UNDERSTANDING SPECIAL STAINS

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MELANIE DOBROMYLSKYJ explains some of the most common special stains used in diagnostic laboratories, their main indications and clinical situations where they can aid diagnosis

Summary

Do you want to know why your pathologist has delayed the final report on your case, for the sake of some oddsounding “special” stain? This article aims to summarise the most common special stains used in a diagnostic laboratory setting, their main indications and the corresponding clinical situations where they might be used to aid diagnosis. These include the metachromatic stains, for example Giemsa, used for blood smears, mast cells and some fungal and protozoal infections, stains for infectious agents, such as Gram (bacteria), Warthin-Starry (spirochetes), Ziehl-Neelsen (acid-fast bacteria), periodic acid-Schiff (fungi, glycogen), and stains for materials, such as pigments, fat, amyloid and calcium.

Key words

histology, special stains, infectious agents, neoplasia

THE haematoxylin and eosin (H and E) stain is the routine, everyday stain used in histological sections and will probably be the first slide a pathologist looks at for any given case.

Haematoxylin colours nuclei and a few other objects blue (referred to as basophilic) while the eosin counterstains the cytoplasm in various shades of pink (termed “pale” to “brightly” to “deeply

eosinophilic" depending on the precise shade). Structures do not have to be acidic or basic to be called basophilic or eosinophilic, the terminology is simply based on the affinity to these dyes. Other, endogenous colours may also be present in a section, for example erythrocytes are bright red, melanin is brown-black and other pigments may be yellow, golden-brown or green.

Based on the appearance of this initial section, a pathologist may request further "special" stains for a number of reasons. Some specific tissue structures simply do not stain well with routine H and E stains. For example, the reticular fibres in the liver require a silver stain, such as Gordon-Sweets, to allow better assessment of liver architecture, while Masson's trichrome stains connective tissues, highlighting the presence of fibrosis in some cases of liver disease (Figure 1). Hydrophobic structures also tend to remain clear since these are usually rich in fats; this includes adipocytes and the myelin around neuron axons.

Some special stains aid better identification of poorly differentiated neoplastic cells (such as mast cells or a mucin-secreting neoplasia), or may highlight the presence of infectious agents. Other stains allow more precise identification of deposits such as amyloid, calcium and a variety of pigments.

This article is not intended as an exhaustive list of special stains because there are very many of them – even Table 1 does not list every stain available to the histopathologist – but it will concentrate on the ones most often used in routine histopathology cases.

Metachromatic stains

Metachromatic stains are those that have the ability to produce different colours with various histologic or cytologic structures. Examples include Giemsa, Wright and Diff-Quik stains.

The Giemsa stain has multiple purposes within the diagnostic laboratory. It is one of the classic stains for peripheral blood smears and bone marrow specimens, and is also useful for staining some fungal species such as *Histoplasma* and some intracellular protozoa, such as *Leishmania.*

Another major use of Giemsa, as well as other metachromatic stains, such as toluidine blue and astra blue, is the identification of mast cells because it stains the cytoplasmic granules present in mast cells a magenta to purple colour. This may aid further differentiation of a round cell tumour as being of mast cell origin, especially in some of the more poorly differentiated tumours where the granules may not be obvious in routine H and E sections.

In other cases, where increased numbers of mast cells are present in a lesion that is otherwise inflammatory in appearance, a Giemsa stain can help reveal how many mast cells are present, whether they form clusters, how pleomorphic they are, whether they contain mitotic figures and whether the pathologist should be suspicious of an underlying neoplasm. The Giemsa stain can also help when assessing mast cell tumour margins and scar line resections; although it is

important to remember it cannot distinguish between neoplastic mast cells and the non-neoplastic mast cells that are migrating to the site of the tumour, attracted by the release of histamine and other bioactive substances.

Stains for infectious agents

Gram and Warthin- Starry stains

Gram staining is a method for highlighting the presence and morphology of bacterial populations within a section; it also differentiates bacterial species into two large groups – Gram-positive and Gram-negative – based on the chemical and physical properties of their cell walls. It detects peptidoglycan, which is present in a thick layer in Gram-positive bacteria and results in a blue/ purple colour. Gram-negative bacteria generally have a thin layer of peptidoglycan between two membranes, which results in a pink/red colour. Not all bacteria can be definitively classified by this technique, thus forming Gram-variable and Gramindeterminate groups as well.

Gram-positive bacteria include many well-known genera, such as *Bacillus, Listeria, Staphylococcus, Streptococcus, Enterococcus and Clostridium,* while Gram-negative bacteria include, among others, *Bordetella, Campylobacter, Enterobacter, Escherichia coli, Helicobacter, Pasteurella, Pseudomonas* and *Salmonella.*

However, Gram staining is not always reliable in histological sections and follow-up culture and full identification of potentially significant bacterial populations seen on histology is usually recommended.

Warthin-Starry is a silver nitrate-based staining technique used to detect spirochetes, such as *Leptospira* and *Borrelia,* as well as *Helicobacter* species present in gastric biopsies.

Ziehl-Neelsen stain

The Ziehl-Neelsen (ZN) stain, also known as the acid-fast stain, is a special stain used to identify acid-fast organisms, mainly mycobacterial species such as *Mycobacterium bovis, M paratuberculosis and M avium.* These may be present within suspicious lesions in very low numbers, which means identification on routine H and E sections is often nearly impossible. Specialised cultures or molecular techniques, such as PCR, are required to confirm the identification of *Mycobacterium* species and to rule out other acid-fast bacilli (although other types of bacilli are extremely unlikely) and also to precisely identify the species of *Mycobacterium* involved. There is some zoonotic potential from *Mycobacterium* species, adding importance to accurate identification and reporting of such infections. The ZN stain can also be used to identify intranuclear lead inclusion bodies.

Wright’s stain

Leishmaniasis generally causes a chronic inflammatory response of granulomatous nature, due to the parasite targeting phagocytic cells, such as macrophages. It can also provoke a type IV hypersensitivity response. Several forms of leishmaniasis occur, including cutaneous, mucocutaneous and visceral forms. Within the dog, the intracellular parasite is detected by Wright's stain on blood smears, lymph node aspirates or in histological sections, where it assumes a non-flagellated form.

Periodic acid-Schiff

Periodic acid-Schiff (PAS) is a staining method used to detect glycogen and other polysaccharides in tissues. Probably the most common use of PAS in diagnostic pathology is for fungal infections, such as *Malassezia,* fungal hyphae and *Candida* (living fungi). Other stains, especially silver stains, are also used to detect fungal organisms, for example Grocott's methenamine silver stain (fungi both dead and alive).

The presence of glycogen can be confirmed in tissue sections by using diastase to digest the glycogen from a section, then comparing a diastase-digested PAS section with a normal PAS section. If the positive-staining material present in the PAS slide is glycogen, then it will be absent from the corresponding location on the diastase-digested slide. This technique can be used on liver sections to confirm intracytoplasmic material is glycogen, as opposed to fat, and can be used on neurological tissues to distinguish glycogen storage diseases from other types of storage disease (Figure 2).

Stains for amyloid

Amyloid is a fairly homogeneous, nondescript eosinophilic material on routine H and E stained sections, and its presence needs to be confirmed by special stains such as Congo red. This stain colours amyloid an orangered colour, and when viewed under a polarised light the material demonstrates apple-green birefringence.

Amyloid may be present in a range of diseases. Primary amyloidosis is the most common systemic or generalised form, and is most often due to plasma cell or B-cell dyscrasias, such as multiple myeloma and other monoclonal B-cell proliferations. These can result in increased amounts of immunoglobulin light chain (AL amyloid), the basis for the type of amyloid present in these cases.

Secondary or reactive amyloidosis is the form that is generated from increased amounts of the acute phase protein serum amyloid A (AA amyloid) and is generally associated with chronic inflammatory conditions or neoplasia, although it can also be idiopathic.

Familial amyloidosis is a hereditary and systemic condition that can affect various organ systems. It is seen in Abyssinian cats and Shar Pei dogs (Figure 3), where deposition occurs in the kidney, and in Siamese cats, where it occurs in the liver.

Stains for calcium

The presence of calcium in tissues is confirmed by the von Kossa stain, which colours calcium black.

Pathological calcification of tissues falls into two broad categories, dystrophic (serum calcium levels are normal, but the tissue is not) and metastatic (serum calcium levels are increased, but the tissues are normal).

Dystrophic calcification can occur in necrotic tissues, such as in the centre of granulomas in tuberculosis and Johne's disease. It can also be seen in the skin of dogs, for example calcinosis cutis associated with hyperadrenocorticism (Cushing's disease) and calcinosis circumscripta (occurring in the skin and other soft tissues) associated with sites of repeated trauma.

Metastatic calcification can be seen associated with renal failure (secondary hyperparathyroidism), vitamin D toxicosis (ingestion of calcinogenic plants, rodenticides or even the owner's tube of psoriasis cream – Figure 4), primary hyperparathyroidism, pseudohyperparathyroidism (release of parathyroid-related protein from certain tumours) and destructive bone lesions.

Pigment stains

Masson- Fontana

Melanocytic tumours can represent a diagnostic challenge. Poorly melanised or amelanotic tumours can vary markedly in their cellular morphology, and in the absence of melanin as a clue, they can resemble a large number of other tumours. Masson-Fontana, a stain that detects melanin pigment, can aid diagnosis in poorly melanised tumours by highlighting the presence of any small amounts of melanin present in the sections.

Permanganate bleach

Large amounts of melanin pigment in the tumour cells can obscure both nuclear and cellular morphology, making histological assessment of malignancy difficult. Bleaching sections with permanganate bleach removes the colour of the pigment and allows clearer assessment of nuclear morphology and the mitotic index, important features for distinguishing between malignant or benign tumours when taken together with anatomical location.

Other pigment stains

Various stains are available to help the pathologist distinguish between pigments present in histological sections.

For example, the Dunn-Thompson stain for haemoglobin may be of use in cases of intravascular haemolysis leading to deposition of haemoglobin within renal tubules. The Fouchet stain for bile pigments may be used in cases of cholestasis (Figure 5). Perl's Prussian blue is used to highlight the presence of haemosiderin and to distinguish it from other pigments. Examples include the presence of haemosiderin in alveolar macrophages seen with passive chronic congestion of the lungs (so-called "heart-failure cells") and when excess iron is released during red blood cell breakdown, for example as seen with auto-immune haemolytic anaemia.

Fat stains

Various stains are used to detect the presence of fat in a section, although these require the use of frozen tissue sections. This is because the normal routine processing of tissues removes the vast majority of fat from sections, simply leaving an empty space in cells and tissues. Pathologists may be suspicious that these spaces previously contained fats, either in the form of lipid, lipoproteins or triglycerides, but the need to perform special stains, such as oil red O and Sudan III to confirm its presence.

For example, the presence of lipid in a poorly differentiated sarcoma may aid the further diagnosis of the tumour as a liposarcoma. Presence of lipid in vacuolated hepatocytes may aid diagnosis of a hepatic lipidosis, as opposed to a disease involving hepatic storage of other substances, such as glycogen. Figure 6 demonstrates the use of oil red O staining to highlight fat in vacuoles of neoplastic cells; this section is from one of multiple abdominal tumours in a horse that presented with marked and recurrent ascites.

Oil red O stain on frozen sections together with immunohistochemical stains and electron microscopy confirmed the diagnosis of a lipid-rich mesothelioma in this case, the first report of this type of tumour in a horse.

Reference

- 1. Dobromylskyj M J, Copas V, Durham A, Hughes T K and Patterson-Kane J C (2011). Disseminated lipid-rich peritoneal mesothelioma in a horse, *Journal of Veterinary Diagnostic Investigation* **23**(3): 615-618.

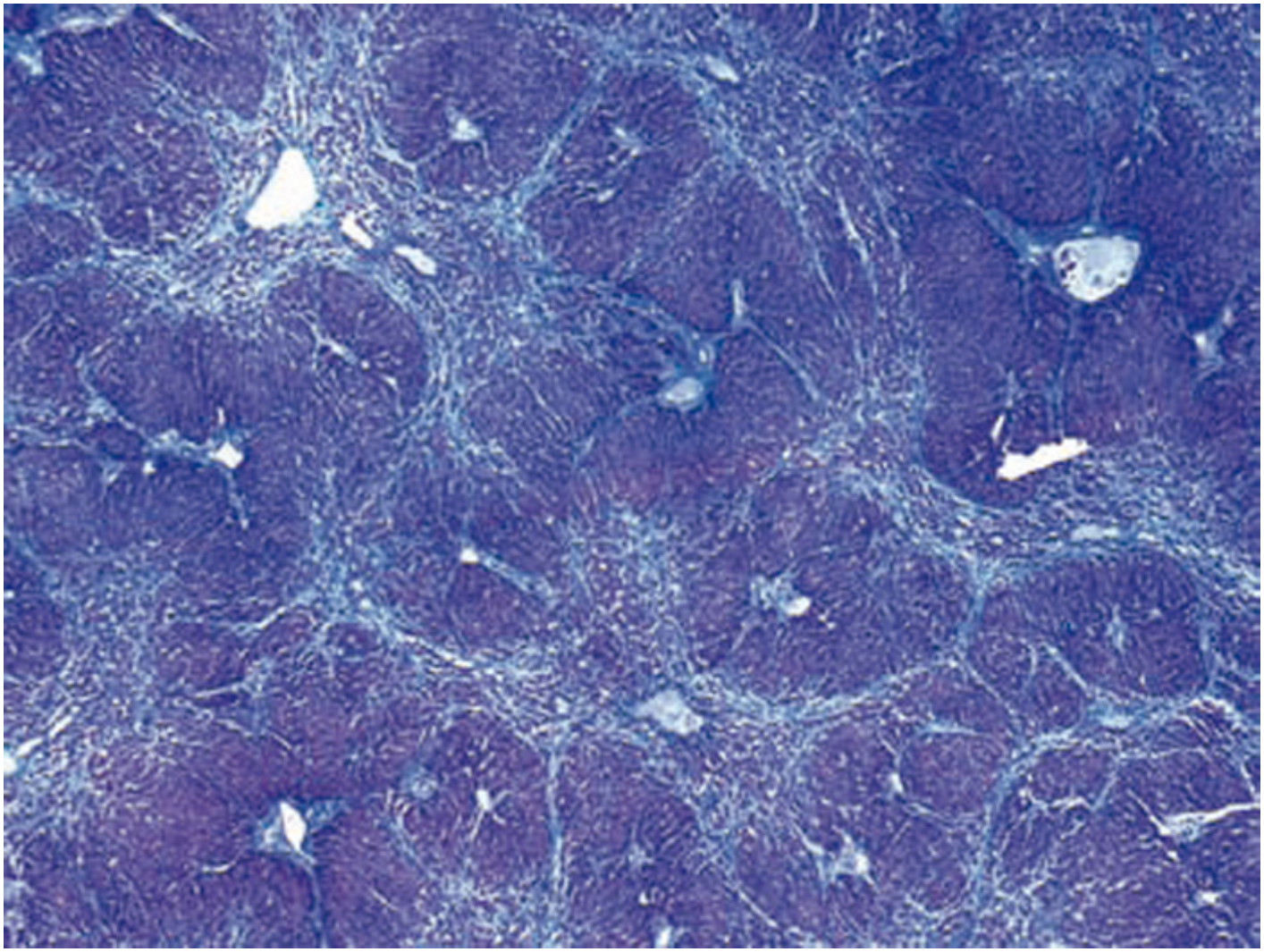
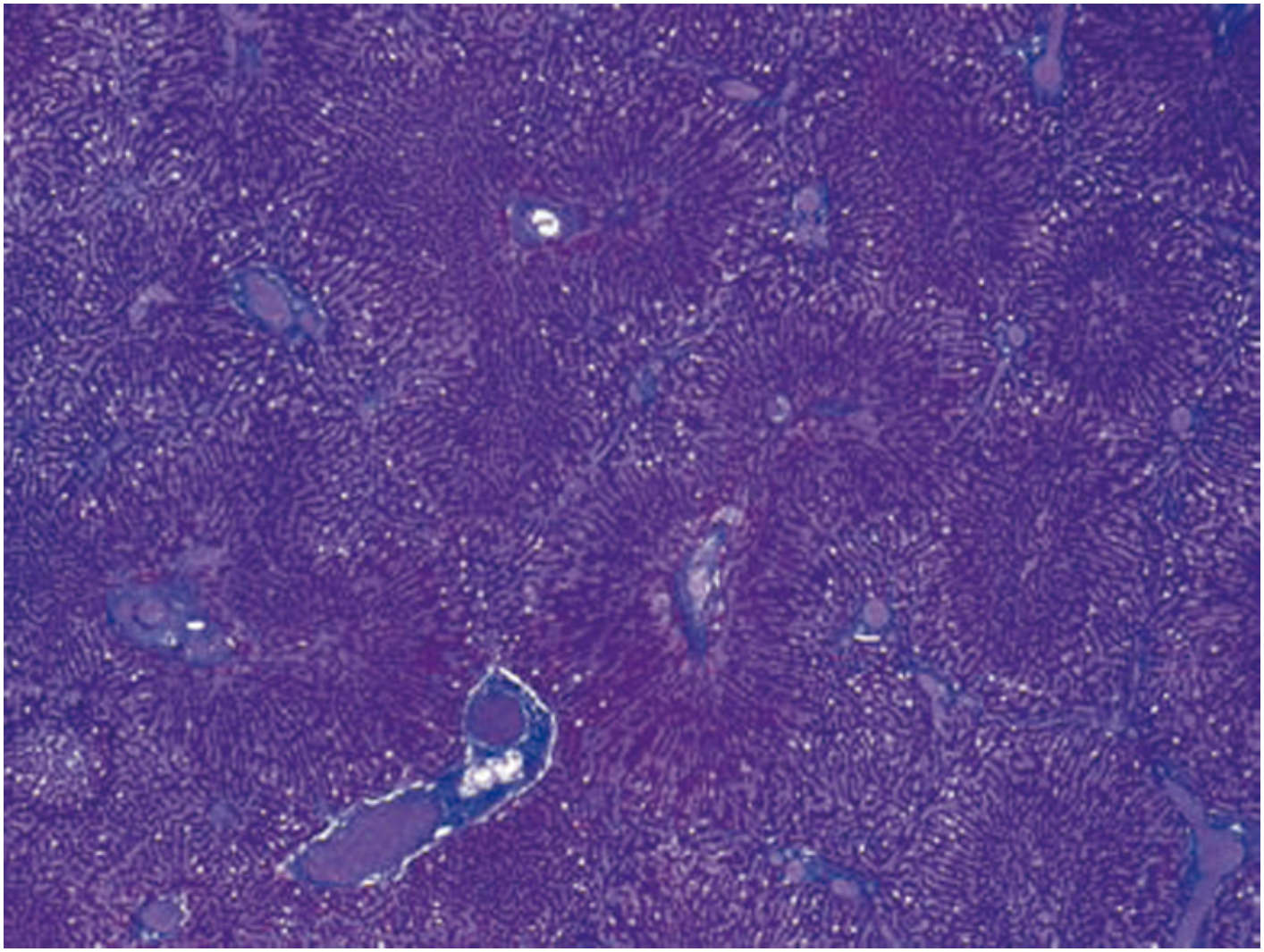


Figure 1. Masson's trichrome. a) Section from the liver of a bongo antelope in a zoological collection, with chronic diffuse hepatic fibrosis. Liver, Masson's trichrome, 4x. b) section from a normal liver for comparison 4x.



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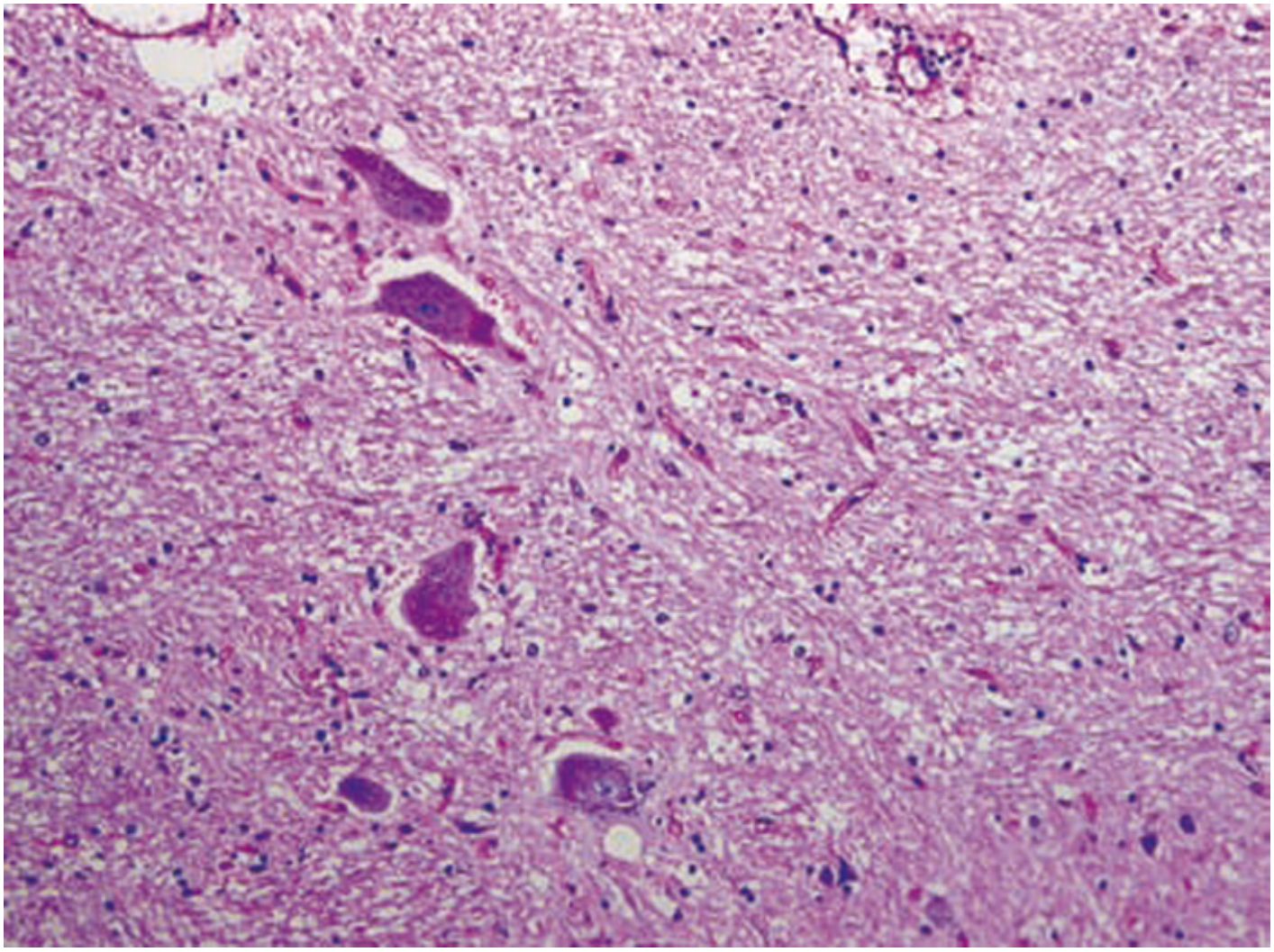


Figure 2. Periodic acid-Schiff stain of a storage disease. Section from the brain of a dog with a glycogen storage disease. PAS-positive material is present within multiple neurons. This case was submitted to the Veterinary Diagnostic Services, University of Glasgow. Brain, PAS, 10x.

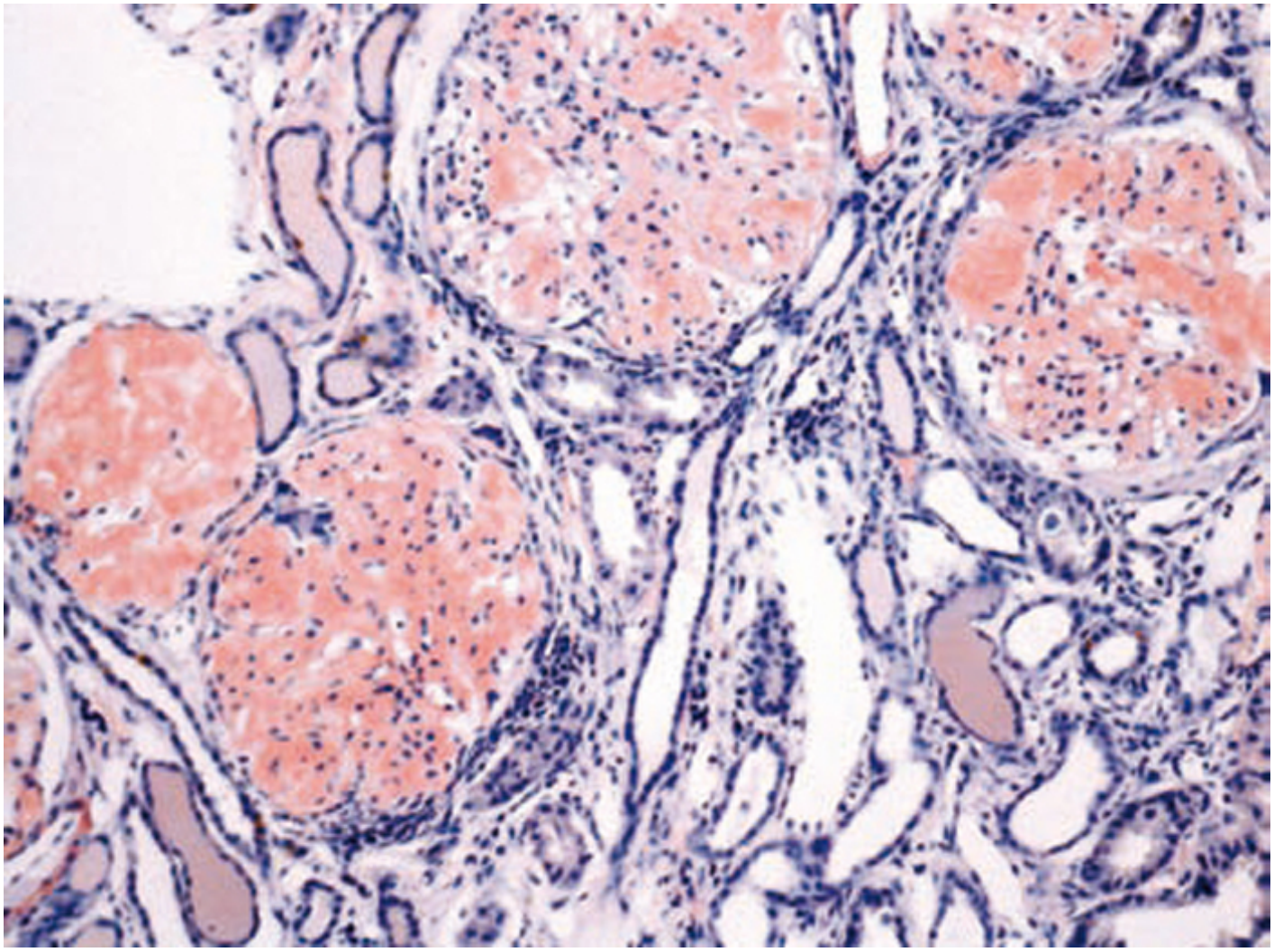


Figure 3. Congo red stain. Section from the kidney of a four-year old Shar Pei dog with renal amyloidosis. Congo red staining confirms the material present within the glomeruli (which is eosinophilic on H and E) is amyloid, colouring it red. Kidney, Congo red, 40x.

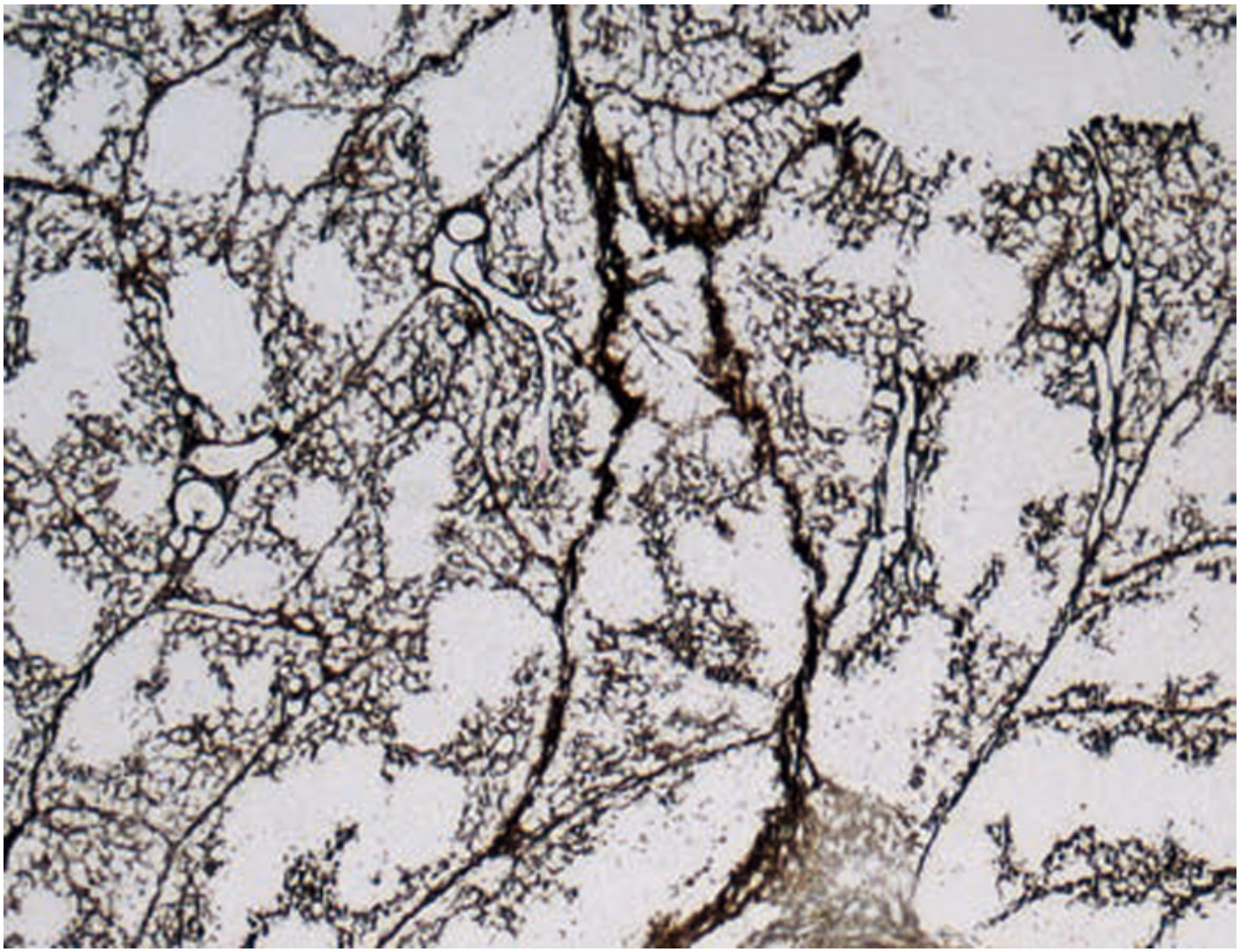


Figure 4. Von Kossa stain. Section from the kidney of a puppy that had ingested its owner's tube of psoriasis cream, resulting in vitamin D toxicosis and widespread, severe calcification of multiple tissues. A von Kossa stain of the mesenteric adipose tissues colours the extensive calcium deposits black. von Kossa, 20x.

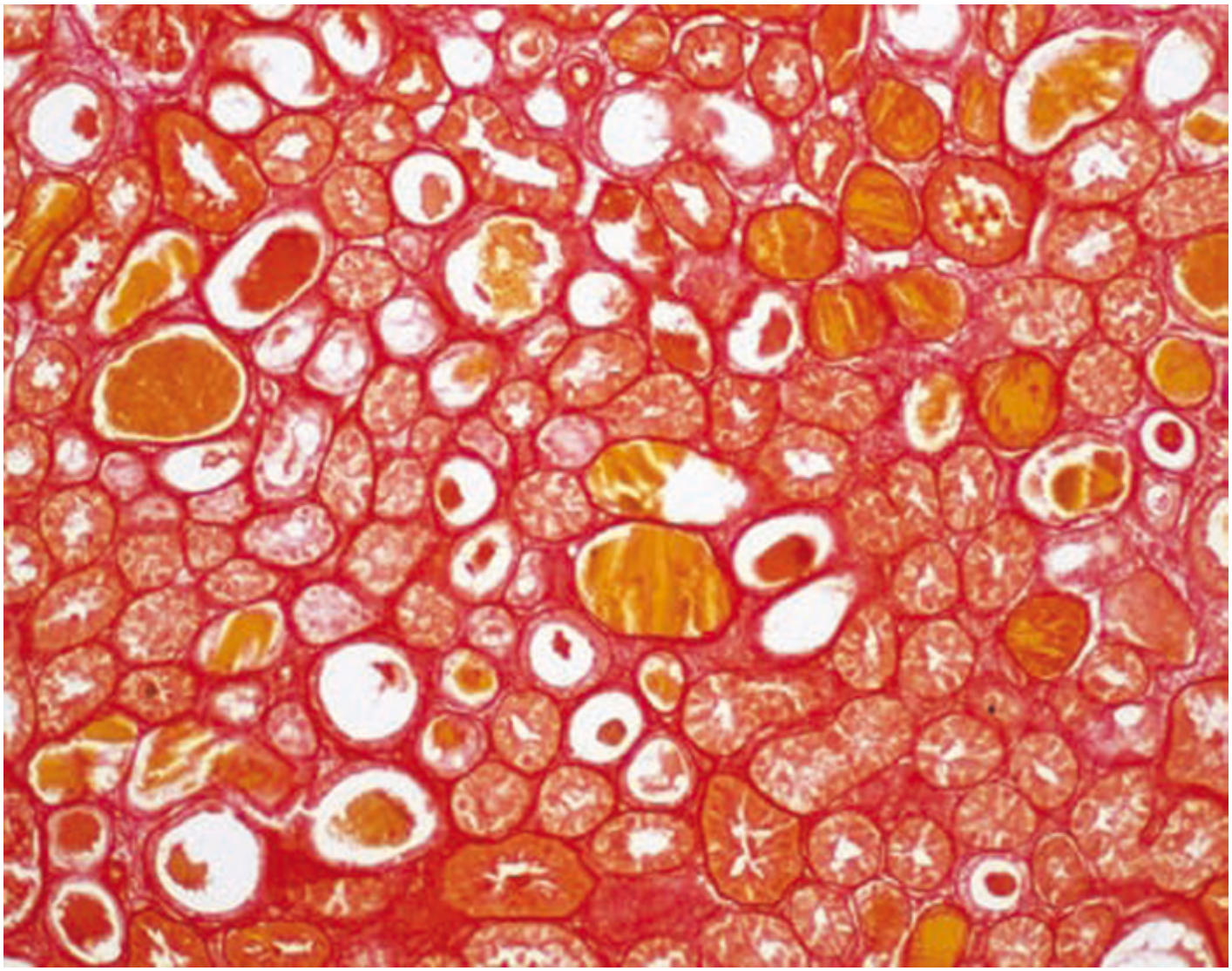
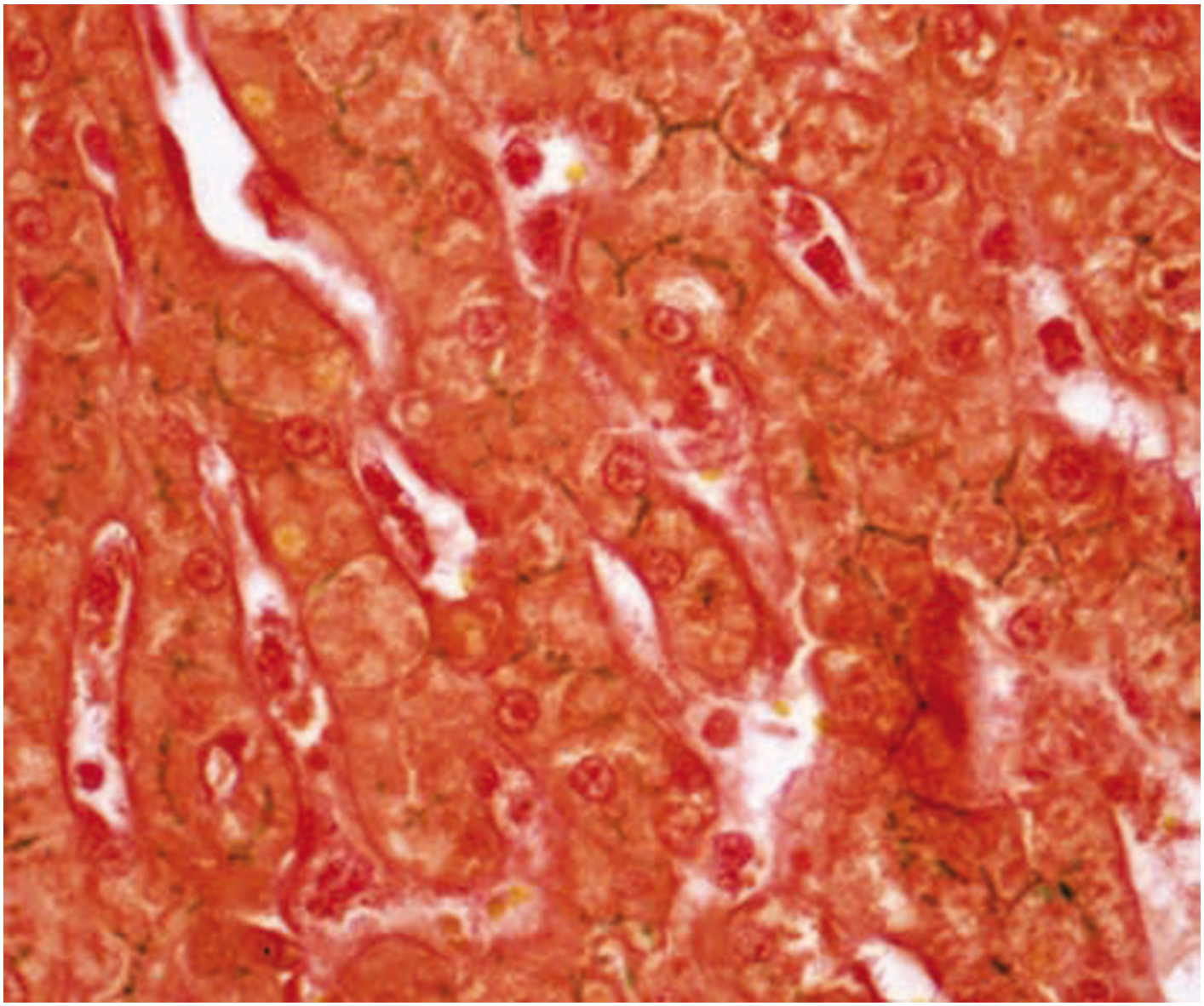


Figure 5. Fouchet stain. Sections from the kidney and liver of a tapir. a) kidney: many renal tubules are distended by bright orange-red, amorphous to crystalline material, which stains yellow with a Fouchet stain (20x), allowing differentiation between haemoglobin and bile, which stains emerald green with Fouchet, as in b). section from the liver, 40x. Final diagnosis in this case was of a haemoglobinuric nephrosis.



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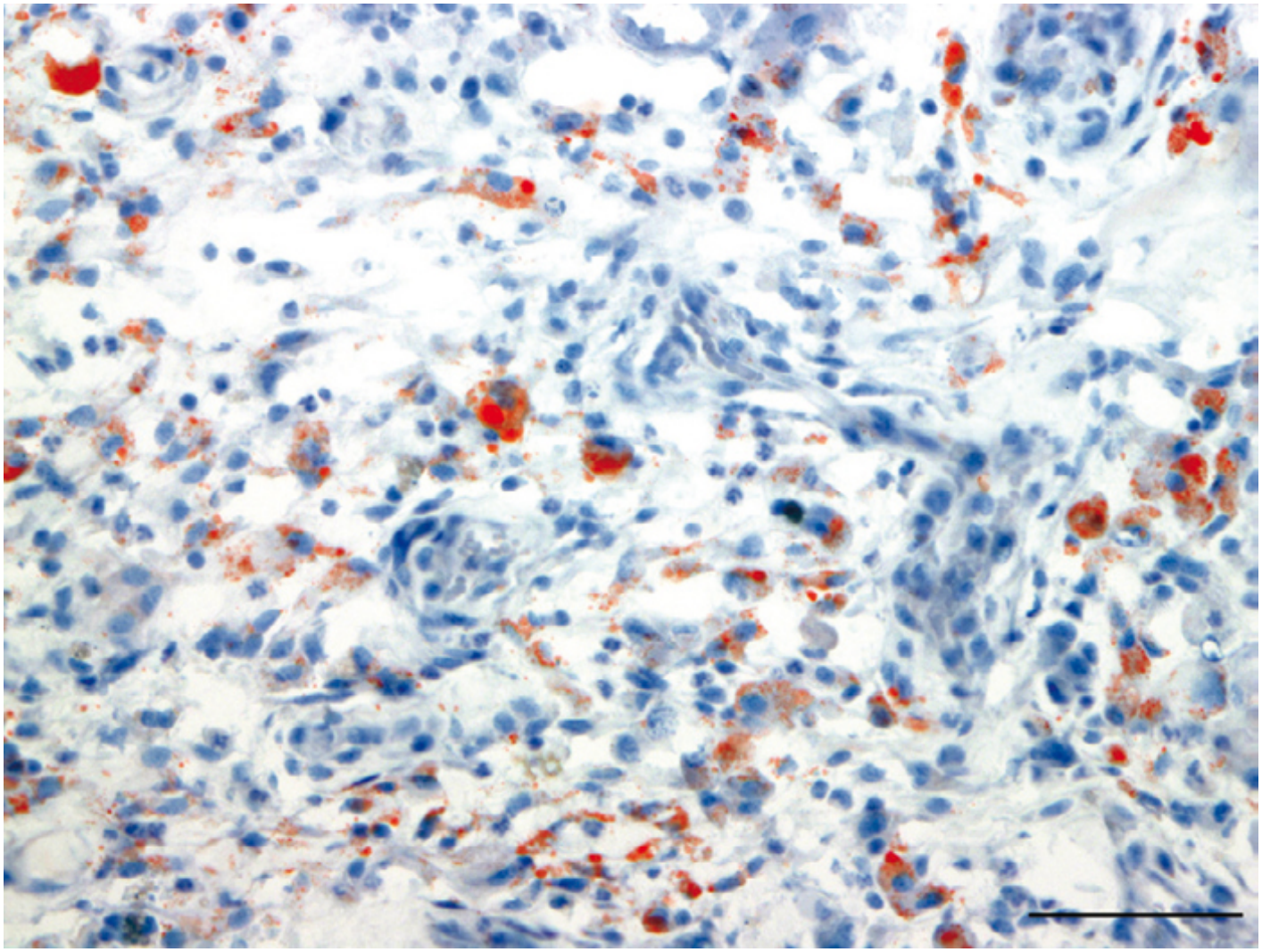

Figure 6. Oil red O. Section from one of multiple abdominal tumours in a horse that presented with marked and recurrent ascites. Oil red O stain on frozen sections reveals positive-staining (red) vacuoles within many tumour cells, which together with immunohistochemical stains and electron microscopy confirmed the diagnosis of a lipid-rich mesothelioma, the first reported case in a horse (submitted to the Veterinary Diagnostic Services, University of Glasgow) [1]. Abdominal mass; Oil red O, 20x.

Metachromatic dyes	
Giemsa	Blood smears, mast cells, infectious agents
Toluidine blue, astra blue	Mast cells
Alcian blue/safranin	Different types of mast cell
Infectious agents	
Gram or Gram/Twort	Gram +/- bacteria
Ziehl-Neelsen	Acid-fast bacteria, iron inclusion bodies
Periodic acid-Schiff (+/- diastase digestion)	Fungi (living)
	Glycogen
Grocott's methenamine silver stain	Fungi (dead and alive)
Warthin-Starry	Spirochetes
Phloxine/tartrazine	Some viral inclusions
Leishman	Blood cells, parasites
Wrights	Leishmania
Macchiavello	Rickettsiae, some viral inclusions
Giemsa	Trypanosomes, Plasmodium, Chlamydia, Histoplasma
Materials and tissue components	
Congo red, sirius red, toluidine blue	Amyloid
Rubeanic acid, rhodanine	Copper
von Kossa, alizarin red	Calcium
Oil red O, Sudan III, Scharlach red	Lipids, triglycerides, lipoproteins
Alcian blue	Acid mucins (proteoglycans)
Alcian blue/PAS	Acid and neutral mucins
High iron diamine/alcian blue	Sulphated and carboxylated mucins
Masson's trichrome	Connective tissues
Miller's	Elastin
Gordon and Sweets	Reticulin, liver structure
Gomori hexamine silver	Basement membranes
Haematoxylin van Gieson	Collagen, muscle
Martius scarlet blue	Fibrin, connective tissue
Safranin O/fast green	Cartilage
Solochrome cyanine	Myelin (non CNS)
Luxol fast blue/cresyl violet	Myelin, Nissl substance (CNS)
Methyl green pyronin	DNA, RNA
Specific cells	
Aldehyde fuchsin	Pancreatic islet cells
Barrett's	Pituitary cells
Carbol chromotrope	Eosinophils
Grimelius	Argyrophil cells
Naumenko/Feigin	Astrocytes
Pigment stains	
Masson-Fontana	Melanin
Permanganate bleach	Removes melanin
Dunn/Thompson	Haemoglobin
Fouchet	Bile pigments
Perl's Prussian blue	Ferric iron
Turnbull	Ferrous iron
Schmorl	Lipofuscin

Table 1. A selection of special stains available to the histopathologist