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REVIEW ARTICLE

Histological Patterns of Hepatotoxic Injury in *Cavia Porcellus* and Methods to Detect Them: A Review

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ABSTRACT

Histology is of invaluable importance in medical science. It allows thorough analysis and characterization of pathological changes in clinical and research studies. The main limit of classic pathology is the correct interpretation of complex morphological data relations and interconnections. For this reason, modern medicine is moving towards automated systems and, ultimately, artificial intelligence. *Cavia Porcellus*, commonly referred to as Guinea Pig is an excellent model for human medicine, especially for what concerns liver disease. The present review aims to provide a comprehensive guideline of the histological techniques available to produce quality samples for innovative diagnostic tools (computational pathology) on hepatic lipidosis characterization. To date, hepatic lipidosis is indeed one of the most common secondary disorders worldwide without a cure.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common obesity-related complications and the most common chronic liver disease affecting humans worldwide (Teng *et al.*, 2023). The wide spectrum of fatty liver diseases and their intricate relationship with metabolic syndrome leaves unanswered questions requiring further studies to be able to design effective treatments (Mahjoubin-Tehran *et al.*, 2021; Pouwels *et al.*, 2022). The complexity of liver lipidosis etiology and pathophysiology requires the integration of many clinical data and parameters from different organs. Storage and interpretation (in terms of correlation) become therefore challenging (Ching *et al.*, 2018). The application of automated tools in medical sciences has dramatically increased the ability to extract quantitative information from histological samples, especially by finding and extracting hidden information from routinely available data (Abdelsamea *et al.*, 2022; Hatta *et al.*, 2023). Computational pathology represents a promising tool to achieve a better-integrated solution to the large and diversified amount of data required for diagnostic formulation (Cui and Zhang, 2021; Shafi *et al.*, 2023) Prassas *et al.*, 2024). *Cavia Porcellus* (domestic cavy) is a great model for the study of fatty liver given the high degree of common features with human pathology. In cavies, hepatic lipidosis is mainly triggered by anorexia following a period of feeding on a high-energy diet and it can lead the animal to death (Deogburn *et al.*, 2016; Skat-Rordam *et al.*, 2021; Lintrup *et al.*, 2024). Anorexia can be a consequence of changes in diet, gastrointestinal tract stasis, dietary lack of vitamin C or dental problems that cause pain during mastication (Hoefer, 2001). However, pain, in general, can induce inappetence, therefore, the spectrum of anorexia's primary causes is wide and often difficult to trace back in the antemortem animal. For this reason, post-mortem analysis of liver organs can provide a resourceful amount of information allowing for tracing back the primary cause of the animal pathology and its possible triggers (Minarikova *et al.*, 2015). Recent studies show how the spontaneous development of non-alcoholic steatohepatitis (NASH) in this species seems to correlate to breeder-dependent variation (Brunt, 2007; Minarikova *et al.*,

2015). The fact that liver lipidosis occurs naturally in cavies makes this species a valuable alternative source of information, helping to address ethical concerns related to the use of other animals.

In the following sections of the article, key pathological changes associated with liver lipidosis are described alongside the histological techniques to detect them.

Steatosis: Steatosis is a condition characterized by the accumulation of triglycerides in the cytoplasm of hepatocytes. Steatosis becomes pathological when more than 5% of the hepatocytes are affected. In its pathological form it is relatively benign and reversible, however it can progressively lead to inflammation and tissue injury which may lead to liver failure (Geisler and Renquist, 2017)

Steatosis in humans and cavies: Hepatocytes distended with lipid droplets are a signature feature of steatosis. There are two types of steatosis; macro vesicular with hepatocytes containing single or multiple large fat vacuoles pushing the nuclei toward the periphery of the cells and micro vesicular, a more severe condition with finely divided vacuoles and the hepatocytes nuclei preserved in the central position (Brunt, 2007; Gluchowski *et al.*, 2017). The following methods are used for detection of steatosis:

Hematoxylin and Eosin: The first line of histological staining used to detect steatosis is Hematoxylin and Eosin (H&E). Hematoxylin, a colorant resembling a basic dye, stains the nuclei of cells in blue or purple color while eosin stains eosinophilic structures generally composed of intracellular or extracellular proteins (Fischer *et al.*, 2008). It allows identifying the structure of the fat vacuoles to distinguish between macro (Fig. 1A) and micro-vesicles (Fig. 1B) typologies.

It uncovers lobular inflammation and marks the characteristic cytoplasmic inclusions like Mallory bodies (MDB). MDB are important because they seem to show a different localization according to the etiology of liver lipidosis (Aishima *et al.*, 2010). In general, the development of these inclusions indicates a failure in intracellular protein quality control (Denk *et al.*, 2024). MDB formation is related to several liver diseases including, among others, hepatitis B & C, NAFLD, and copper toxicosis (Basaranoglu *et al.*, 2011; Kayacetin and Basaranoglu, 2015).

Other signs of hepatocyte injury visible with H&E are eosinophilic hyaline inclusions (Fig. 1C). The origin of these deposits is not clear, but they might originate from endocytosis or retention of endoplasmic reticulum material (Aishima *et al.*, 2010).

Early and reversible stages of cholestasis may also be detected with H&E. Cholestasis (Fig. 1D) is characterized by impaired bile flow because of bile duct obstruction or excretory failure of hepatocytes (Onofrio and Hirschfield, 2020). Hemosiderin (Fig. 1E) is a brown, granular pigment made of aggregates of ferritin molecules derived from the breakdown of erythrocytes in case of iron overload (Crichton *et al.*, 2002). It appears as brown droplets with H&E staining and is mostly found in Kupffer cells of the liver (Bloomer and Brown, 2019).

A critical feature of liver lipidosis that can also be identified with H&E is the typology of cell death (apoptosis or necrosis and different typologies of the two classes) present in the sample. Cell death is a crucial step in the progression of liver disease because of inflammation and fibrosis. Several typologies of cell death have been observed in the context of liver pathology including apoptosis, necrosis, necroptosis, autophagy, pyroptosis, and ferroptosis (Shojaie *et al.*, 2020). Depending upon the kind of cell death triggered, the outcome of liver injury will be different however it is challenging to clearly distinguish among different types of cell death with the sole tool of morphological analysis. Nevertheless, there are diagnostic criteria regarding cell death in H&E-stained sections (Elmore *et al.*, 2016a). For instance, apoptotic cells (Fig. 1F) are usually found scattered along the tissue rather than organized in clusters, and their size is consistently reduced with small pyknotic single nuclei or fragmented ones (karyorrhexis) (Elmore *et al.*, 2016a). In the liver, the cytoplasm of apoptotic cells is usually more hyper-eosinophilic than in lymphocytes (Elmore *et al.*, 2014). The morphological features of necrosis (Fig. 1G) are more strictly dependent on the nature of the insult. However, two very generic common features are nuclear swelling and pale cytoplasm (Elmore *et al.*, 2016b).

Necrosis of the tissue can lead to the progression of liver fibrosis and nodular formations (Zuñiga-Aguilar and Ramírez-Fernández, 2022). Proliferated smooth endoplasmic reticulum ultrastructure can appear in hepatocytes because of toxic injury or viral infection (Maiers and Malhi, 2019). Cells displaying this pathological change will have a “ground-glass” (Fig. 1H) appearance in H&E-stained sections (Boone *et al.*, 2023).

Grade of fat content: The balance between the metabolization of peripheral lipids and lipid clearance within the liver is disrupted in the case of anorexia. The lipids in excess are deposited as intra-hepatocellular droplets (Heeren and Scheja, 2021). Within the first 24 hours of food deprivation, the glycogen storage in the liver is depleted. Subsequently, liver cells begin to break down fatty acid to produce ketone bodies, molecules easily transported outside the tissue and used as an immediate source of energy through oxidation (Berg, 2011; Cantrell and Mohiuddin, 2023). Higher concentrations of ketones in the blood can lead to fatal dehydration (Koeslag *et al.*, 2003).

Lipids imbalance in humans and cavies: In humans, dyslipidemias are most commonly secondary to other metabolic disorders (unhealthy lifestyle, obesity or diabetes mellitus) but can also be genetically determined (primary or familial dyslipidemias) (Pirillo *et al.*, 2021). In cavy’s, ketosis usually develops in females as a side effect of pregnancy (“pregnant toxicosis”). Toward the end of the gestational period, the animals lose their appetite and tend to display decreased motility leading to abnormal energy metabolism with hypoglycemia, hyperlipidemia, and ketosis (Riggs, 2009). In males and non-pregnant females, this phenomenon is still poorly understood, and it comes

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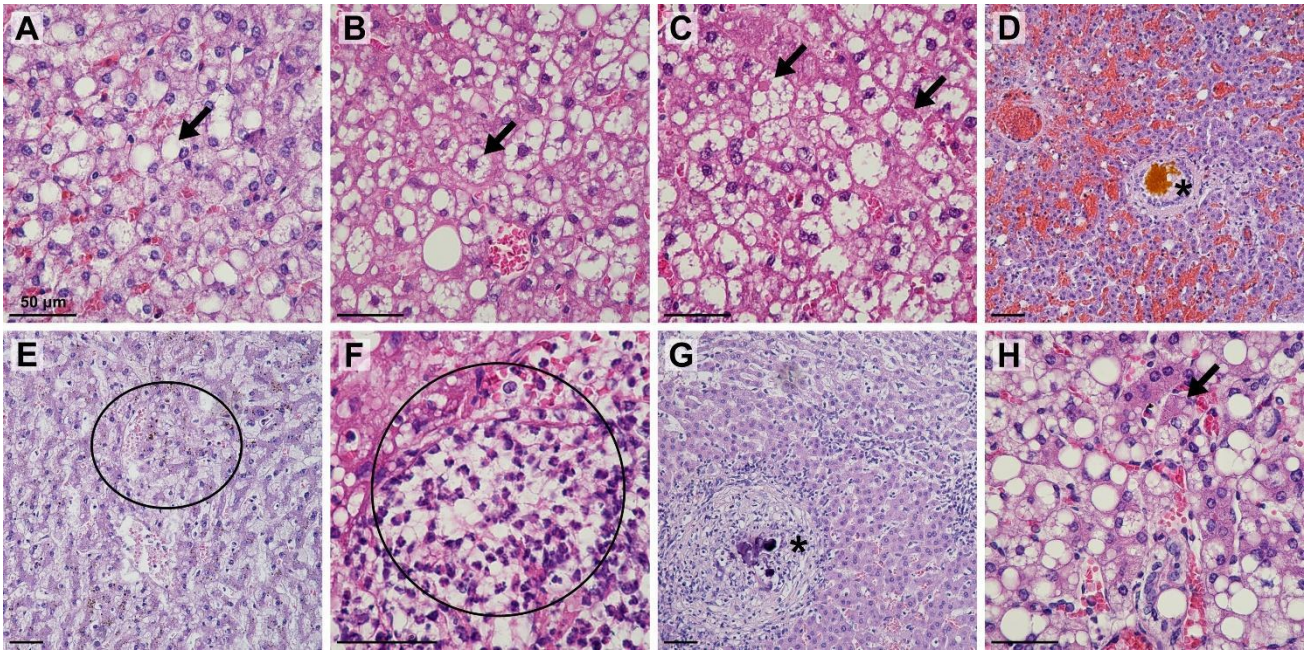

Fig. 1: Hepatic Lipidosis, liver, (*Cavia Porcellus*) 400x (H&E). a) Macro vesicular steatosis with single lipids vacuoles: thin arrow. b) Micro vesicular steatosis with finely divided vacuoles: arrow. c)Hyaline inclusions: thin arrow. d) Cholestasis: asterisk. e) Hemosiderin: outlined area f) Apoptosis: outlined area. g) Necrosis: asterisk indicating necrotic area. h) Ground-glass hepatocytes: thin arrow.

because of general inappetence and anorexia (Schmid *et al.*, 2020). More recent studies identify obesity (mainly caused by improper diet regimen with high-energy feeding) as a determining factor for ketosis (Shi *et al.*, 2020). Despite differences in etiology, humans and cavies present common pathological features in histological sections. The following methods are used for lipid content evaluation:

Oil red O: Oil red O is a dye used to assess the triacylglycerol (TAG) content in steatotic liver (Riva *et al.*, 2018) that will appear as red droplets in histological samples. The histological mechanism of staining the lipids is a function of the physical properties of the dye that is more soluble in fat than in the vehicular solvent (Lilllie and Ashburn, 1943).

Periodic Acid Schiff (PAS): Periodic Acid Schiff (PAS) is a specialized staining method used to detect polysaccharides like glycogen (Fig. 2) (Vyas *et al.*, 2022). The periodic acid reagent oxidizes the "carbon to carbon" bonds in the tissue sample forming aldehydes. In turn, the newly formed aldehydes react to the fuchsine-sulfurous acid giving the typical magenta color (Dubowitz *et al.*, 2020). Glycogen will appear as violet droplets in the histological sample.

Fibrosis: Fibrosis is a dynamic phenomenon triggered by inflammation and chronic injury (Ipsen *et al.*, 2020). There is strong evidence that cell death of hepatocytes initiates inflammation and fibrosis in fatty livers (Arjmand *et al.*, 2020). Fibrosis begins in a specific area and progresses outwards as thin fibrous septa that can then extend to adjacent portal tracts or central venules. This process is known as bridging fibrosis, a pathological stage common to several chronic liver diseases (Arjmand *et al.*, 2020; Ipsen *et al.*, 2020). The first stage is characterized by fibrotic expansion in some portal areas. At stage two, fibrosis is present in every portal area. The progression to stage three shows occasional portal-to-portal bridging. Stage four is defined by marked portal-to-portal bridging while at stage five, occasional nodules start to appear. The recurrence of nodules marks the progression to the last stage: complete cirrhosis (Arjmand *et al.*, 2020).

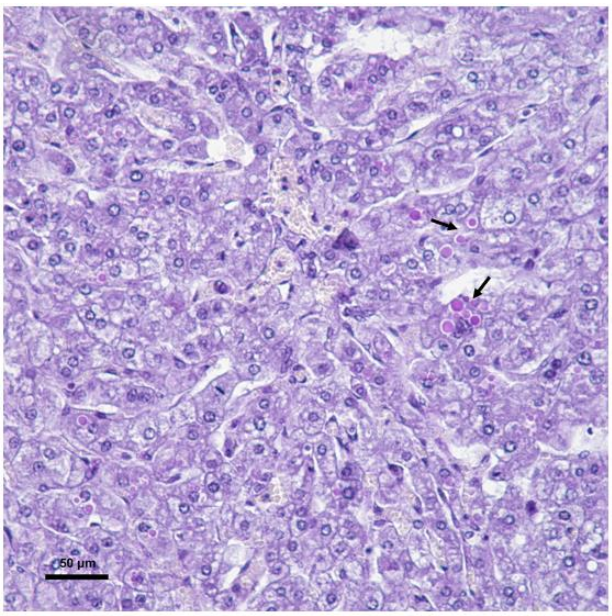

Fig. 2: Hepatic Lipidosis, liver, (*Cavia Porcellus*) 200x (PAS). The distribution of glycogen within the organ and inside the cell appears depleted. Single droplets in sparse hepatocytes are visible: arrows.

Fibrosis in humans and cavies: In humans, liver fibrosis might be the consequence of different kinds of injuries such as metabolic syndromes, viral infection, autoimmune diseases, toxic poisoning, alcoholic &non FLD, and many

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others (Lv *et al.*, 2023; Mehal, 2023; Pellicano *et al.*, 2023). Cavies represent an excellent model for NASH induced by diet. The development of advanced hepatic fibrosis (one of the hallmarks of this condition) in these animals happens indeed within a relatively short time frame (Ipsen *et al.*, 2021). The main causes of fibrotic liver damage in pet cavies are contact with toxins like aflatoxins and some classes of drugs (i.e. itraconazole, azathioprine, diclofenac, amoxicillin-clavulanate, ivermectin) or chronic liver failure because of the prolonged hepatic lipid accumulation in steatotic animals (Sheen and Pellet, 2021). The following methods are used for fibrosis detection:

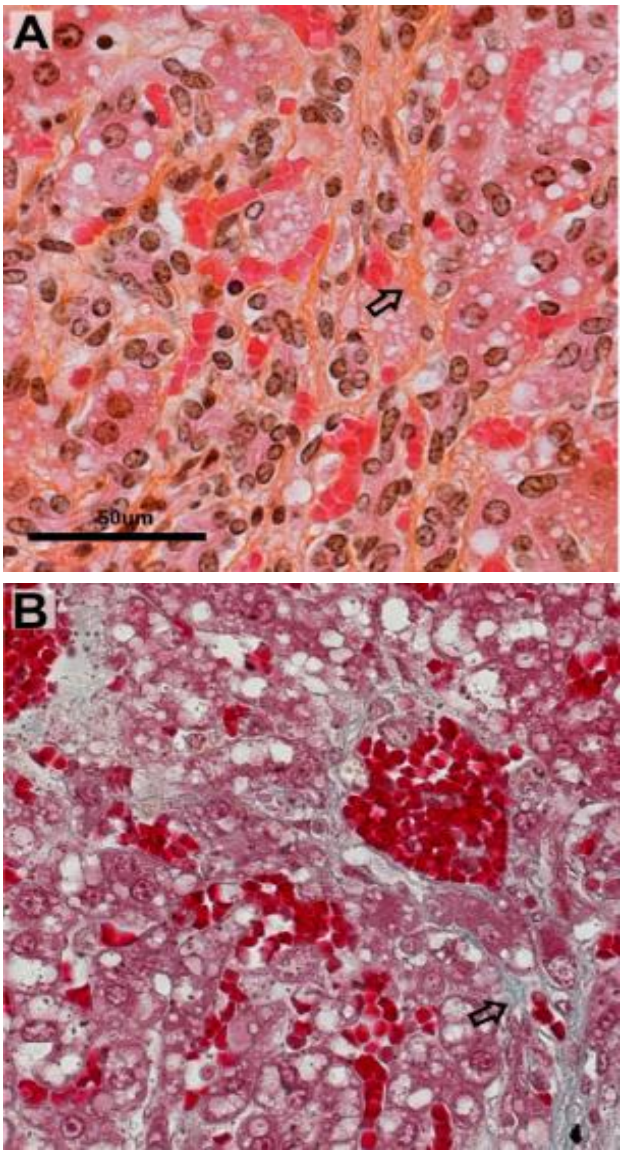

Fig. 3: Hepatic Lipidosis, liver, (*Cavia Porcellus)* 400x (HES Masson Trichrome). a) HES: the outline arrow points to the fibers of collagen type I stained in yellow saffron delimiting the sinusoids. b) Masson trichrome: the outline arrow points to the fibers of collagen type I delimiting the sinusoids, stained in green by the phosphotungstic acid.

Hematoxylin Eosin and Saffron (HES): HES (Fig. 3A) is a trichromatic staining procedure for routine use in histological laboratories that facilitates the identification of four main types of collagen fibers (I, II, III, and IV) (Ceccopieri *et al.*, 2021). The acidity of saffron's water-soluble carotenoids allows the staining of collagen's acidophilic structure (Bathaie *et al.*, 2014). The most performing application of HES in liver samples is obtained for the detection of reticulin fibers surrounding sinusoids (collagen type III).

Masson-goldner trichrome: Masson-Goldner Trichrome (Fig.3B) uses three dyes to selectively stain muscle, collagen fibers, fibrin, and erythrocytes (Luna, 1968). The tissue is first stained with Biebrich Scarlet, an acid dye binding with the acidophilic tissue components. Once the phospho-acids are applied to the tissue, the Biebrich Scarlet reagent is pulled out from the collagen creating a link for the interaction with aniline blue. The less permeable tissue components retain the red dye (Onyije *et al.*, 2017). The procedure allows detecting the collagen type I deposition along the sinusoids surrounding the hepatocytes (Fig. 4B), (25). Furthermore, it detects long-standing fibrosis associated with the formation of mature elastic fibers derived from the collapsing of the tissue's normal architecture during acute hepatitis with necrosis (Boyd *et al.*, 2020).

Picrosirius Red: Picrosirius red (Fig. 4) is a chemical dye characterized by an elongated structure and birefringent properties (Lattouf *et al.*, 2014). In tissue samples, it is bound to various molecules but only when bound to collagen it assumes a specific configuration. It takes a parallel position to the collagen fibrils enhancing their natural birefringence (De Padilla *et al.*, 2021). The method allows histological Visualization of collagen I and III fibers. It helps study tissue remodeling caused by connective tissue-related pathologies. Indeed, it allows the differentiation of collagen types, because the spectrum of absorption of polarized light of the dye strictly depends on the orientation of the collagen bundles (Velidandla *et al.*, 2014; Rittié, 2017; López De Padilla *et al.*, 2021).

Gordon and sweets: Gordon and Sweet's reticulin staining method detects the deposition of collagen type III (Fig. 5A-B) (Gordon and Sweets, 1936). The principles of the staining reaction include firstly tissue oxidation, then sensitization with the iron alum facilitating the bond to the silver. Formalin is then used to reduce the silver to its visible metallic state. The method gives the possibility to assess the gross architecture of the specimen (Gordon and Sweets, 1936). In areas of recent hepatocellular necrosis and fibrosis, it shows condensation of reticulin fibers (Hall *et al.*, 2021) while in case of progression to necrosis, the collapsing of reticulin fibers.

Special inclusions: Fatty livers often show accumulations of endogenous or exogenous pigments such as lipofuscin, hemosiderin, porphyrins, bile, iron, or copper (Churukian, 2008). Pigments can be present in hepatocytes or more often, in Kupffer cells and may be more visible in portal areas. Identification of hepatic pigments usually requires multiple special stains. An exhaustive pathological analysis of liver special inclusions should describe the morphological features of the pigmentation, the cell type involved, the lobular distribution, and any structural change that might be associated with the pigment

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deposition. The severity grade should be assigned based on the relative amounts of pigment present (Gopinath and Mowat, 2014).

Lipofuscin: Lipofuscin is a light brown granular pigment, mainly found within the cytoplasm of Kupffer cells or surrounding the bile canaliculi of centrilobular hepatocytes (Terman and Brunk, 1998; Snyder and Crane, 2023). It originates from the residues of cell membrane degradation in lysosomes. Lipofuscin accumulation is generally age-related, but oxidative stress can also increase the formation of this pigment (Terman and Brunk, 1998; Snyder and Crane, 2023). Oxidative stress plays an essential role in fatty liver syndrome. Indeed, an overload of free fatty acids can lead to an electron leakage during mitochondrial β-oxidation resulting in the generation of lipid peroxides (Raja *et al.*, 2022). Those radicals are responsible for hepatic membranes, proteins, and DNA damage (Koek, Liedorp, and Bast, 2011; Raja *et al.*, 2022).

Iron: Iron overloading is frequently observed in chronic liver diseases, regardless of etiology (Hsu *et al.*, 2022; Kowdley, 2016). Iron can be toxic if present in an excessive amount because it can severely damage cells and tissues with the acceleration of the Fenton chemical reaction that generates noxious reactive oxygen species (ROS) (Galaris *et al.*, 2019). Iron accumulates as hemosiderin in Kupffer cells and hepatocytes (Hayashi *et al.*, 2017).

Iron inclusions in humans and cavies: Iron overload might lead to the formation of liver inclusions inside the macrophages as isolated organelles or in the form of hemosiderin (Hayashi *et al.*, 2017). In humans, excessive levels of iron can be caused by genetic conditions (like genetic non-hemochromatosis iron overload disorder), alcohol consumption, or metabolic syndromes (Hayashi *et al.*, 2017). In cavies elevated iron levels are associated with diet-induced fatty liver disease (Ye *et al.*, 2013).

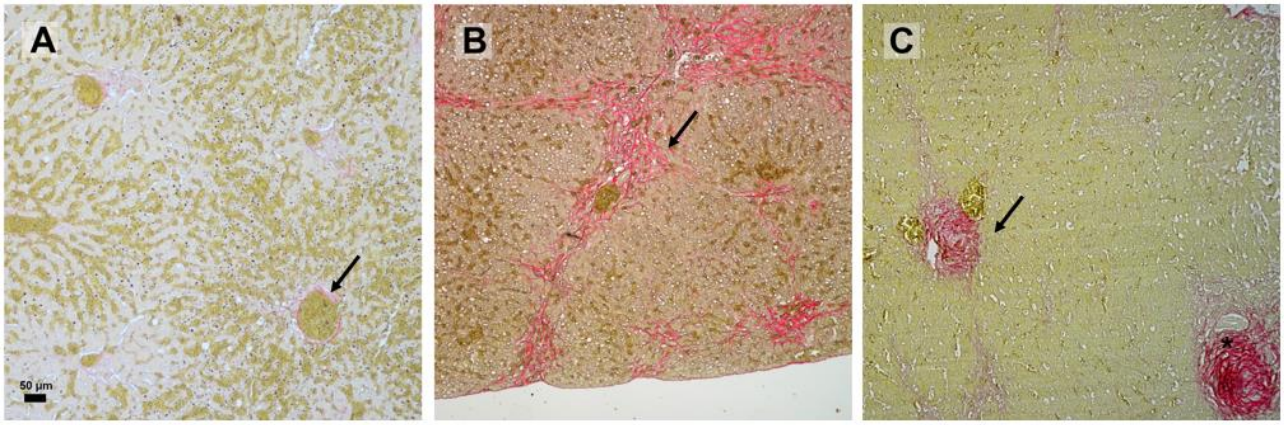

Fig. 4: Hepatic Lipidosis, liver, (*Cavia Porcellus)* (Picrosirius red). a) 10x, early fibrosis with inflammation presents only around the portal areas: arrow. b) 10x, late-stage degeneration with bridging fibrosis: arrow; c) 20x, end-stage fibrosis around portal areas (arrow) and nodular formations (asterisk).

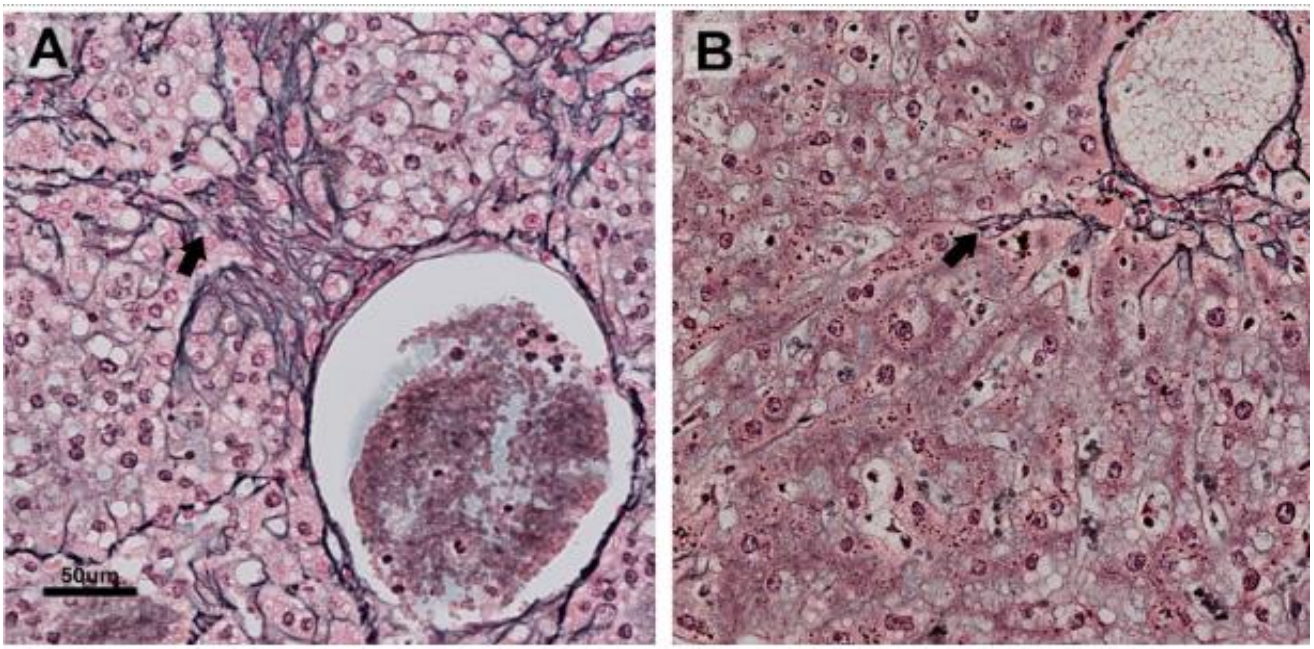

Fig. 5: Hepatic Lipidosis, liver, (*Cavia Porcellus)* 200x (Gordon and Sweet's). a) The band-like area of the collapsed reticular network (thick arrow) around the central vein indicates necrosis. b) The collapse or absence of reticulin fibers (thick arrow) corresponds to areas of cell loss.

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Bile: Bile is an essential, aqueous secretion that is formed in the hepatocytes by filtration in response to osmotic gradients (Boyer, 2013). Downstream, it is then modified through the secretory and absorptive process of the bile duct epithelium (Hundt et.al., 2024). In fatty liver, the impairment of the bile flows with the accumulation of bile residues in and out of the bile ducts can be observed (Gottlieb and Canbay, 2019). Recent studies in human medicine are evaluating the possible correlation between fatty liver and cholestasis mainly focusing on the changes in bile acid composition observed in patients (Gottlieb and Canbay, 2019).

Bile flow impairment in humans and cavies: Hepatic lipidosis in cavies (like in most mammals) can lead to cholestasis due to the increased uptake and reduced clearance of fatty acids leading to oxidative stress inflammation and fibrosis (Hawkins and Bishop 2012). The fibrotic process may disrupt the canalicular membrane, impairing transporters' function (Irwin M. Arias, 2009; Aseem *et al.*, 2023).

Copper: In the liver, copper is bound to ceruloplasmin, a major copper-carrying protein in the blood. Approximately fifty percent of copper is excreted through the biliary system, and the remaining half is excreted through other gastrointestinal secretions (Linder, 2020; Royer and Sharman, 2023). Copper is required as an important catalytic cofactor in redox reactions of many proteins, however, when present in excess, free copper ions can cause damage to cellular components causing oxidative stress but also DNA damage and reduced cell proliferation (Gao and Zhang, 2023). Copper is an iron regulatory factor. Imbalances in its concentration can cause pathologic iron accumulation, often observed in steatotic livers (Royer and Sharman, 2023).

Copper inclusions in humans and cavies: In newborn cavies, copper levels are physiologically higher (Srai *et al.*, 1986) resembling the levels observed in humans' Wilson disease (a rare, autosomal recessive disorder) (Immergluck and Anilkumar, 2023).

The following methods are used for the detection of special inclusions:

Cresyl fast violet: Cresyl Fast violet (Fig. 6A) is a histochemical method with the potential to stain a wide spectrum of tissue structures, pigments, and microorganisms without requiring a differentiation step (Puchtler and Sweat, 1964). The bile pigments react to cresyl violet, taking an olive-green color in formalin-fixed tissue samples. This coloration of the pigment gives high contrast against the background and the cell nuclei are stained in different shades of blue and violet. The downside of using this method is that the cresyl violet reagent is very expensive and there is poor reference literature concerning its use for bile detection.

Hall's staining: Hall's staining method (Fig. 6B) allows the detection of bilirubin pigment in liver samples, and it is considered the main method employed for bilirubin detection in tissue slides (Sheehan and Hrapchak, 1980). The Faouchet's reagent included in the protocol oxidizes bilirubin to biliverdin showing a distinctive olive-green color (Sheehan and Hrapchak, 1980).

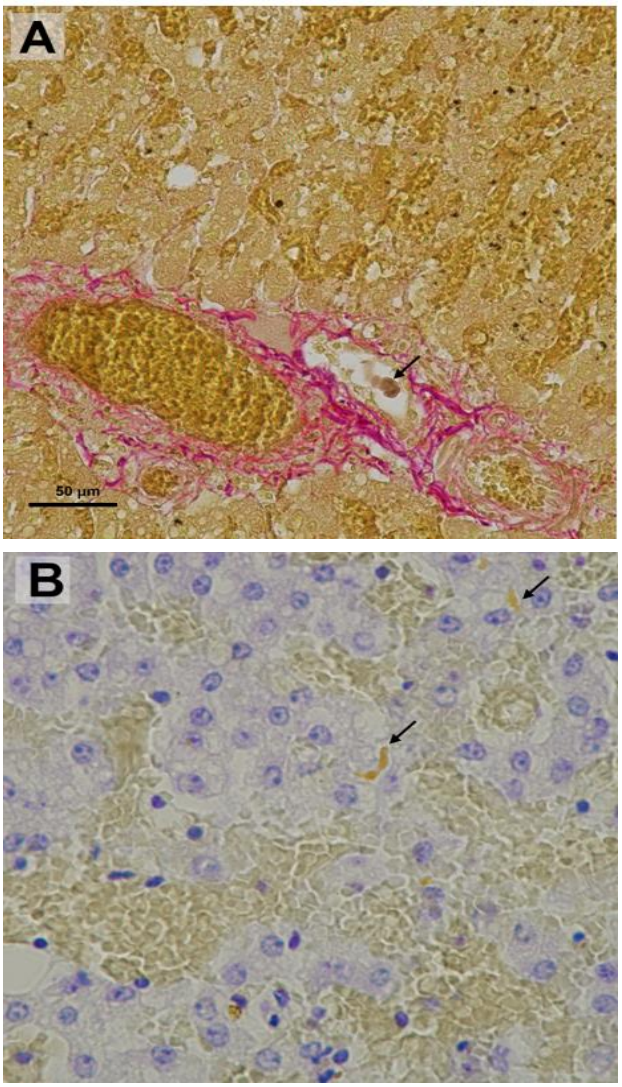

Fig. 6: Hepatic Lipidosis, liver, (*Cavia Porcellus*) 200x. (Hall's staining, Cresyl Violet) a) Hall's staining: Bile residues visible in brown-green color inside the canaliculus: arrow b) Cresyl Violet: Bile residues in the sinusoids visible in olive green color: arrows.

Rhodanine B: Rhodanine B stain is used to evaluate the copper level (Ludwig *et al.*, 1979). The dye stains the proteins binding to the copper and is considered the most reliable among commercially available copper stains. It allows for the most reproducible results that also have a linear relationship with the copper levels measured by atomic absorption spectroscopy (Irons *et al.*, 1977). A disadvantage of the method is that rhodanine-stained tissue sections tend to lose coloration with time (Thornburg *et al.*, 1985).

Prussian blue: The Prussian Blue staining method (Fig. 7A) evaluates the iron content, which is crucial in identifying oxidative stress (Galaris *et al.*, 2019). During the staining process, the ferric ions (+3) present in the tissues combine with the acidic solution of ferrocyanides resulting in the formation of a bright blue pigment called 'Prussian blue" or ferric ferrocyanide (Churukian, 2008).

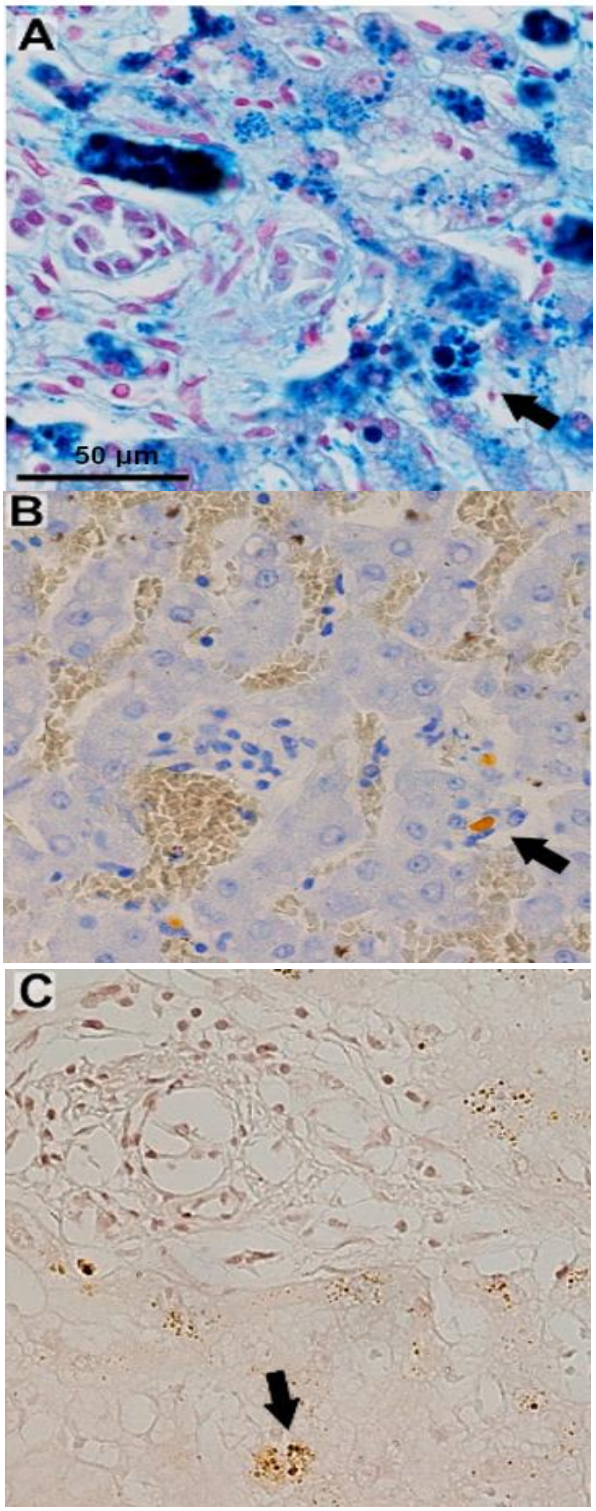

Fig. 7: Hepatic Lipidosis, liver, (*Cavia Porcellus)* 400x (Pearl's Prussian blue, Cresyl Violet, Orcein). a) Pearl's Prussian blue: The blue pigments within the cells (arrow) indicate iron 3+ deposition. The sample shows an example of an overload of iron called hemochromatosis. b) Cresyl Violet: The yellow pigments (arrow) indicate the copper-binding proteins within the hepatocytes' lumen c) Orcein: The brown pigments (arrow) indicate copper-associated proteins sparse around the tissue.

Orcein: Orcein (Fig. 7B) is used for several purposes in this context: as an alternative to rhodanine B to stain copper-associated proteins and to identify the presence of hepatitis B surface antigens (Sheehan and Hrapchak, 1980). Orcein stains "copper-associated protein" rich in sulfhydryl groups however several studies defined it as an inconsistent method for the diagnosis of copper toxicosis (Thornburg *et al.*, 1985; Henwood, 2003).

Conclusions: A correct diagnosis of hepatic injury requires histological staining, especially for chronic liver diseases where the main method to evaluate treatment effectiveness is through biopsy. The introduction and approval of automated whole slide imaging scanners allow the integration of anatomical, clinical, and molecular pathology rendering diagnostic quality. It, however requires training on high-quality representative images of pathological samples. Given the increasing interest and urgency in expanding the knowledge of liver lipidosis, the authors hope that this paper may represent a valuable technical guide for the generation of high-quality training material for automated tools in veterinary and human medical research.

Authors contribution: CC executed the staining, analyzed and tissue sample, and wrote the manuscript JP supervised the experiments and proofread and validated the manuscript: JS-K helped with the performance of the staining: KK-K helped with the performance of the staining: PK conceived and designed the study: JK, MF and JD examined the animals and collected the samples: TP cooperated in the design of the study and supervised the animals' examination and sample collection.

Disclaimer: All the histopathological images are from authors' personal archive and have neither been published nor will they be published elsewhere

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