioning

Microtome section at 4 µm, at multiple levels. Thicker sections are useful for assessment of cortical architecture, for example in Nissl- and in NeuN-stained sections in cases of suspected cortical dysplasia.

6.5 Routine staining

Microtome sections: H&E stains as routine and LFB/Nissl stain to delineate the anatomy of hippocampal subfields, amygdala and assessment of neocortical architecture where this is required by the context of the case and adequacy of the material.

6.6 Further investigations

Special stains and immunohistochemistry to be performed according to initial findings on H&E sections. For example:

- NeuN, neurofilament, GFAP, MAP2, CD34 and vimentin stains are useful in the assessment of focal cortical dysplasia[12]
- GFAP in the confirmation of hippocampal sclerosis, NeuN in the assessment of granule cell dispersion and subtype of sclerosis,[13] and dynorphin or ZnT3 (zinc transporter 3) immunohistochemistry (in preference to Timms stain) in the identification of axon

sprouting.[13] If low-grade tumour is suspected, tumour datasets and appropriate immunohistochemistry panel should be followed.

- inflammatory markers in suspected encephalitis, viral markers where appropriate.

6.7 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- biopsy dimensions
- description of the histological findings
- interpretation of the findings amounting to specific or differential diagnosis
- comparison with any previous biopsy
- any recommendation for re-biopsy if inadequate for diagnosis; for example, in small, poorly orientated or incomplete and fragmented hippocampal specimens confirmation or subtyping of the pattern of hippocampal sclerosis may not be possible[13]
- conclusion
- SNOMED codes.

[Level of evidence B – see section 3.6 and references below.]

7 Temporal artery biopsy

See the relevant section in The Royal College of Pathologists' *Tissue pathways for cardiovascular pathology*.[14]

7.1 Fixation and embedding options

Fix in formalin and embed in paraffin. The artery should be oriented transversely.

7.2 Sectioning

Microtome section at 4 µm, at multiple levels.

7.3 Routine staining

Analyse the biopsy on multiple transversely oriented levels stained with H&E and EVG (to look for vessel wall destruction and internal elastic lamina disruptions and duplications).

7.4 Further investigations

If inflammation is identified, characterise further by immunohistochemistry for the inflammatory markers (CD3, CD20, CD68), particularly for recognising steroid-treated temporal arteritis.[15]

7.5 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- biopsy dimensions
- description of the histological findings
- interpretation of the findings amounting to specific or differential diagnosis
- comparison with any previous biopsy
- any recommendation for re-biopsy if inadequate for diagnosis
- conclusion
- SNOMED codes.

[Level of evidence GPP – the recommendations in this section are based on the expert opinions of the authors.]

8 CSF for cytological assessment

CSF cytology serves several different purposes and largely depends on the clinical settings and the expectations of the clinical teams. It can be used to determine if there are malignant cells in the CSF, where it has an important role in the investigation, diagnosis and staging of suspected or established malignancy.[16] CSF examination by microbiology, immunology and chemistry laboratories has an important role in the investigation of non-neoplastic disease. Other indications for CSF cytology are non-neoplastic disease, for example neutrophilia in most causes of meningitis, mixed neutrophils and lymphocytes in sarcoidosis and tuberculosis or lymphocytes in encephalitis.

8.1 Specimen submission and immediate handling

Specimens may be received as fluid from wards, theatre or from other diagnostic department, when multiple tests are being performed on CSF (e.g. cell count, protein concentration). Specimens should be handled in a microbiological safety cabinet. For high-risk samples, consider decontamination steps following local procedures and policy. For the investigation of malignant cells in the CSF, it has been reported that false-negative CSF cytology results correlate with a small CSF volume being withdrawn. A further finding of this study was the importance of immediate submission to the laboratory for examination.[17]

8.2 Preparation – options and routine staining

A standardised volume of CSF should be examined. The speed, time and acceleration of the machine must be adjusted so that an optimum yield of morphologically preserved cells in a monolayer can be obtained. Additional slides may be prepared for subsequent immunohistochemical tests, for example to confirm the epithelial nature of atypical cells.

The number of additional slides can be adapted to the cell count, which should be done prior to the CSF cytospin.

The following should be considered:

- preparation of tinctorial staining such as H&E, Giemsa or other suitable preparations on one slide. Consider preparing an additional two or more slides and methanol fix, when fluid volume is sufficient.
- if clinical differential diagnosis includes neoplastic spread in the CNS, prepare several spare slides if possible to enable subsequent immunohistochemical stainings to characterise atypical cells
- high red blood cell counts are usually an indication of blood contamination
- save supernatant if larger volumes (e.g. exceeding 3 ml) are sent; this can be further used for molecular biology (e.g. NGS) or biochemistry.

8.3 Additional investigations

In some cases, further special stains (e.g. gram, PAS, Grocott and Ziehl-Neelsen) may be required for investigation of infectious diseases. Perls' stain should be done where the clinical suspicion is superficial siderosis. Mucicarmine or PAS or other adequate stains should be done when cryptococcal meningitis is suspected. Immunohistochemistry may be required for suspected lymphomas or other malignancies e.g. metastatic carcinomas or glial tumours.

8.4 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- presence/absence of tumour/atypical cells
- optional: cell counts (total red blood cells and white blood cells)
- description of the cytological findings, such as increased granulocytes, macrophages and blood contamination
- interpretation of the findings amounting to specific or differential diagnosis. The reports include a descriptive report of the cells present, whether extraneous cells or contaminants are present (e.g. chondrocytes in lumbar puncture specimens or glial tissue in external ventricular drainage specimens).
- any recommendation for repeat CSF tap if inadequate for diagnosis. In cases where the specimen is insufficient in volume and a further specimen is necessary, this is also indicated on the report to alert the clinician. In addition, in cases where there has been any delay in processing (for example if the specimen has been misdirected) with deterioration of specimen, a note on the quality of the specimen is included for the clinician and the possibility of an additional specimen may be suggested.
- comparison with concurrent or previous biopsy or CSF examination
- conclusion
- SNOMED codes.

[Level of evidence GPP – the recommendations in this section are based on the expert opinions of the authors.]

9 Bone and soft tissue from skull or axial skeleton

See The Royal College of Pathologists' *Tissue pathways for bone and soft tissue pathology.*[18]

10 Samples for investigation of genetic disorders including neurometabolic and paediatric disorders

Histopathology from a variety of tissue samples can provide sensitive, specific and rapid diagnosis in neurogenetic disease and can be particularly helpful for metabolic diseases and diseases of childhood. The choice of sample should be the least invasive that can be used to diagnose the suspected disorder (e.g. many of the lysosomal storage disorders [LSD] can be investigated by the examination of a blood film). The examination of some of these samples is highly specialised and referral to regional or national centres is recommended.

10.1 Skin and subcutaneous tissues

See The Royal College of Pathologists' *Tissue pathways for dermatopathology* (August 2016) for general diagnostic approaches.[19]

Samples for investigation of suspected:

- neuronal ceroid lipofuscinosis (NCL; including Batten's disease, Kuf's disease) – fixation in glutaraldehyde with epoxy resin embedding for EM, skin formalin fixation, wax embedding and immunohistochemistry for subunit C of mitochondrial ATP synthase and saposin D (sphingolipid activating protein D)
- Lafora body disease – fix skin bearing apocrine glands (preferable) or eccrine glands in formalin followed by wax embedding and staining with PAS ± diastase and/or Lugol's iodine to detect inclusions; fixation in glutaraldehyde with epoxy resin embedding for EM; photography for documentation recommended as the Lugol's stain may fade rapidly
- Niemann-Pick disease type C – fixation in glutaraldehyde followed by epoxy resin embedding for EM; frozen tissue for fibroblast culture (mandatory); formalin fixation and paraffin embedding for light microscopy (to look for characteristic but non-specific foam cells) only if plenty of tissue[20]
- CADASIL – skin fixed in formalin and embedded inparaffin wax; fixation in glutaraldehyde with epoxy resin embedding for EM to look for granular osmiophilic material; stain paraffin-embedded tissue with PAS to look for granular deposits in the media of small arteries and arterioles; immunostaining with antibody to the extracellular domain of NOTCH3 is possible
- rabies – nuchal skin biopsy, immunostaining for rabies virus. Skin biopsy specimen should be 5–6 mm in diameter, taken from the back of the neck at the hairline and contain at least ten hair follicles https://www.cdc.gov/rabies/specific_groups/doctors/ante_mortem.html). The biopsy specimen should be of sufficient depth to include the cutaneous nerves that are abundant at the base of the hair follicles, thus increasing the probability of detecting the rabies virus because of its neurotropic nature. For more information on rabies in the UK see: www.gov.uk/government/collections/rabies-risk-assessment-post-exposure-treatment-management.

10.2 Peripheral blood

10.2.1 Clinical indication and specimen collection

The examination of blood films is only offered in a small number of specialist centres but provides a very rapid and, in several diseases, very specific test. The clinical context will usually be in the investigation of suspected storage disorders, principally LSD, for example Batten's disease (NCL), Salla disease, I cell disease, β galactosidase deficiency (GM1 gangliosidosis), mucopolysaccharidoses, Niemann-Pick disease, fucosidosis, mannosidosis, Wolman's disease and certain glycogenoses. For example, it can provide a specific diagnosis in Wolman's disease and some NCL, or can provide a diagnosis where no biochemical test is available (some forms of NCL).

10.2.2 Specimen handling

A sample of EDTA preserved whole blood should be received by the laboratory. Heparinised whole blood is also acceptable. Check for infection risk label and also assess risk from clinical information provided. EDTA blood is stable for days at room temperature or at 4°C, which facilitates transport to specialist centres. However, the sample must not be frozen, as this will cause artefacts. Several blood films (typically six) are prepared. For the initial assessment, one of the smears is prepared with a Romanowsky stain.

10.2.3 Routine examination

The blood film should be examined at the thin end of the film near the tail, rather than the thicker region. The blood film should be examined in a systematic manner, with particular attention to the detection and morphological characteristics of the lymphocytes. The optimal number of lymphocytes to be examined is uncertain but if fewer than 20 are seen in the first film, a second film should be examined.[21]

10.2.4 Ultrastructural examination

A buffy coat should be prepared for EM if vacuolated lymphocytes are seen in the blood film or in all cases of certain diseases (e.g. all suspected NCL cases should be examined by EM). Ultrastructural examination of inclusions in lymphocytes may allow a specific diagnosis and has particularly aided the identification of variant subtypes of NCL. At least 100 lymphocytes should be examined, as in some variants of NCL, only a minority contain diagnostic material.[22]

10.2.5 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- description of the cytological findings – comments should be made as to the size and extent of the vacuolation
- interpretation of the findings amounting to specific or differential diagnosis
- any recommendation for further investigation – in all patients, a diagnostic test should be undertaken to confirm a specific diagnosis
- conclusion
- SNOMED codes.

[Level of evidence B – see section 3.6 and references below.]

10.3 Bone marrow

Bone marrow aspirate is helpful in the diagnosis of some LSD, e.g. where vacuolated lymphocytes are not a feature on blood films. In particular, it can assist in the diagnosis of

Niemann-Pick and Gaucher diseases. Aspirates should be prepared and stained by the standard haematological protocols.

[Level of evidence GPP – the recommendations in this section are based on the expert opinions of the authors.]

10.4 Hair

10.4.1 Clinical indication and specimen collection

The examination of hair samples is a very specialised test but may help in a wide range of paediatric disorders. Abnormalities of hair shafts may be seen in conditions such as Menkes, Netherton's, trichothiodystrophy, Chediak-Higashi, Griscelli or uncombable hair syndrome.[23]

Most abnormalities of the shaft are best diagnosed in cut samples of hair as plucking may cause unwanted breakages at the sites of trichorrhexis invaginata in Netherton's syndrome, thus rendering the sample non-diagnostic. The hairs should be cut close to the scalp.

Disorders of the hair follicle can only be diagnosed on a sample of plucked hair containing the root. There are a number of suggested methods for obtaining such samples, e.g. hair samples may be obtained by plucking; this may be carried out either by gripping fewer than ten hairs between a finger and the thumb, or by gripping a couple of rows of hairs with a needle holder at the base, and pulling sharply.[23]

10.4.2 Immediate handling

Hairs are dry mounted (without mounting medium) by placing a rectangular frame with double sided sticky tape edges (e.g. Frame-Seal incubation chamber) on a microscope slide. The hairs are lined up in parallel order securing one or both ends to the sticky edge(s) of the frame. A cover glass is then placed over the Frame-Seal containing the strands of hair.[23]

If Chediak-Higashi or Griscelli syndrome is suspected, a mountant (DPX) is used to give a clearer view of the specimen.[12]

10.4.3 Routine examination

Samples examined using routine light microscopy and under polarised light. It is important to examine as many strands of hair as possible because not every hair may demonstrate morphological abnormalities in the lengths examined.

10.4.4 Ultrastructural examination

In some centres, scanning EM is used to define the shape of the hair shaft but this is technically challenging and not suitable for routine use.

10.4.5 Report content

To include:

- clinical and demographic information received with the biopsy (gender, date of birth, age at biopsy)
- description of the morphological findings
- interpretation of the findings amounting to specific or differential diagnosis – morphological changes described as being compatible or indicating specific diagnoses must be interpreted in the light of appropriate clinical information
- any recommendation for further investigation – in all patients, a diagnostic test should be undertaken to confirm a specific diagnosis
- conclusion

- SNOMED codes.

[Level of evidence B – see section 3.6 and references below.]

10.5 Urine

10.5.1 Clinical indication and specimen collection

Urine examination can provide a rapid and accurate diagnosis in metachromatic leukodystrophy. A 10 ml sample of fresh urine (not the first of the day) is recommended.

10.5.2 Immediate handling

Three cytospins should be prepared and air-dried and fixed in formalin vapour. The samples should be stained overnight in 0.01% Toluidine Blue.

10.5.3 Routine examination

Routine light microscopy reveals crystals of sulphatides, which appear red/brown and should be confirmed to show green birefringence.

10.6 Other samples

Brain biopsies are rarely undertaken in the investigation of genetic and metabolic disorders in modern practice but are occasionally encountered in paediatric patients with undiagnosed encephalopathic illnesses. The samples should be treated as above but with an emphasis on preserving material for EM and for viral and other microbiological investigation.

Muscle biopsies are frequently taken in paediatric patients with complex undiagnosed neurological disorders to investigate for mitochondrial disease (see section 2).

[Level of evidence GPP – the recommendations in this section are based on the expert opinions of the authors.]

11 Criteria for audit

The content and timeliness of histopathology reports should be audited against the recommendations in these guidelines.

In addition, audits should be performed against The Royal College of Pathologists' key performance indicators (KPIs) (see *Key Performance Indicators – Proposals for implementation* [July 2013] on www.rcpath.org/resourceLibrary/key-performance-indicators---proposals-for-implementation-.html):

- histopathology cases (surgical pathology, cancer biopsies) should be reported, confirmed and authorised within seven and ten calendar days of the procedure
  - standard: 80% of cases (surgical pathology, cancer biopsies) must be reported within seven calendar days and 90% within ten calendar days
- no specific guidelines exist for the reporting turnaround of diagnostic muscle and nerve biopsies. These have to be agreed locally with the clinical team to meet their clinical requirements and should be documented in the departmental quality manual. These turnaround times should be audited against the locally agreed targets.

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Appendix A Summary table – explanation of grades of evidence

(modified from Palmer K *et al. BMJ* 2008;337:1832)

Grade (level) of evidence	Nature of evidence
Grade A	At least one high-quality meta-analysis, systematic review of randomised controlled trials or a randomised controlled trial with a very low risk of bias and directly attributable to the target cancer type or A body of evidence demonstrating consistency of results and comprising mainly well-conducted meta-analyses, systematic reviews of randomised controlled trials or randomised controlled trials with a low risk of bias, directly applicable to the target cancer type.
Grade B	A body of evidence demonstrating consistency of results and comprising mainly high-quality systematic reviews of case-control or cohort studies and high-quality case-control or cohort studies with a very low risk of confounding or bias and a high probability that the relation is causal and which are directly applicable to the target cancer type or Extrapolation evidence from studies described in A.
Grade C	A body of evidence demonstrating consistency of results and including well-conducted case-control or cohort studies and high-quality case-control or cohort studies with a low risk of confounding or bias and a moderate probability that the relation is causal and which are directly applicable to the target cancer type or Extrapolation evidence from studies described in B.
Grade D	Non-analytic studies such as case reports, case series or expert opinion or Extrapolation evidence from studies described in C.
Good practice point (GPP)	Recommended best practice based on the clinical experience of the authors of the writing group.

Appendix B AGREE guideline monitoring sheet

The tissue pathways of The Royal College of Pathologists comply with the AGREE II standards for good quality clinical guidelines. The sections of this tissue pathway that indicate compliance with each of the AGREE II standards are indicated in the table.

AGREE standard	Section of guideline
Scope and purpose	
1 The overall objective(s) of the guideline is (are) specifically described	Foreword, 1
2 The health question(s) covered by the guideline is (are) specifically described	Foreword, 1
3 The population (patients, public, etc.) to whom the guideline is meant to apply is specifically described	Foreword
Stakeholder involvement	
4 The guideline development group includes individuals from all the relevant professional groups	Foreword
5 The views and preferences of the target population (patients, public, etc.) have been sought	n/a
6 The target users of the guideline are clearly defined	1
Rigour of development	
7 Systematic methods were used to search for evidence	Foreword
8 The criteria for selecting the evidence are clearly described	Foreword
9 The strengths and limitations of the body of evidence are clearly described	Foreword
10 The methods for formulating the recommendations are clearly described	Foreword
11 The health benefits, side effects and risks have been considered in formulating the recommendations	Foreword
12 There is an explicit link between the recommendations and the supporting evidence	4–10
13 The guideline has been externally reviewed by experts prior to its publication	Foreword
14 A procedure for updating the guideline is provided	Foreword
Clarity of presentation	
15 The recommendations are specific and unambiguous	4–10
16 The different options for management of the condition or health issue are clearly presented	4–10
17 Key recommendations are easily identifiable	4–10
Applicability	
18 The guideline describes facilitators and barriers to its application	Foreword
19 The guideline provides advice and/or tools on how the recommendations can be put into practice	2–10
20 The potential resource implications of applying the recommendations have been considered	Foreword, 1
21 The guideline presents monitoring and/or auditing criteria	11
Editorial independence	
22 The views of the funding body have not influenced the content of the guideline	Foreword
23 Competing interest of guideline development group members have been recorded and addressed	Foreword