Pathologic Assessment of Equine Hepatic Disease

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Abstract

Hepatic disease is considered a common finding within equine practice. Despite frequency of diagnosis, aetiology of specific cases often remains unknown. As a result, clinicians may struggle to offer their clients a prognosis for affected horses. In 2003 a scoring system was devised for equine liver biopsies that intended not only to provide assessment of damage, but also prognosticate on the basis of that damage. Since that time, this system has not been reviewed. This study consisted of a review of the hepatic scoring system that is currently in place for equine hepatic tissue, assessed an extended fibrosis scoring system, and investigated the utility of image analysis in equine hepatic cases. Agreement between image analysis results and those results provided by a trained anatomic pathologist were determined. As both postmortem and biopsy tissue was used in this study, the impact of tissue sample type was also considered for all aspects of scoring.

A total of fifty-three cases were submitted for analysis from centres in England, Scotland and Ireland. Of these, twenty-six cases were known to be being investigated for hepatic disease. Twenty-two cases had ante-mortem diagnoses of extra-hepatic disease and five cases had no known ante-mortem diagnoses. Samples were collected over a period of eight years (2010-2017) with follow-up data for 19 horses after original sample submission (averaging 14.5 months) with the remaining thirty-four cases lost to follow-up.

None of the aspects of the traditional scoring system were found to be significant with regards to an ante-mortem diagnosis of hepatic disease nor did they provide information with regards to prognosis. Of the aspects of the proposed extended grading system, mild centrilobular fibrosis was found to be protective with regards to a diagnosis of hepatic disease and no aspect was found to be significant with regards to clinical outcome. Image analysis was found to be in agreement with pathologist driven assessment of hepatic tissue, but similarly, did not aid in diagnosis or prognostication with regards to hepatic disease. While tissue sample type did not impact anatomic pathologist driven tissue assessment, a difference was seen between image analysis results of biopsy versus post-mortem material using Sirius Red, collagen III and smooth muscle actin staining.

While the results from this study were not concordant with previous study, the utility of biopsy results in a clinical practice is not under question. Instead, biopsy results should be considered a useful tool in a hepatic work-up and utilised in conjunction with other clinical data to inform clinical decision making.

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Abbreviations

Abbreviation	
ECM	Extracellular Matix
HSC	Hepatic stellate cell
SMA	Smooth muscle actin
SEC	Sinusoidal endothelial cell
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitors of metalloproteinases
IASL	International Association for the Study of Liver
TDAV	Theiler's disease associated virus
SBA	Serum bile acids
GGT	Gamma glutamyl tranferase
ALKP	Alkaline phosphatase
AST	Aspartate transferase
SD	Sorbitol dehydrogenase

GDH	Glutamate dehydrogenase
RBC	Red blood cell
EDTA	Ethylenediaminetetraacetic acid
Alb	Albumin
A:G ratio	Albumin:globulin ratio
Ca	Calcium (non-ionised)
CK	Creatinine kinase
Glob	Globulins
Trig	Triglycerides
Chol	Cholesterol
Na	Sodium
Cl	Chloride
K	Potassium
Na:K ratio	Sodium: potassium ratio
NA	Not applicable

H&E	Haematoxylin and eosin
PPB	Perls Prussian Blue
SR	Sirius Red
Coll I	Collagen I
Coll III	Collagen III
ROI	Region of interest
GI	Gastrointestinal disease
Multi	Multisystemic disease
Musc	Musculoskeletal disease
CV	Cardiovascular disease
Fact	Horses going to the factory
GUVS	Glasgow University Veterinary School

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is a result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Jennifer Hollyer

Chapter 1 Introduction

1.1 Overview of Equine Hepatic Disease

While epidemiological studies have yet to be carried out, hepatic disease is considered to be common in the horse (West, 1996, DeNotta and Divers, 2020). Suspected liver disease is generally diagnosed on the basis of clinical signs and alterations in hepatic biochemical parameters, while biopsy is recommended and currently considered the gold standard for confirmation of hepatic changes, morphological or aetiological diagnosis, and prognostication (Durham et al., 2003c, Rendle, 2010). Often elevations in biochemical markers of hepatocellular damage and cholestasis are seen without presence of clinical signs suggesting subclinical disease is not uncommon, however, hepatocellular enzymes may be increased with other diseases (e.g. gastrointestinal disease) (DeNotta and Divers, 2020). Clinical signs associated with liver failure are documented (West, 1996), though organ failure is considered less common (DeNotta and Divers, 2020).

Despite hepatic disease being widespread in practice, diagnosis and particularly prognostication can be challenging. To aid with prognostication in hepatic cases, Durham et. al. devised a liver scoring algorithm in 2003, which categorised and weighted specific histological changes with respect to severity of disease and case outcome. While this liver grading system was developed in 2003, it has yet to be reviewed. Since that time, advances in imaging and imaging software with regards to histological study have shown promise in creating a more quantitative analysis of histological sections, as opposed to the solely qualitative analysis carried out by anatomic pathologists.

1.2 Anatomy and physiology of the liver

The liver is the largest organ in the body, and constitutes approximately 1-1.5% of total body weight in large herbivores (Brown et al., 2017, Singh et al., 2018). Grossly the liver is divided into lobes, with right, left, quadrate and caudate lobes being recognized in the horse. This gross division into lobes, however, does not appear to

reflect homologies between areas of the liver, and instead this feature is more dependent on vasculature (Singh et al., 2018).

The liver is fed by two vessels, the portal vein and the hepatic artery, though the exact contribution of each vessel to total blood supply in the liver is not certain (Singh et al., 2018). The portal vein is thought to supply 70-80% of total afferent hepatic blood, with the remainder being contributed by the hepatic artery (Brown et al., 2017). Vessels that enter the liver from the hepatic artery are essentially end arteries, which divide along with vessels from the portal vein to eventually empty into the hepatic sinusoids, where blood from both portal vein and hepatic artery mix (Brown et al., 2017, Singh et al., 2018). Both the hepatic artery and portal vein are fed by vessels from the digestive system (digestive tract, pancreas and spleen). Blood leaving the liver is collected in central veins, which coalesce into a few large hepatic veins which then empty into the caudal vena cava (Singh et al., 2018).

The location and blood supply to the liver are crucial when considering the organ function. The liver is the seat of metabolism in the body and plays a role in the metabolism of carbohydrate, protein, fat, bilirubin, xenobiotics, as well as having a role in urea synthesis, detoxification, and immunity via the presence of B and T lymphocytes, Kupffer cells (members of the macrophage family that are found in hepatic sinusoids),natural killer lymphocytes and natural killer T lymphocytes, and the release of acute phase proteins by hepatocytes (Brown et al., 2017, Singh et al., 2018). The liver is located below the diaphragm and straddles the blood stream that drains the gastrointestinal tract, assuring that the products of digestion flow directly into hepatic cells (Singh et al., 2018).

Furthermore, the liver acts as a secretory organ, and secretes bile. Unlike other species, the horse has no gallbladder for bile storage and instead an enlarged duct system conducts bile to the cranial duodenum on a papilla that is shared with the pancreatic duct (Singh et al., 2018).

The traditional functional unit of the liver is the hexagonal hepatic lobule that is 1-2mm wide. The lobule is centered around the central vein and a portal tract can be found at each of the lobule's angles. The portal tract consists of a bile duct, branches of the portal vein and hepatic artery, nerves, and lymphatic vessels within a collagenous stroma (Figure 1.1). The border of a portal tract consists of discontinuous

hepatocytes and is termed the limiting plate. Areas of the lobule are divided into regions termed periportal, midzonal and centrilobular (Figure 1.2) (Brown et al., 2017).

If considered as a bile secreting gland, the liver can be thought of as being divided into acini by the branches of the portal vein and hepatic artery that dissect the parenchyma. In this division, the branches of the portal vein and hepatic artery are at the centre of each acinus with the terminal hepatic venule at the periphery. Within the acinus there are three zones: zone 1 is closest to the afferent blood from the portal vein and hepatic artery, zone 2 is adjacent to zone 1 and zone 3 borders the hepatic venule. Bile flows from zone 3 canaliculi of the hepatocytes to zone 1 and then into the interlobular bile ducts in the portal areas (Figure 1.2) (Brown et al., 2017).

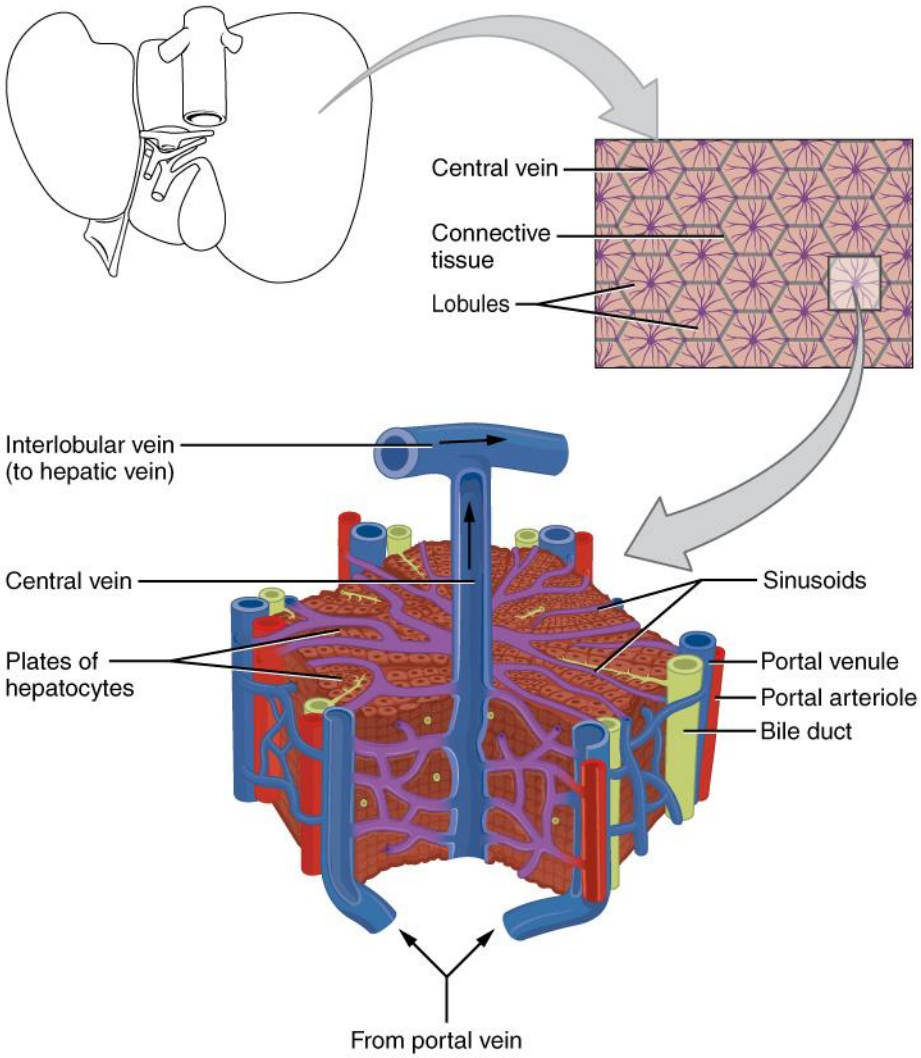

Figure 1.1 The structure of the hepatic lobule. The hexagonal lobule has portal triads at each point, with hepatocytes radiating from a central vein, all supported by a fine connective tissue structure. (http://cnx.org/content/col11496/1.6/, 2013).

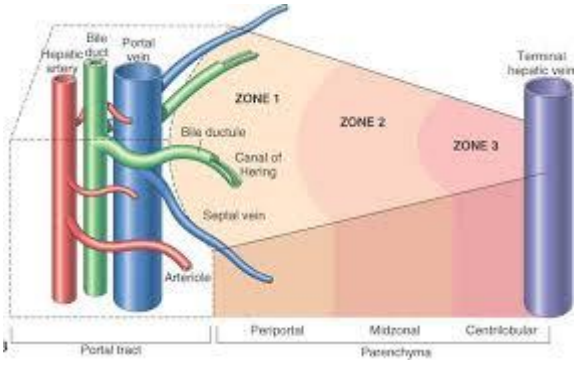

Figure 1.2 the structure of hepatic acinus with zones 1-3 moving from the portal area towards the terminal hepatic vein. This manner of describing the subunits of the liver is best utilised when considering the liver as a bile secreting organ, as bile flows from zones 3-1 becoming more concentrated as it reaches the terminal hepatic vein. (Brown, 2006).

Hepatocytes are organised as branching plates that are one cell thick and radiate from the terminal hepatic venule. Between these plates of hepatocytes is an area known as the sinusoid which is lined by fenestrated endothelial cells. It is via the fenestrae in the sinusoids that exchange between the plasma and hepatocyte can occur (i.e. uptake of nutrients from plasma and secretion of hepatocellular products). Plasma passes from the sinusoids into a gap between the sinusoids and the hepatocytes known as the space of Disse, where plasma products can be taken up by the microvilli on the hepatocellular surface and also where hepatocellular products can be exocytosed (Figure 1.3). It is also within the space of Disse that hepatic stellate cells are found. These cells normally contain stores of vitamin A, but with injury, vitamin A stores are lost and these cells are activated into a myofibroblast which can increase the synthesis of extracellular matrix (ECM) components leading to hepatic fibrosis. The sinusoids are supported by collagens III, IV and XVIII as well as other extracellular matrix components, with this support structure collectively referred to as the reticulin. Disruption to the sinusoids or the space of Disse has a significant impact on hepatic function (Brown et al., 2017).

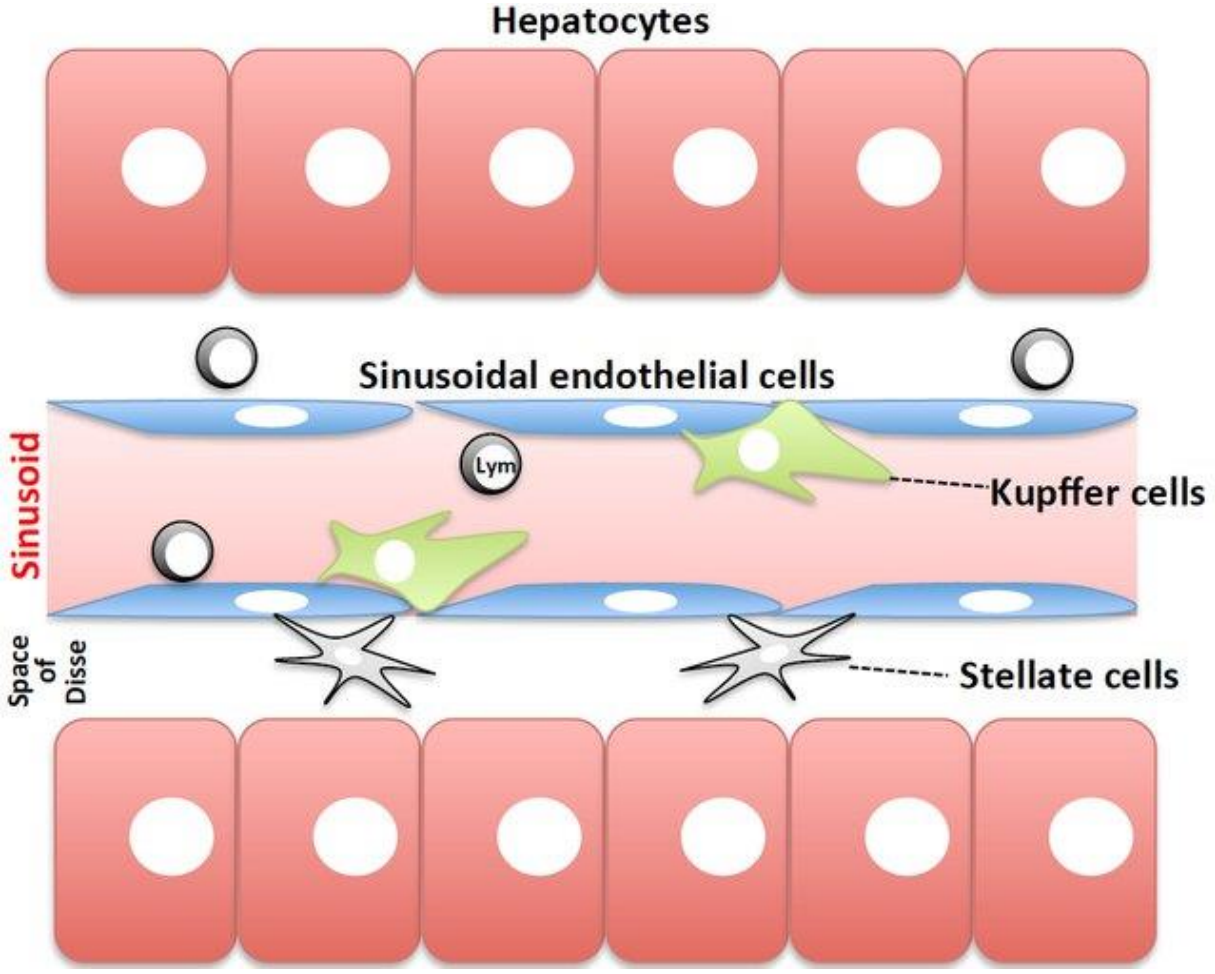

Figure 1.3 A schematic of hepatic sinusoids. The sinusoidal endothelial cells are fenestrated allowing plasma, but not blood cells, to pass into the space of Disse. Here, there is an exchange between the plasma and the hepatocytes with hepatocellular products being excreted into the passing plasma and plasma constituents being taken up by the hepatocytes. (Tsutsui and Nishiguchi, 2014).

Bile flow is in the opposite direction of blood flow that allows for the concentration of bile. Bile canaliculi begin in centrilobular areas and drain into the canals of Hering outside the limiting plates, which in turn drain into cholangioles which converge into interlobular bile ducts in portal areas. Interlobular bile ducts empty into lobar ducts that converge to the hepatic duct (Brown et al., 2017).

Bipotential progenitor cells are thought to be found in the cholangioles. These cells can become hepatocytes or biliary epithelial cells and are activated during times of injury or nutritional deficits. Activated cells form islands initially at the edges of the limiting plate. Proliferation of these islands is known as ductular reaction and marks severe hepatic injury (Haque et al., 1996,Haruna et al., 1996).

1.3 Hepatocellular response to injury

The pathological changes associated with hepatic insult include influx of inflammatory cells, necrosis/apoptosis, fibrosis, and finally cirrhosis. Initially, insult to the liver will lead to an inflammatory response, with a subsequent recruitment of inflammatory cells to the injured site. Frequently, the pattern and type of inflammatory lesions (i.e. the accumulation of inflammatory cells) present not only indicate the of duration of injury, but can also indicate of the causative agent of liver disease. Description of inflammatory cells present, in what proportion as well as anatomical distribution of these inflammatory cells provides a morphologic description of hepatic disease. If cellular injury is severe enough, the cell will undergo necrosis or apoptosis.

Cellular degeneration and necrosis is categorised into the following patterns, which again aid in interpretation of aetiological agent: random, zonal, bridging, or massive necrosis which affects either one entire lobule or contiguous lobules. Zonal necrosis is further broken down into the following categories: centrilobular (often associated with severe anaemia or right sided heart failure), paracentral (often associated with a toxin or severe anaemia), midzonal (often associated with aflatoxicosis), or periportal (often associated with toxins i.e. phosphorus). Any necrosis is followed by regeneration of the hepatic tissue (hepatocytes, bile epithelium, endothelium, sinusoidal lining). This process often occurs by replication of mature hepatocytes, however this may also occur via ductular reaction (Section 1.2). Repetitive injury may lead to nodular regeneration which distorts hepatic architecture and often lacks normal function. (Brown et al., 2017).

Hepatic fibrosis, or increase in overall hepatic ECM, is a common result of chronic hepatic injury. Fibrosis is considered the best prognostic indicator for hepatic disease (Durham et al., 2003c) owing the impact fibrosis may have on hepatic function. As such, some patterns of fibrosis can have a greater impact on hepatic function than others (e.g. perisinusoidal fibrosis which alters the microanatomic structure of the sinusoids thereby altering the ability of the liver to absorb, secrete and synthesize its products) (Brown et al., 2017). Furthermore, some patterns of fibrosis are indicators of aetiology in a disease process. For example, centrilobular fibrosis is considered a common finding in toxic damage, owing to the anatomy of the liver. Hepatocytes in this area are the site of metabolism for many drugs. This pattern of fibrosis is also

present with passive congestion of blood seen with other diseases (e.g. right sided heart failure), which again reflects hepatic anatomy. This can be compared to periportal fibrosis can result from chronic inflammation or toxicants that do not require cytochrome P450 (an enzyme responsible for the metabolism of a multitude of compounds) for their metabolism (Brown et al., 2017). Other patterns of fibrosis exist which extend across multiple lobules and include: 1)bridging fibrosis, which links portals tracts, and may be present with more severe injury, 2) biliary fibrosis (affecting bile ducts within the portal triad), 3) focal/multifocal fibrosis which can be seen with insults such as parasitic larval migration, 4) and diffuse fibrosis which effects all parts of the hepatic lobule (Brown et al., 2017).

Cirrhosis is a term used to describe liver that has undergone sufficient diffuse injury such that normal liver and lobular architecture is lost. Cirrhosis is an irreversible event (Brown et al., 2017).

1.4 Mechanism of fibrosis

Clinically, liver injury is detected via serum markers and haematological parameters, and is confirmed, graded and staged via histological evaluation of biopsy material. While serum markers are able to detect the presence of injury, correlation with severity of disease is not well established (Durham et al., 2003a, McGorum et al., 1999, West, 1996) and it would be invalid to prognosticate using these markers alone. Biopsy with histopathological evaluation is better placed to determine severity of disease as well as prognosis (Durham et al., 2003b, Durham et al., 2003c). One of the key elements of scoring is assessment of hepatic fibrosis, specifically portal fibrosis as it is considered one of the best prognostic indicators of hepatic disease (Durham et al., 2003c, Malhi and Gores, 2008).

Fibrosis is the upregulation of ECM including matrix proteins, basement membrane proteins, proteoglycans and carbohydrates (e.g. hyaluronic acid) (Gressner and Weiskirchen, 2006, Friedman, 2008, Rostami and Parsian, 2013). Human research and murine models have shown that this process is directed by the morphological and functional shift in hepatic stellate cells (HSCs). These cells, residing in the space of Disse, are normally quiescent and act as vitamin A stores. In response to injury, the

cells lose their retinol stores and begin to express α-smooth muscle actin (SMA). This process, termed transdifferentiation, converts the quiescent HSCs into a myofibroblast which is capable of producing collagen I along with other components of ECM, cytokines, chemokines, and becomes contractile, allowing for phagocytosis (Friedman, 2008, Gressner and Weiskirchen, 2006, Friedman, 1993, Gressner, 1995).

HSCs do not act in isolation; other fibrogenic cells in humans and murinae play a role (Friedman, 2008). Bone marrow derived fibrocytes will migrate to the site of injury and differentiate into myofibroblasts in response to TGF-β1 (Kisseleva et al., 2006). Portal fibroblasts, circulating fibrocytes and cells derived from epithelial-mesenchymal cell-transition will also add to the fibrogenic cellular pool. The degree to which each of these cell types contributes to the overall fibrotic state may reflect aetiology (Friedman, 2008, Wells et al., 2004, Jhandier et al., 2005, Forbes et al., 2004, Beaussier et al., 2007). Furthermore, liver sinusoidal endothelial cells (SECs), react to liver injury and produce fibronectin (activating HSCs), as well as collagen IV, proteoglycan, and factors that activate transforming growth factor, which is itself fibrogenic (Rostami and Parsian, 2013).

Progressive fibrosis is not only a strong prognostic indicator, but also is a major feature of chronic liver disease (Malhi and Gores, 2008). While acute injury may signal for fibrogenesis, chronic disease is required for significant accumulation. Inflammatory infiltrate into the liver as well as metabolic disease such as haemachromatosis signal for fibrosis (Friedman, 2008). Research suggests that sustained hepatocellular apoptosis, which is present in all forms of liver injury, as well as necrosis, are also drivers for fibrogenesis (Malhi and Gores, 2008). Apoptosis may result in the presence of inflammatory infiltrate, further stimulating the fibrogenic pathway. Production of reactive oxygen species (reactive molecules formed from oxygen such as peroxidases, superoxidases, etc.) results from inflammatory, metabolic and apoptotic injury, and act as mediators, which, in turn, induce cytochrome P450 2E1 and lead to pericentral injury and fibrosis (Friedman, 2008, Castillo et al., 1992, Chitturi and Farrell, 2001, De Minicis and Brenner, 2007). The process creates a cyclical pathway, whereby the induced factors of apoptosis further fuel fibrogenesis. Necrosis, on the other hand, may be the result of higher concentrations of an injurious agent. The effect of necrosis on the stimulation of fibrogenesis relative to apoptosis is uncertain (Friedman, 2008, Parola and Robino, 2001, Jaeschke, 2006).

While fibrosis has long been thought to be an irreversible event, current research has shown that removal of the injurious agent can result in resolution or regression of the fibrosis. The presence of proteases and collagenases is required for the constant turnover of ECM in normal liver. Matrix metalloproteinases (MMPs) function primarily to degrade ECM substrates with HSCs and hepatic macrophages are known sources for these proteinases (Hernandez-Gea and Friedman, 2011, Iredale, 1997, Henderson and Iredale, 2007, McCrudden and Iredale, 2000).

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous proteinase inhibitors which act as one level of regulation on MMPs and prevent ECM degradation. In response to liver injury, expression of both MMPs and TIMPs is increased, reflecting an increased turnover and remodeling of ECM. During resolution of fibrosis, however, levels of specific MMPs and TIMPs are generally reduced. These pathways are being investigated as potential targets for anti-fibrotic therapy in human medicine (Hernandez-Gea and Friedman, 2011, Knittel et al., 1999, Murphy et al., 2002, Duffield et al., 2005).

The complex homeostatic control of ECM has yet to be fully elucidated. Other mechanisms of fibrotic regression have also been investigated with regards to control of apoptosis of myofibroblasts (e.g activated HSCs and other recruited fibroblasts). In particular NF-κβ signaling has been investigated with regards to its role as both proinflammatory and profibrogenic signaling pathway as well as its ability to confer apoptotic resistance (Watson et al., 2008, Tergaonkar, 2006, Chakraborty and Mann, 2010, Elsharkawy and Mann, 2007, Oakley et al., 2003, Hernandez-Gea and Friedman, 2011). Other molecules are also under investigation for their role in both myofibroblast apoptosis regulation and fibrosis (Hernandez-Gea and Friedman, 2011).

1.5 Aetiologies of hepatic disease in the horse

Aetiologies of hepatic disease in the horse are numerous. Toxic, infectious-including bacterial, parasitic and viral agents, Theiler's disease, metabolic and neoplastic causes of liver disease are reported, though aetiological agents are often unknown. While incidence of hepatic disease has not been quantified to the author's knowledge, owing to the fact they graze, horses are thought to be at an increased risk for liver

disease (West, 1996). As clinical outcome can range from complete resolution of a hepatopathy to death, prognostic indicators are of particular importance to both practitioners and owners.

Toxic causes include pyrrolizidine alkaloid toxicosis, *Trifolium hybridum, Panicum coloratum*, and *Indospicine* hepatotoxicosis/hepatitis; all related to ingestion of plants. Dietary copper and iron toxicosis are reported and may relate to dietary supplementation or naturally occurring soil/herbaceous content. Horses are also at risk from mycotoxins which may be present in feedstuffs (e.g. aflatoxicosis relating to moldy corn ingestion) (Bergero and Nery, 2008).

Megalocytosis, necrosis and fibrosis are reported in cases of pyrrolizidine alkaloid heptotoxicosis (Curran et al., 1996, Bergero and Nery, 2008). Hepatitis and photosensitivity are reported in cases of *Trifolium* and *Panicum* hepatitis, while *Indospicine* ingestion inhibitis protein synthesis by acting as an arginine inhibitor. Iron and copper accumulate in the liver, and excessive loads can induce liver failure. Necrosis, biliary hyperplasia and fibrosis are associated with mycotoxin ingestion (Bergero and Nery, 2008).

With regards to bacterial and parasitic infectious causes of hepatic disease in the horse, cholangiohepatitis, destruction of parenchyma and inflammation of the bile ducts, are the predominant pathological lesions seen. The literature describes bacterial agents: *Escherichia coli, Salmonella sp., Pseudomonas, Actinobacillius equuli, Clostridia sp., Pasteurella sp,* and parasitic agents: *Strongyle sp.* and *Parascaris equorum* (due to larval migration) being associated with hepatic disease. *Fasicola hepatica* is an infrequent parasite of horses as they appear to have a degree of resistance to the trematode, however, the liver is the affected organ in cases of infestation (Bergero and Nery, 2008).

Viral causes of hepatic disease have been a recent addition to the literature. Hepaciviruses associated with hepatic disease have recently been reported in the horse. The first hepacivirus discovered was termed “equine hepacivirus” and is most closely related to hepatitis C virus. It is known to cause transient elevations in liver enzymes; however, longstanding chronic hepatitis does not appear to be a consequence of infection. The second two viruses that belong to this family were detected during an outbreak of Theiler’s disease in the United States. Both of these

viruses are members of the Pegivirus family, and despite being discovered in association with a specific form of hepatic disease, do not appear to be hepatotrophic—in other words, their discovery and the presence of hepatic disease appear coincidental at this time. One is termed “Theiler’s disease associated virus” (TDAV) while the other has been called “equine pegivirus” (Divers and Tomlinson, 2020).

The association of the previously mentioned Pegiviruses with Theiler’s disease, lead to investigation of other the presence of other viruses in association with the disease. Theiler’s diease, also known as serum sickness, or serum hepatitis, causes acute hepatic failure. A type III hypersenstivty reaction occurs 10 days to 10 weeks post injection with equine serum (most frequently in association with tetanus antitoxin administration). Lymphocytic foci and widespread hepatic necrosis (Smith et al., 1991), and arteritis are characteristic of the disease. Recently, a new parvovirus has been identified in the serum and liver of a horse that died of Theiler’s disease. The same virus was found in the tetanus antitoxin that was administered to this horse prior to development of disease. Current research is assessing this newly described equine parvovirus’ role in the development of Theiler’s disease (Divers et al., 2018, Divers and Tomlinson, 2020).

Hyperlipidaemia, a metabolic dysfunction, is a common cause of hepatic disease in the horse. This condition arises as a secondary complication to another underlying disease. Depositions of fat within the liver overwhelm beta-oxidation pathways and prevent gluconeogenesis thus leading to a fatal outcome without supportive treatment (Bergero and Nery, 2008).

Finally, primary hepatic neoplasia is uncommon in the horse; however, metastases from other sites may occur (Bergero and Nery, 2008). Extra hepatic disease may lead to hepatic pathology. Lesions likely vary depending on the underlying disease process (West, 1996).

1.6 Clinical signs of hepatic disease in the horse

Clinical signs of hepatic disease are not dependent on aetiology and commonly include weight loss, anorexia, dullness and depression. Less commonly reported are signs of jaundice, tachycardia, intermittent pyrexia, abdominal pain, ventral oedema, clotting deficiency, muscle fasciculations, diarrhoea or constipation. Photosensitization, dysphagia, encephalopathy and haemorrhages appear to be associated with severe or end stage disease (West, 1996).

Interestingly, clinical signs associated with hepatic failure often appear suddenly. As failure requires loss of 75% or more of the liver capacity (West, 1996), substantial acute insults, but more often accumulated damage from chronic disease, is implicated. Onset of liver failure may appear acute owing to the non-specific clinical signs associated with less severe hepatic disease. As these clinical signs can also can be associated with non-hepatic diseases—e.g., intestinal disease, parasitism, dental disease, endocrine disease, etc. (West, 1996), there may be a delay in diagnosing primary hepatic insults and administering hepatic support. Furthermore, these extra-hepatic diseases often have an impact on hepatocellular health and should also be considered when assessing clinicopathological data, which may further confound the diagnosis of primary hepatic complaints.

1.7 Common blood results associated with liver disease in the horse

As clinical signs are non-specific and subclinical disease may be prevalent, the use of haematological and biochemical markers for hepatic disease is necessary. Common serum markers for hepatic and biliary health include total serum bile acids (SBA), bilirubin, gamma glutamyl transferase (GGT), alkaline phosphatase (ALKP), aspartate transferase (AST) (Gupta et al., 2019), sorbitol dehydrogenase (SD), glutamate dehydrongenase (GDH), urea, globulins and, if available as a stable side test, ammonia. Many of these serum markers are not specific to the liver however, and are produced in other tissues. Furthermore, all of these biochemical markers may be elevated in response to extra-hepatic disease. Haematology is often run alongside biochemistry in suspected cases of liver disease and may show inflammatory changes. While changes in chemistries and haematology may add support to a suspicion of

hepatic disease when considered alongside clinical signs and imaging results, the prognostic value of these markers is questionable (Durham et al., 2003a, McGorum et al., 1999, West, 1996).

SBA has been found to positively correlate to portal and parenchymal inflammation, portal fibrosis, haemosiderin deposition in Kupffer cells (section 1.2), nuclear changes (anisokaryois progressing to megalocytosis), and histologic score (Dunkel et al., 2015). SBA appears to remain elevated longer than GDH in horses that recovered from hepatic necrosis (West, 1996), likely due to the relatively short half-life of GDH in the horse (12-24 hrs) (DeNotta and Divers, 2020), and is thought to remain elevated in horses that have terminal hepatic disease. One study found that SBA >20 µmol/ l equated to an increased risk of non-survival (Durham et al., 2003a), however a separate study found a low specificity associated with single measurements of SBA as a prognostic indicator of survival, which may relate to resolution of disease (Dunkel et al., 2015). Elevated globulins and decreased albumin and urea also suggest an increased risk of non-survival (Durham et al., 2003a).

Of the serum markers, ALKP appears to have the greatest prognostic value (Durham et al., 2003a); however, its specificity for hepatic disease is low owing to its production in bone, intestine, kidneys, placenta, mammary tissue, and neutrophils (Walton, 2013). GGT, which is specific for liver disease, was found to have prognostic significance for values >399 IU (Durham et al., 2003a) and appears to correlate with severity of clinical signs in some studies (West, 1996). The enzymes AST and GDH did not appear to offer prognostic value (Durham et al., 2003a), however, elevation in GDH has been associated with chronic liver disease. The degree of elevation of this analyte correlates poorly with severity of hepatic disease. Elevated ammonia appears to be both sensitive and specific for liver disease, however it can fluctuate widely over the course of a day (West, 1996), decreasing its prognostic utility. As testing for blood ammonia must be done stable side owing to highly variable results with delayed analysis and/or improper sample handling, it may not be available to many equine practitioners. Specifically, delayed sampling may lead to falsely elevated results from the release of ammonium from labile proteins in plasma or production for ammonium from erythrocytes and leukocytes, or falsely decreased results from the escape of ammonia from improperly stoppered tubes/air in blood tube (Stockham and Scott, 2008).

Leukocytosis, most commonly represented as a neutrophilia, occasionally with a left shift, is often found in conjunction with hepatic disease (West, 1996). Occasionally anaemia was reported, though was attributed to other underlying disease processes (West, 1996), whereas RBC > $10x10^{12}$/L was associated with a significant increase in risk for non-survival (Durham et al., 2003a).

1.8 Performing a liver biopsy in the horse

Combinations of clinical signs, biochemistry, haematology and imaging are useful in pinpointing the area of disease but are often unreliable in providing an aetiology and prognosis. The current gold standard for providing this type of information is histopathological examination of biopsy material (Rendle, 2010).

Biopsy is generally performed on the right side, though, if indicated, may be performed on the left with ultrasound guidance. If focal pathology exists, this area is targeted, however, if pathology appears diffuse, areas away from hepatic vessels with reasonable depth of hepatic tissue are chosen. The procedure is performed under standing sedation with local anaesthetics (Rendle, 2010).

Biopsy is contraindicated if hepatic abcesses are present or suspected. Complications following biopsy include haemorrhage, colic, peritonitis, pneumothroax, pleuritis, haemothorax and cellulitis. The risk of haemorrhage in the presence of coagulation abnormalities is considered minimal (Rendle, 2010). The question of whether a representative sample of tissue has been taken is a major limitation of this procedure. In human medicine, biopsy length of 15mm with more than 5 portal tracts is required for histological assessment (Manning and Afdhal, 2008). The optimal biopsy length for histological assessment has yet to be determined for the horse, and further investigation is required to determine if similar criteria are required.

1.9 Systems for staging human liver disease

Biopsy provides confirmation of organ injury, may provide definitive diagnosis of aetiology and can also be used for grading and staging a disease. Several grading

paradigms have been developed for assessing human liver biopsies, whose grades and stages vary between systems. The concepts of grading and staging are divided in that grading provides an indication of how quickly a disease is progressing to end-stage, while staging is the measure of how far disease has progressed to end stage. In other words, the grading reflects a rate and the severity of the underlying disease (often denoted by inflammatory component), while staging reflects a measure of the present injury (which relates to degree of fibrosis). As inflammation becomes more marked, grade increases, and as fibrosis increases from none to portal expansion, to bridging, to early cirrhosis, to established cirrhosis, stage increases. Both measures are meant to inform prognosis and treatment, though, in human medicine, the prognostic capability of grading and staging is questionable (Goodman, 2007).

The creation of different scoring systems relates to the variation in lesions characteristic of specific diseases, and focus on septal and portal fibrosis, biliary hyperplasia, and inflammation (Neuman et al., 2016).

Necroinflammatory conditions, such as viral hepatitis, can be graded and staged using simple systems such as the International Association for the Study of Liver system (IASL), Metavir score, or the Batts-Ludwig system which assess the degree of piecemeal necrosis and parenchymal injury as well as fibrosis. These systems award scores that can fall into descriptive categories (i.e. mild, moderate, marked) or numeric categories. However, systems for describing chronic hepatitis can be more complex and include many categories for assessment. Most commonly used are the Histology Activity Index (Knodell score) and Ishak score. The grade and stage may be reported separately or may be added together with the Knodell score but are reported separately with Ishak scoring. These staging and grading systems are most useful for statistical analysis of large cohort therapy trials and are not considered useful in the management of individual patients due to lack of reproducibility (Goodman, 2007).

Staging and grading systems have also been developed for hepatic steatosis, however understanding of the relationship between histopathological features and disease progression is less well understood. Brunt et. al. (1999) established a three grade system that looked at fat, hepatocyte ballooning, and inflammation. Kleiner et.al. (2005) devised a system looking at non-alcoholic steatosis, termed NAFLD Activity Score to be used in large clinical investigations (Goodman, 2007, Kleiner et al., 2005).

Other scoring systems for varying types of fatty liver disease also exist (Brunt, 2016). Chronic cholestatic, primary biliary cirrhosis and primary sclerosing cholangitis do not have corresponding grading systems as of present (Goodman, 2007). Specifics of many of the grading and staging systems can be found in “Practical Hepatic Pathology: a Diagnostic Approach” (Guido, 2018, Lackner, 2018).

1.10 Image analysis techniques

Given the longevity of the existence of liver scoring systems in human medicine, the question of reproducibility of scores (grade, stage and total score) between different pathologists (interobserver) as well as the same pathologist (intraobserver) has been examined. Studies in this vane often use Kappa analysis to show agreement between each assessment (Mohamadnejad et al., 2010). In one study that compared the Knodell score to another proposed system of liver scoring (the Scheuer score) it was found that fibrosis scores were reproducible in both systems—i.e. they had good agreement with regards to intra- and interobserver variation, with the Scheuer scoring system having slightly better Kappa values than the Knodell system. However, with regards to inflammation, the Knodell system was not very reproducible, and the Scheuer system performed well when examining severe inflammation (Goldin et al., 1996). When considering grading systems, it is thought that systems that reduce the number of categories tend to have higher reproducibility amongst pathologists, however, systems that have higher numbers of categories provide more information with regards to the disease process, and may be more clinically useful (Goodman, 2007). In attempts to decrease the amount of intra and interobserver variation, automated assessment of histopathological material has been considered (i.e. image analysis).

Image analysis is the process of scanning a histopathological slide to generate a computer image. Computer software is available to allow for the assessment of specific pathological lesions. Lesions are detected by the user, with thresholds for what constitutes the lesion of interest (often based on colour pixel intensity). At present, image analysis appears to have utility with various types of tissue assessment

from hepatic fibrosis to assessment of mitotic rate in breast cancer (Bedossa et al., 2003, Calvaruso et al., 2009, Huang et al., 2014, Mohammed et al., 2012)

1.11 Current method of liver scoring in the horse

In an attempt to standardise the assessment of liver pathology and offer a degree of prognostication, Durham et al. (2003) devised a grading system for equine liver biopsies. This system grades histological lesions on the degree of fibrosis, irreversible cytopathology, haemosiderosis, inflammatory infiltrate in the portal area and bile duct proliferation. Scores were generated using statistical analysis of specific pathological change and assessed their impact on risk of non-survival past 6 months. Total liver scores range from 0-14, with horses with total liver scores between 2-6 being 12 times less likely to survive 6 months versus those horses with a liver score of 0, and horses with scores between 7 -14 being 50 times less likely to survive. Fibrosis appears to be the most useful prognostic indicator, with periportal and bridging fibrosis being particularly significant as indices of poor prognosis. Biliary hyperplasia was also found to have strong prognostic value (Durham et al., 2003c).

The scoring system was designed to grade diffuse hepatic pathology and is inappropriate for use with focal lesions and neoplastic disease. Scores often correlated well with corresponding necropsy material (Durham et al., 2003c) and animals with moderate fibrosis, severe haemosiderosis or severe biliary hyperplasia were likely to have ultrasonographic abnormalities detected, suggesting targeted biopsy is achievable (Durham et al., 2003a).

If changes are not visible or appear focal on imaging, the question of whether or not a biopsy is representative remains. Interpretation and prognostication of focal pathology is further complicated if an aetiological agent cannot be determined. With these questions in mind, the poor correlation of haematological and biochemical parameters with severity of hepatic disease and the known propensity for subclinical disease, the clinical information generated by biopsy examination should be scrutinised with regards to the impact on prognosis.

1.12 Summary

Owing to their increased risk as grazers, liver disease is not an uncommon finding in horses (West, 1996). At present, liver biopsy acts as a gold standard with regards to prognostication (Durham et al., 2003a, Durham et al., 2003b, Durham et al., 2003c, Rendle, 2010). The histopathological information generated by reading biopsies is often useful clinically, however, based on findings from human medicine, intra and interobserver variation in histopathological assessment has the potential to create a known bias. Use of quantitative methods for assessing pathological changes in histological samples may decrease the level of these biases.

1.13 Aims

To the author's knowledge, molecular pathways of fibrosis in the horse have yet to be examined and presence of key players, such as HSCs, has yet to be investigated. This project assumed a similarity in these pathways between equids and humans. As such, the aim of this thesis is two pronged:

1) Perform a traditional manual histopathological assessment of equine hepatic tissue whereby:
    - the current liver scoring system for equine liver biopsy (Durham et al., 2003c) and post-mortem material is reviewed
    - compare the scoring system (Durham et al., 2003c) when using biopsy vs necropsy material
    - assess the utility of an extended fibrosis score
    - use several histochemical stains to examine fibrosis in equine hepatic tissue
2) Perform computerised histopathological assessment of equine hepatic tissue looking specifically at fibrosis and haemosiderin deposition to:

- compare scoring results (both traditional and extended) with those generated using image analysis
- assess the impact of biopsy vs post-mortem material on image analysis results

Chapter 2 Materials and Methods

2.1 Sample collection and tissue handling

2.1.1 Geographic areas of sample collection and sample types acquired

Samples for this project were collected over the period of eight years (2010-2017) from five institutions—three equine hospitals, one laboratory, and one abattoir (horse meat factory). All samples used were collected either during clinical investigation or post-mortem examination and included hepatic tissue (either biopsy or post mortem tissue) and blood samples. The study was both retrospective and prospective depending on cases submitted by each institution.

Samples were submitted from two equine hospitals in England (Liphook and Rossdales Equine Hospitals), one Scottish equine hospital (Weipers Equine Hospital) as well as horses that were euthanased and underwent post mortem at the University of Glasgow with the proper consent (Ethics number 25a/13), one laboratory in Ireland (Irish Equine Centre), and one horse meat factory (abbatoir) in Ireland (Shannonside Foods). Twenty-two cases were submitted in total from the English hospitals, twenty cases were submitted from Scotland, and a total of eleven cases were submitted from Ireland, making a total of fifty-three cases included in the study.

2.1.2 Tissue collection and sample description

Fifty-three liver tissue samples were obtained from cases being investigated for hepatic disease, extra-hepatic disease or during routine post-mortem examination. These samples were comprised of twenty-seven biopsy samples and twenty-six post-mortem samples. Forty-eight cases had known diagnoses.

Biopsies were performed on the right side under standing sedation and regional anaesthesia utilising ultrasound guidance. Post-mortems were performed within 48 hours of death by trained anatomic pathologists. Upon collection, all tissue samples were immediately placed in 10% buffered neutral formalin for at least 24 hours prior to histological analysis.

2.1.3 Blood sampling and haematological and biochemical assessment

The results of haematological and biochemical tests that were requested for clinical monitoring at the time of case presentation were used for the present analysis. Blood samples were taken via jugular venipuncture and added to heparinised, ethylenediaminetetraacetic acid (EDTA) or serum tubes.

Heparanised samples were spun using a Beckman-Coulter J6-M floor standing centrifuge (Beckman Coulter, Wycombe, UK) at 3697.8 g for 15 minutes, while serum samples were left to clot. Supernatant was removed and utilised for analysis. After separation, the plasma/serum samples were stored at -20°C until retrospective biochemical analysis due to a change in analysers during the course of the project. Twenty-one heparinised and serum samples underwent routine biochemical analysis using a Dimension Xpand (Siemens, Frimley, UK) which included the analytes listed in Appendix 2. The majority of these samples were taken from horses undergoing clinical investigations ante-mortem, however, three samples were taken at the point of euthanasia by captive bolt and pithing.

Routine haematological analysis was performed on the Advia 120 analyser which was operating using Advia 2120 software (Siemens, Frimley, UK) for five EDTA samples. Alongside machine analysis, Romanowsky stained blood smear examinations were performed for all haematological assessments. This included manual white cell differential counts up to 200 cells. All haematological samples were analysed at the time of initial submission from ante-mortem investigations.

2.1.4 Diagnoses and disease categories

Sample inclusion criteria was based on availability of equine liver tissue, and where possible, a corresponding serum or heparanised blood sample. As such, horses undergoing investigations for any disease type was included in the study to allow for the inclusion of both diseased and healthy liver samples. Pre-mortem clinical diagnoses were used to generate disease categories in statistical analysis in cases that

came from equine hospitals (i.e. English and Scottish cases). Post-mortem diagnoses were considered for the generation of disease categories in statistical analysis in cases from the Irish Equine Centre; incidental findings were ignored with regard to classifying disease type. Horses that were going to the horse meat factory were considered their own category with regards to "disease categorization" in statistical analysis as they would have to be fit for human consumption, however a clinical history was unknown.

For statistical analysis, diseases were given broad category titles. In total, eleven disease/reason for investigation categories were considered: hepatic disease, gastrointestinal disease, multiple body systems affected, ill thrift, musculoskeletal disease, skin disease, cardiovascular disease, dental disease, ocular disease, factory, and unknown. For example, the musculoskeletal category could include horses that had tendon injuries, fractures, or osteoarthritis. A full list of specific clinical diagnoses can be found in Appendix 1.

2.1.5 Clinical outcomes

Clinical outcomes were collected over the period of sample collection (2010-2017). Clinicians from the referral hospitals were contacted to enquire if horses had returned to the hospital or if the referring veterinary surgeon had provided further case feedback, with outcome data collection ending in 2017. Cases that had been subjected to euthanasia at one of the three equine hospitals that submitted samples were noted in hospital records. Post-mortems were carried out on these individuals as requested, and post-mortem results were reviewed. Clinical outcome was known for thirty-four cases (ten biopsy and twenty-four necropsy), with twenty cases having an unknown clinical outcome.

Cases that were submitted from Ireland were all post-mortem samples. Clinical information such as age, breed, sex, etc. as well as diagnosis/cause of death was taken from the post-mortem reports that were provided from the Irish Equine Centre. No clinical information (age, breed, sex, etc) was available from the samples taken from the horse meat factory.

Three categories were devised for outcome based on the follow-up data: 1) death due to hepatic disease (n=5), 2) survival without repeat clinical signs (n=6) and 3) death due to extra-hepatic disease (n=23).

The timespan which the follow-up data covered, was in part reflective of the submission date/collection date of the samples. The data ranged investigation, i.e. died/euthanased on the during investigation (0 months), to 5 years after clinical investigation (average follow up time was 14.5 months).

2.2 Staining techniques of fixed samples

2.2.1 Haematoxalyin and eosin

After adequate fixation, sections were cut at 2 microns and dewaxed and rehydrated through three changes of graded alcohols, before being washed in water. Sections were then added to Gills haematoxylin for 5 minutes. After washing with water, sections were differentiated with 1% acid alcohol. Eosin was added for 5 minutes. Sections received a final wash in water and were then dehydrated, cleared and mounted on to glass slides with glass coverslips using DPX media.

2.2.2 Perls Prussian Blue

Tissue samples were fixed and sectioned as described in section 2.2.1. Sections were dewaxed in histoclear for 2 minutes, washed with water and then treated with 2% potassium ferrocyanide and 2% hydrochloric acid for 10 minutes. After washing in water, sections were then counterstained with 1% safranin for 30 seconds before dehydrating, clearing and mounting on glass slides with glass coverslips using DPX media.

2.2.3 Sirius Red

Tissue samples were fixed, sectioned and dewaxed as described in section 2.2.1. Sections were added to Weirgerts iron haematoxylin for 10 minutes. Sections were then washed in water for 10 minutes before being treated with Sirius Red stain for 5 minutes. Sections were quickly dipped in water before dehydrating, clearing and mounting on glass slides with glass coverslips using DPX media.

2.2.4 Immunohistochemistry

Immunohistochemical techniques were utilised to look at collagens I, III and smooth muscle actin (SMA). All staining was performed at room temperature with Tris buffer

pH 7.5 and Tween being used for rinsing. Briefly, antigen was retrieved using the heat-induced epitope retrieval unit, Menapath (Menarini, Firenze, Italy) with sodium citrate buffer (pH 6) for 1 minute 40 seconds at 125°C at full pressure (SMA and III) or treated with Proteinase K (RTU) (Dako S3020) for 15 minutes (collagen I). Sections were then loaded on the Dako autostainer (Ailgent, California, USA) and rinsed with buffer. Dako Real™ Peroxidase blocking solution was applied for 5 minutes before a second buffer rinse. Sections were treated with primary antibodies in Dako universal diluent for 30 minutes and then rinsed twice in buffer. Secondary reagents were then applied, followed by 2 buffer rinses and 2 treatments with 3,3'-Diaminobenzidine (DAB) (Dako K5007). Table 2.1 provides information on primary and secondary antibodies as well as the dilutions which were used for staining. Sections were rinsed in water and then counterstained with Gills Haematoxylin for 27 seconds. A final wash in tap water was performed before dehydration, clearing and mounting on glass slides with glass coverslips using DPX media.

Protein	Clone Number	Secondary Antibody	Dilution
SMA	1A4 Dako Ref MO 851	Mouse	1:200
Collagen I	Abcam Ref Ab23730	Rabbit	1:800
Collagen III	Abcam Ref Ab7778	Rabbit	1:200

Table 2.1 Primary protein targets, clone numbers, type of secondary antibody and dilution factor used for the immunohistochemical staining techniques.

2.3 Liver Scoring

2.3.1 Standard liver scoring

Liver samples were submitted to a single European Board Certified anatomic pathologist who was blinded to clinical history and horse identification for liver grading. Sections were initially scored using haematoxylin and eosin (H&E) and Perls Prussian Blue (PPB), the latter for haemosiderin detection. Liver samples were initially scored in a similar manner to that described previously (Durham et al., 2003c). Briefly, sections were scored on degree of portal fibrosis, irreversible cytopathology, inflammatory infiltrate, haemosiderin accumulation, and biliary hyperplasia (Table 2.2).

Pathological change	Absent	Mild	Moderate	Severe
Portal Fibrosis	0	0	2	4
Irreversible Cytopathology	0	1	2	2
Inflammatory Infiltrate	0	0	1	2
Haemosiderin Accumulation	0	0	0	2
Biliary Hyperplasia	0	0	2	4

Table 2.2 Schematic for liver scoring system based on observed histopathological changes. For each category of change a score of 0 to 2 (irreversible cytopathology, inflammatory infiltrate, and haemosiderin accumulation) or 0,2 or 4 (fibrosis, biliary hyperplasia) may be allocated. Thus, the minimum cumulative score is 0 while the maximum is 14 (Durham et al., 2003c).

Initial scoring consisted of descriptive terminology from the anatomic pathologist to assess each category of the scoring rubric. The descriptive terms used (none, mild, moderate and marked/severe) were then translated into numerical scores as described by Durham et al. (2003) (Table 2.2). Durham et al. statistically weighted each category of pathological change in order to aid in prognostication, therefore scorings varied between categories. Irreversibly cytopathology and inflammatory infilatrate could be allocated scores of 0 to 2, while haemosiderin accumulation could be scored as either a 0 or 2. Portal fibrosis and biliary hyperplasia were considered to have a greater impact on prognosis and therefore scores of 0, 2 or 4 were allocated dependent on the extent of damage accumulated.

Portal fibrosis was scored in the same manner as that described by Durham. Briefly, only portal fibrosis was considered. Scores were defined as 0 (absent to mild fibrosis, where mild fibrosis was considered to expand the portal tract by twice its normal size), 2 (moderate fibrosis—the portal tract was expanded by 3 times its normal size), or 4 (marked fibrosis—the portal tract was expanded by four times its normal size) (Durham et al., 2003c).

Each type of irreversible cytopathology (amyloidosis, necrosis and megalocytosis) was considered individually with pathology described as none, mild, moderate and marked by the pathologist. A score of 0 was given to the descriptor of none, a score of 1 to minimal and a score of 2 to moderate and marked. If more than one type of

cytopathology was present, extent of the lobule effected was considered when giving a numerical score.

Presence and type of reversible cytopathology was also noted however this did not contribute a score as all reversible changes were scored as 0 in the Durham paper.

Type and location (portal vs centrilobular) of inflammation was noted, though only the severity of portal inflammation was scored as per the Durham algorithm and considered for statistical analysis.

Haemosiderin accumulation was described as minimal/none, moderate, diffuse or marked/severe by the anatomic pathologist. Only haemosiderin accumulation in hepatocytes was considered of pathological significance and thus graded (Durham et al., 2003c). Minimal haemosiderin accumulation was defined as haemosiderin in mainly Kupffer cells, and along with moderate haemosiderin accumulation, equated to a score of 0. Diffuse haemosiderin accumulation was defined as affecting most hepatocytes regardless of intensity and was considered along with marked/severe haemosiderin accumulation to correspond with a score of 2, as more than 50% of hepatocytes were affected in these instances.

2.3.2 Extended liver scoring rubric on haematoxylin and eosin stain

An extended liver scoring/staging was performed. The presence of sinusoidal fibrosis and bridging fibrosis were described as present or absent and were coded as 1 or 0, respectively for statistical analysis. Centrilobular fibrosis was scored subjectively on H&E staining (Table 2.3).

Region of fibrosis	**Severity**			
	Not present	Mild	Moderate	Marked
Centrilobular Fibrosis	0	1	2	4

Table 2.3 The scoring system used for assessing centrilobular fibrosis on H&E staining.

2.3.3 Extended liver scoring rubric on Sirius Red stain

Sections were then reassessed for all forms of fibrosis, i.e. portal, sinusoidal, centrilobular and bridging, and on Sirius Red (SR) staining. Bridging fibrosis was graded on SR staining and given numeric values generated by the pathologist based on criteria they had determined may reflect pathological severity (Table 2.4).

Region of fibrosis	**Severity**			
	Not present	Individual strands extending from portal areas without connection	Majority of portal areas connected by thin bridges	All portal areas connected, often by substantial bridges
Bridging Fibrosis	0	1	2	3

Table 2.4 The scoring system used when assessing bridging fibrosis with SR staining.

Table 2.5 provides a breakdown of numbers of cases included in each portion of this study.

Test type	**Number of cases included**
Liver scoring using current liver scoring method	53
Extended fibrosis scoring	53
Image analysis Sirius Red staining	41
Image analysis Collagen I staining	31
Image analysis Collagen III staining	32
Image analysis SMA staining	31
Image analysis PPB staining	41

Table 2.5 the number of cases included in each histopathological assessment.

2.4 Image analysis

Tissue samples were fixed and stained as described in section 2.2. All sections were batch stained and batch scanned where possible. Slides stained with SR, PPB and for Collagen I (coll I), Collagen III (coll III) and SMA were considered for this portion of the project. Slides were scanned on a Hamamatsu Nanozoomer (Welwyn Garden City, UK) with a 20x lens and 0.75 numerical aperture resulting in a scanning resolution of 0.46 µm/pixel. Images were then transferred to the Leica Digital Hub and analysed using their Tissue Image Analysis module 2.0 (Leica, Milton Keynes, UK). Total tissue area was measured automatically by the software and regions of interest (ROI) were defined manually using a colour pixel thresholding process. This process entailed examination of affected areas of the liver and selecting colour pixel intensity that provided the greatest ROI with the least background inclusion across all stained images using the inbuilt settings on the Leica software. Slides were assessed using a mask that detected the pixel threshold over a small region to assure that the optimal pixel threshold was determined for all slides within a batch stain (Figures 2.1, 2.2). These analysis settings were optimised for each stain, and images were batch analysed for each stain as described by Mohammed et.al (Mohammed et al., 2010). The colour pixel thresholding process was performed by the author under the supervision of an American Boarded anatomic pathologist. Total number of pixels was automatically counted using the Leica software and calculations of "percentage affected area" were performed by dividing ROI area by total tissue area using Microsoft Excel.

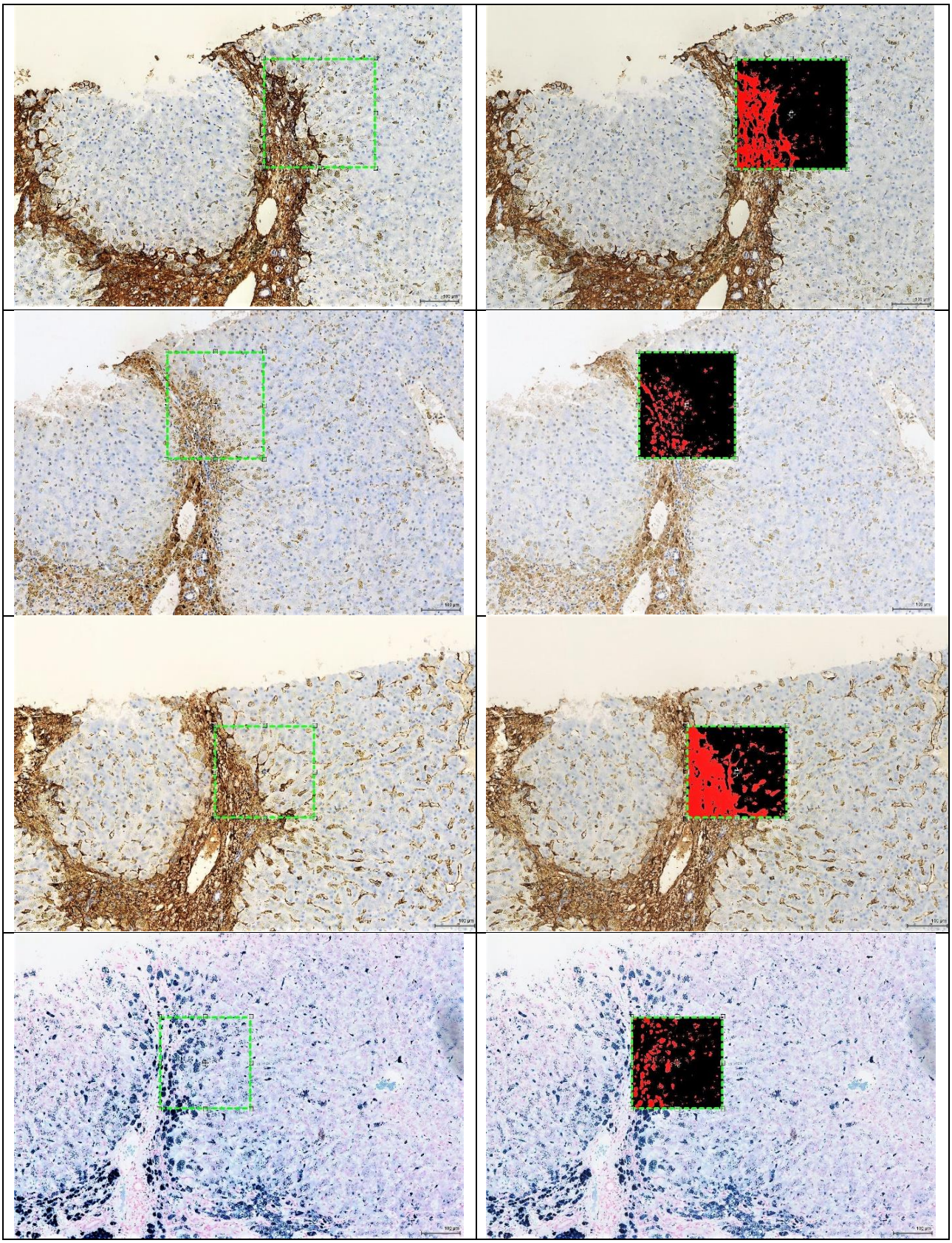

Figure 2.1 Images taken of the same liver sample which has been scanned and the colour pixel threshold used to demarcate a positive area. The top row is stained for collagen I, row 2 is stained for collagen III, row 3 is stained for SMA and row 4 is stained with PPB. The green box in the slides on the left delineates the area of interest where the mask will be applied to assess if colour pixel threshold is appropriate.

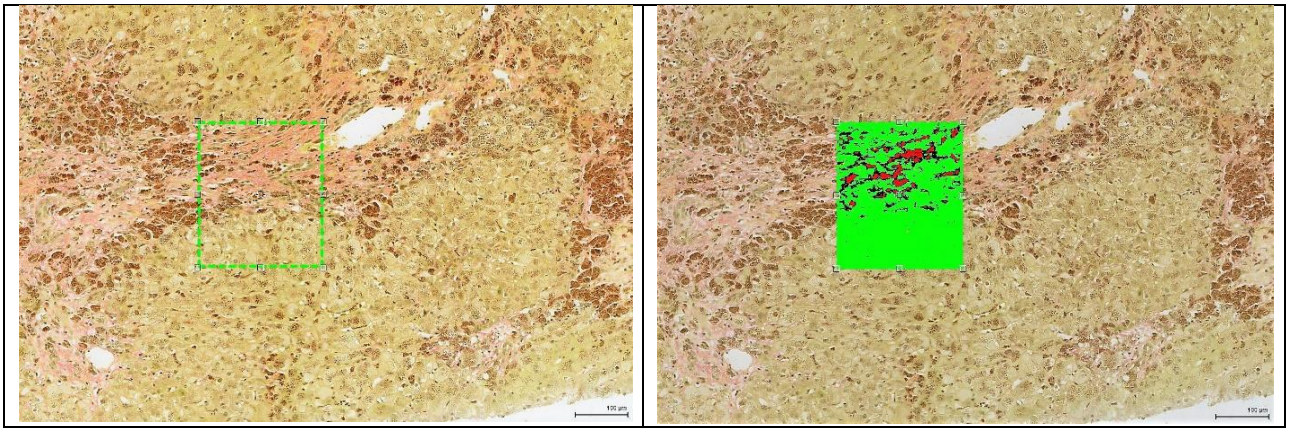

Figure 2.2 Images of one slide taken from slide scanning with and without the application of the colour pixel threshold to a specific area of tissue. Sirius red staining.

2.5 Data management

Data was initially entered into a Microsoft Excel spreadsheet before being uploaded into R (R Core Team (2018)). Tables included in this thesis were generated in Microsoft Word.

2. 6 Missing data

Information collected from liver samples were as follows. Sample type—biopsy or post-mortem material, was recorded, current liver scores and new extended liver scores were recorded as was image analysis data. Furthermore, clinical features including sample origin, sex, breed, age, disease being investigated, and outcome were documented where possible.

Owing to the collection process and availability of information, several of the cases submitted did not have a complete data set, i.e. information described above was not available in all cases, due to incomplete submission information or specific analysis (i.e. image analysis) was not performed. As such, the strength of some of the statistical analyses was affected. All statistical analyses that were performed have the number of observations included to allow the reader to assess the strength of any relationships found within the data.

2.7 Statistical analysis

The statistical methods used in this thesis were similar for both the histopathological as well as the clinicopathological studies. Statistical analysis was carried out using R (R Core Team (2018)). Mean and standard deviation was assessed for age. Breed, sex, country of sample origin, type of disease the horse was being investigated for, and outcome were coded as numerics prior to uploading data to R to allow for statistical analysis. Bar plots, histograms and scatter plots were used to initially look at the descriptive statics for the study cohort and to probe data to look for obvious relationships. Regression analysis was used to look for statistically significant relationships within the data sets. Initially univariable regression analysis was used to

identify statistically significant relationships; all significant relationships (threshold of $p<0.05$) were then considered for inclusion in multivariable models.

Ordered logit estimations were utilised when assessing categorical/ordinal variables, logit estimations were utilised when assessing dichotomous (binary) variables and linear models using maximum likelihood estimates were utilised when assessing (quasi-) continuous variables (Table 2.6 and Table 2.7). Significance was set to a p value of <0.05 and a t value of > 1.96 where ordered logit estimations were used. t-values are the calculated difference represented by standard error, i.e., the greater the t value, the more likely the null hypothesis is false and the greater the chance the results are significant. This is in comparison to a p value which assess the probability of an observation occurring due to random chance. Both p and t values are permutations of the same number.

Outcome	Category
Sinusoidal fibrosis	Absent/present (coded 0 or 1)
Bridging fibrosis (H&E)	Absent/present (coded 0 or 1)
Haemosiderosis	Mild/moderate (coded 0) or marked (coded 2)
Death due to extra-hepatic disease	Absent/present (coded 0 or 1)
Death due to liver disease	Absent/present (coded 0 or 1)
Survival without repeat clinical signs	Absent/present (coded 0 or 1)
Clinically diagnosed liver disease	Absent/present (coded 0 or 1)
Total liver score	0-14 (whole numbers only)
Portal fibrosis score	0, 2, 4
Centrilobular fibrosis score	0-2 (whole numbers only)
Bridging fibrosis (SR)	0-3 (whole number only)
Irreversible cytopathology	0-2 (whole numbers only)
Portal inflammation	0-2 (whole numbers only)
Bile duct proliferation	0, 2, 4
Percentage area affected—Sirius Red image analysis	0.12-29.2%
Percentage area affected—Collagen I image analysis	0.005-7.43%
Percentage area affected—Collagen III image analysis	0.24-12.6%
Percentage area affected—Smooth muscle actin image analysis	0.004-33.1%
Percentage area affected—Perls Prussian Blue image analysis	0.0009-25.5%

Table 2.6 Outcomes and their categories expressed as minimum and maximum values for quasi continuous outcomes used in regression analysis.

Explanatory variable	Category
Percentage area affected—Sirius Red image analysis	0.12-29.2% (continuous variable)
Percentage area affected—Collagen I image analysis	0.005-7.43% (continuous variable)
Percentage area affected—Collagen III image analysis	0.24-12.6% (continuous variable)
Percentage area affected—Smooth muscle actin image analysis	0.004-33.1% (continuous variable)
Percentage area affected—Perls Prussian Blue image analysis	0.0009-25.5% (continuous variable)
Biopsy/post-mortem section	Biopsy= 0, post-mortem section= 1
Sinusoidal fibrosis	Absent/present (coded 0 or 1)
Bridging fibrosis (H&E)	Absent/present (coded 0 or 1)
Haemosiderosis	Mild/moderate (coded 0) or marked (coded 2)
Total liver score	0-14 (whole numbers only)
Portal fibrosis score	0, 2, 4
Centrilobular fibrosis score	0-2 (whole numbers only)
Bridging fibrosis (SR)	0-3 (whole number only)
Irreversible cytopathology	0-2 (whole numbers only)
Portal inflammation	0-2 (whole numbers only)
Bile duct proliferation	0, 2, 4
Death due to extra-hepatic disease	Absent/present (coded 0 or 1)
Death due to liver disease	Absent/present (coded 0 or 1)
Survival without repeat clinical signs	Absent/present (coded 0 or 1)
Sex	Mare, gelding
Age	Foal (<1 year), young horse (1 -5 years), middle aged horse (6 -14 years), old horse (>15 years)
Sample origin	English, Scottish, Irish
Breed	Thoroughbred, Shetland, Highland

Table 2.7 The explanatory variables used in the regression analysis. As only three stallions were present in the study, they were made the referent for the sex. Ages were coded as foal, young horse, middle aged, and old horse, and included the age ranges listed. Breeds were examined by the three most common breeds present in the study. Each category for sex, age, breed, and sample origin was included as an individual variable in univariable or multivariable analyses.

Breed, sex, clinical outcome, country of sample origin and age were coded (Table 2.8) for statistical purposes.

Variable	Categories	Referent
Breed	Thoroughbred, Highland, Shetland	Other breeds
Sex	Mare, Gelding	Stallion
Outcome	Death due to hepatic disease, death due to non-hepatic disease, survival with no repeat clinical signs	Survival with repeat clinical signs
Country of sample origin	England, Ireland, Scotland	Yes/no for each category; Ireland is referent for multivariable regressions
Age	Foal(<1 year), young horse (1 year <= x<=5 years), middle aged (6 years<= x<= 14 years), old horse (>= 15 years)	Yes/no for each category; foal is referent in multivariable regressions
Tissue sample type	Biopsy	Post-mortem section
Hepatocellular haemosiderin deposition	Score of 2 (marked)	Score of 0 (mild or moderate)
Disease	Liver disease	Other disease

Table 2.8 presents all variables that were represented by dummy/indicator variables in regression analyses. Dummy/indicator variables for breeds were chosen based on frequency of breed inclusion within the study.

All regression analyses were run on the entire cohort and the subset of hepatic cases where appropriate.

When relationships were found using univariable analysis, multivariable regressions (linear models using maximum likelihood estimates) were performed to assess impact of data collection methodology on the relationships where appropriate. Models were initially built using all statistically significant explanatory variables across all data sets. Owing to missing data, the number of observations was found to be too low (n=12) to be considered statistically significant. Instead, smaller multivariable models were built looking at the impact of age, breed, sex and sample origin on statistically significant variables ($p<0.05$). Multivariable analysis was also carried out using entire haematological and biochemical profiles as explanatory variables and presence of liver disease as the outcome.

2.8 Ethical approval

Research was conducted with approval from the MVLS ethics committee at Glasgow University. Biopsy samples were collected for clinical monitoring or diagnostic purposes. Post mortem samples were collected from animals that were euthanased on humane grounds. Blood samples used in this project came from excess blood taken for either clinical purposes or within 2 minutes of death in horses that were euthanased. All tissue samples were utilised with owner's consent.

Chapter 3 Histopathological assessment of equine hepatic tissue

3.1 Introduction

3.1.1 Equine liver scoring: current technique

In an attempt to standardise the assessment of liver pathology and offer a degree of prognostication, Durham et. al (Durham et al., 2003c) devised a grading system for equine liver biopsies. This system grades histological lesions on the degree of portal fibrosis, irreversible cytopathology, haemosiderosis, inflammatory infiltrate in the portal area and bile duct proliferation (biliary hyperplasia). Scores were generated using statistical analysis of specific pathological change and their ability to predict non-survival past 6 months. Total liver scores ranged from 0 to 14. Horses with total liver scores between 2 and 6 were 12 times less likely to survive beyond 6 months when compared to horses with a liver score of 0. Horses with scores between 7 and 14 were 50 times less likely to survive past 6 months. Fibrosis appeared to be the most useful prognostic indicator of survival time, with periportal and bridging fibrosis being particularly significant as indices of poor prognosis. Biliary hyperplasia was also found to have strong prognostic value.

The scoring system was designed to grade diffuse hepatic pathology and is inappropriate for use with focal lesions and neoplastic disease. Scores generated from biopsy material often correlated well with corresponding post-mortem material, suggesting that diffuse hepatic disease is well represented by biopsy material (Durham et al., 2003c). Animals with moderate fibrosis, severe haemosiderosis or severe biliary hyperplasia were likely to have ultrasonographic abnormalities detected (Durham et al., 2003a), however, in 61 cases of confirmed equine hepatic disease, only 17 horses had ultrasonographical abnormalities suggesting that the absence of ultrasonographic lesions does not exclude a diagnosis of hepatic disease (Durham et al., 2003b).

3.1.2 Aims of the chapter

The aim of this chapter is to evaluate the current equine liver grading algorithm and a proposed extended equine liver grading algorithm on a cohort of horses with both hepatic and extra-hepatic disease. As both post-mortem and biopsy material was

used for grading, the impact of tissue sample type on liver grading parameters (both current and extended) was evaluated.

3.2 Results

3.2.1 Cohort details

Fifty-three samples were included in this study, each from an individual horse. Ages were known for forty horses and ranged from 6 weeks to 27 years old. The mean age was 11.6 years old, and the median age was 11 years. Twenty-five mares/fillies, nineteen geldings, and three stallions/colts were included. Six horses were of unknown sex. The breed was known for forty-four of the included individuals. The most common breeds included were Thoroughbreds (n=9), Shetlands (n=6) and Highlands (n=4).

The clinical premortem diagnosis was known for forty-eight of the fifty-three individuals included. There were twenty-six hepatic cases (49%), and twenty-two extra-hepatic cases (41.5%), and five cases had an unknown clinical history (9.5%).

Clinical outcome was known for thirty-four of the included individuals. Twenty-three (69.7%) died due to extra-hepatic disease, while five horses died due to hepatic disease (12.1%). The remaining six horses were known to have survived (18.1%) at least six months after initial onset of clinical disease; five of these horses were being investigated for hepatic disease while one had multisystemic disease (initial septic bicipital bursitis which resolved, and presented several months later with a pyrexia of unknown origin).Two of the horses that had hepatic disease had died due to extra-hepatic disease at the time of data collection (both of these horses died over one year after presenting with liver disease), one was found dead in the field after an episode of colic while the other had spinal ataxia. Two of the survivors of hepatic disease were found to have iron toxicity and responded well to treatment and one survivor of hepatic disease had a history of exposure to ragwort. The aetiology of hepatic disease was never determined for the remaining two survivors of hepatic disease.

A full list of clinical features (age, breed, sex, sample origin, clinical diagnosis, outcome, follow-up time) of included cases can be found in Appendix 1.

3.2.2 Liver scoring results

3.2.2.1 Standard liver scoring descriptive results

Total liver scores for the cohort ranged from 1-13. The distribution of total liver scores is provided in Figure 3.1. The means and standard deviations for all graded parameters are shown in Table 3.1.

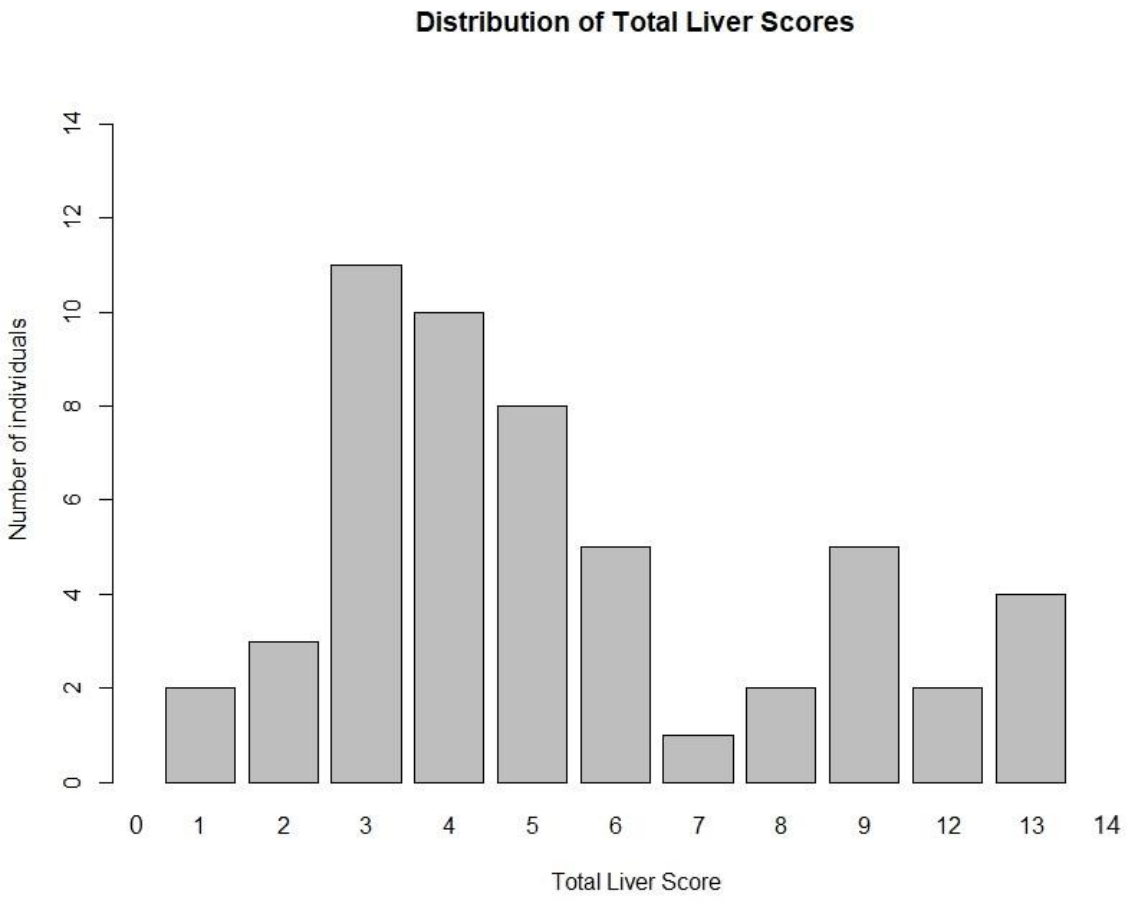

Figure 3.1 Distribution of total liver scores for the fifty-three samples assessed

	Mean	Standard Deviation
Current Liver Scoring		
Total liver score	5.566	3.241
Irreversible cytopathology	0.660	0.581
Inflammatory infiltrate	1.019	0.693
Haemosiderin accumulation	0.604	0.927
Biliary hyperplasia	2.189	1.374
Portal fibrosis	1.094	1.596
Extended Liver Scoring		
Sinusoidal fibrosis	0.774	0.423
Centrilobular fibrosis	0.714	1.099
Bridging fibrosis (H&E)	0.660	0.478
Bridging fibrosis (SR)	1.615	0.844

Table 3.1 Summary statistics for liver scoring of horses included in the study

Within the study cohort, fourteen samples had scores between 7 and 14 (26.4%) and thirty-nine (73.6%) samples had scores between 1 and 6. No samples were scored 0 or 14. The majority of horses that had total liver scores of 7 and above were being investigated for hepatic disease (n=8), however, individuals being investigated for dental disease, multisystemic disease, musculoskeletal disease, and ocular disease (n=1 for each disease category) were also found to have total liver scores of 7 and above (Figure 3.2). The reason for investigation of two horses with hepatic scores of 13 and 9 was unknown. Horses being investigated for hepatic disease had a mean total liver score of 6.2 (standard deviation = 3.3) and horses with extra-hepatic disease had a mean total liver score of 4.6 (standard deviation =2.7).

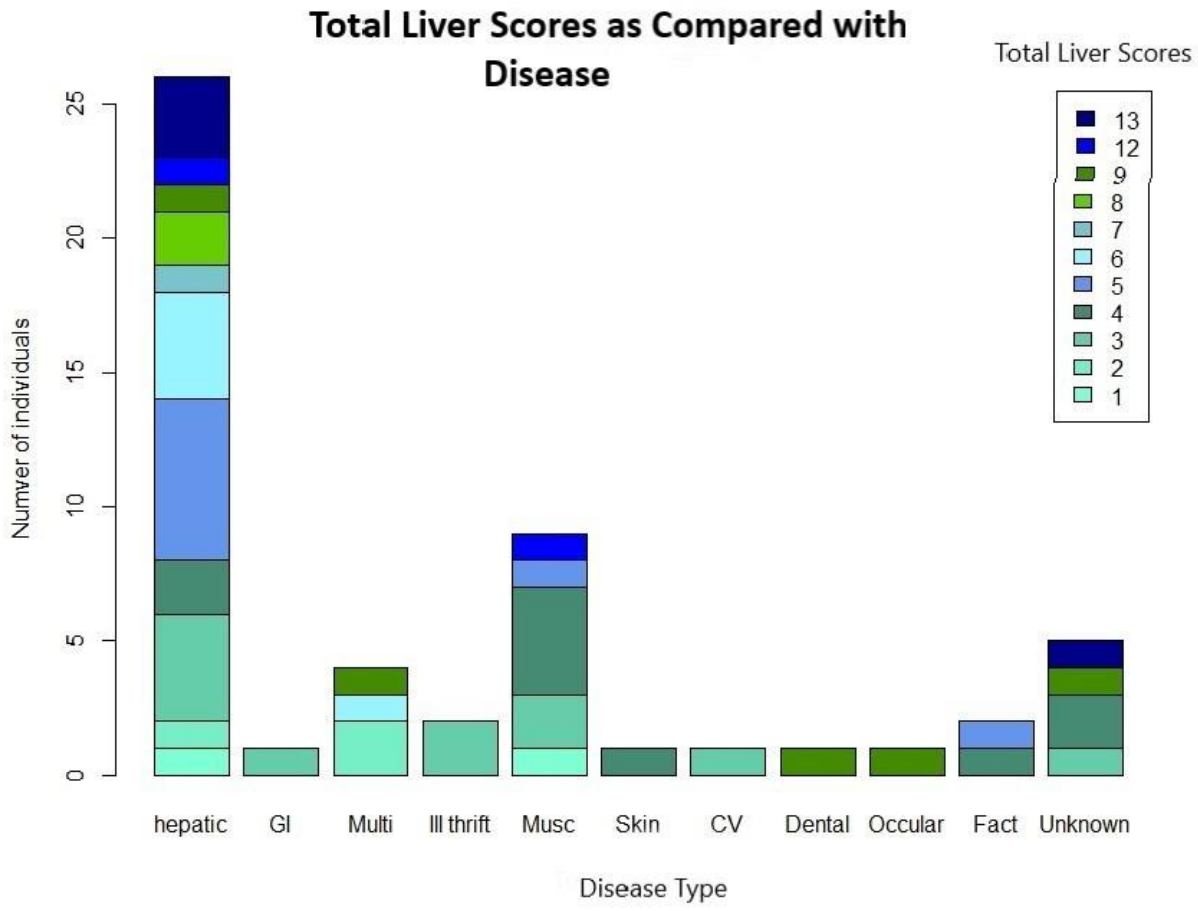

Figure 3.2 Distribution of total liver scores within each disease category. GI= gastrointestinal disease/colic, Multi= multisystemic disease, Musc= musculoskeletal disease (i.e. fractures, arthritis, etc.), CV= cardiovascular disease. Fact= horse meat factory and indicates those horses who went for meat production.

Portal fibrosis is considered to have the greatest prognostic capability of the features graded (Durham et al., 2003c). Of the fifty-three graded samples, ten samples had a portal fibrosis score of 4 (corresponding to severe portal fibrosis) (Table 3.2). Of the twenty-six individuals being investigated for hepatic disease, six had a portal fibrosis score of 4. One horse with musculoskeletal disease and one horse with multisystemic disease also had a portal fibrosis score of 4 (Figure 3.3). The reason for clinical investigation of the remaining two horses who had a portal fibrosis score of 4 was unknown.

FPortal fibrosis score	0	2	4
Number of tissue samples	34	9	10
Inflammatory infiltrate score	0	1	2
Number to tissue samples	12	28	13
Haemosiderin accumulation score	0	2	
Number of tissue samples	37	16	
Biliary hyperplasia score	0	2	4
Number of tissue samples	10	28	15
Irreversible cytopathology score	0	1	2
Number of tissue samples	21	29	3

Table 3.2 The number of horses with each score in the standard liver scoring rubric.

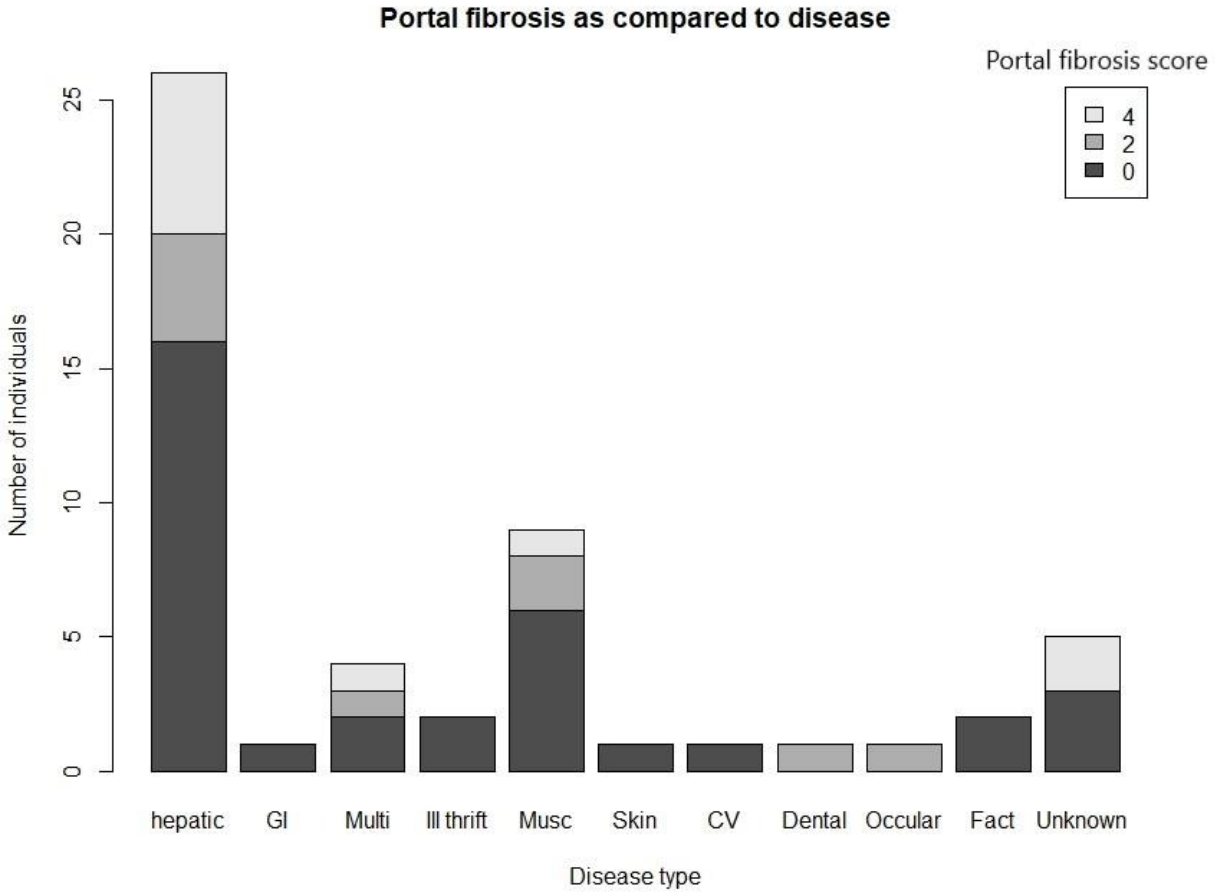

Figure 3.3 Distribution of portal fibrosis scores across the different disease categories. GI= gastrointestinal disease/colic, Multi= multisystemic disease, Musc= musculoskeletal disease (i.e. fractures, arthritis, etc.), CV= cardiovascular disease. Fact= factory which indicates those horses who went for meat production.

Samples with total liver scores of 7 and above were found to have portal fibrosis scores of 2 or 4 (moderate to severe fibrosis). While the majority of horses with total liver scores of 1-6 (n=39) had a portal fibrosis score of 0, four individuals with a portal fibrosis score of 2 (10.3%) and one with a portal fibrosis score of 4 (2.6%) were found within this group.

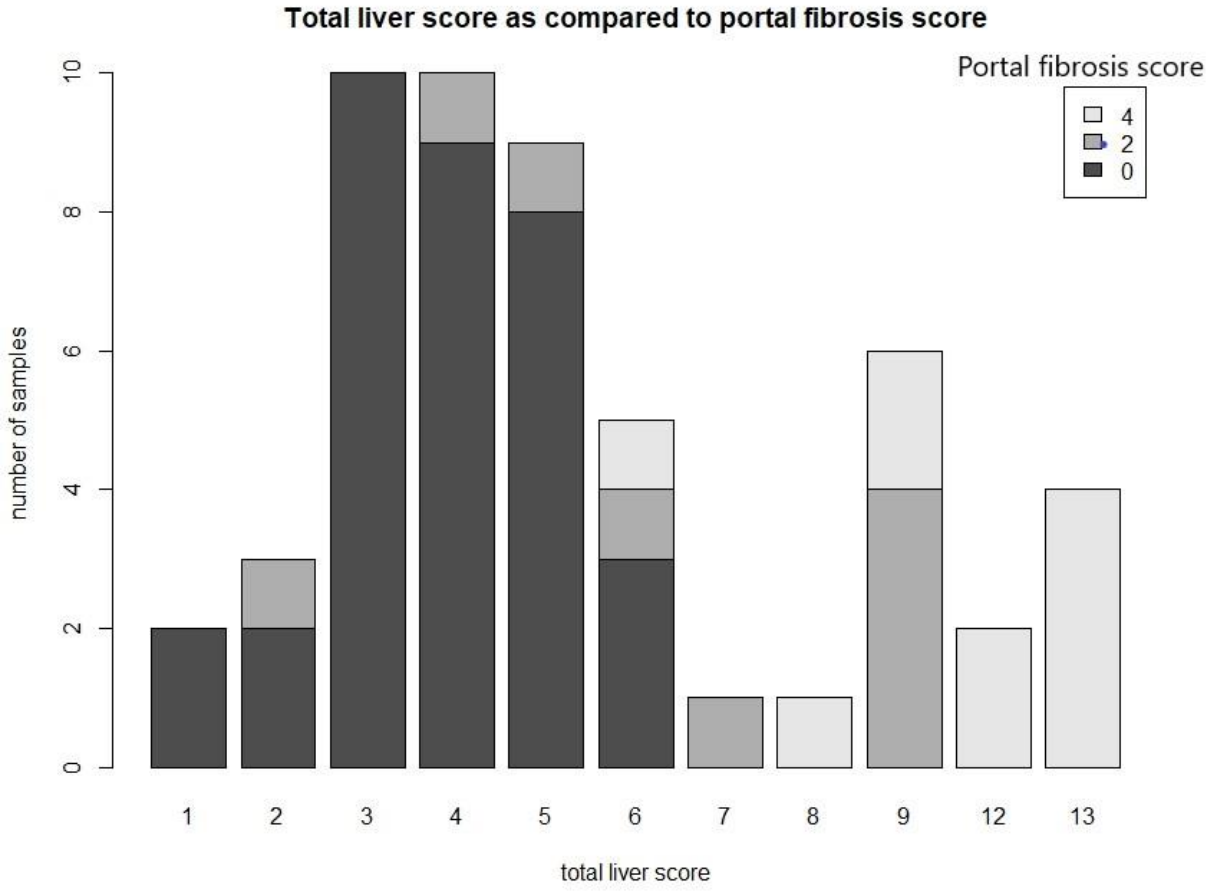

Figure 3.4 The distribution of portal fibrosis scores across total liver scores.

Contrary to expectation, the highest total liver scores were associated with either survival from hepatic disease or with death due to extra-hepatic disease. Of the fourteen horses with a total liver score greater than 7, only 2 died due to hepatic disease (14.3%), while four survived (28.6%) and four died due to extra-hepatic disease (28.6%). The other four horses in this group had an unknown outcome. Interestingly, two horses died due to hepatic disease that had a total liver score less than 7 (Figure 3.5).

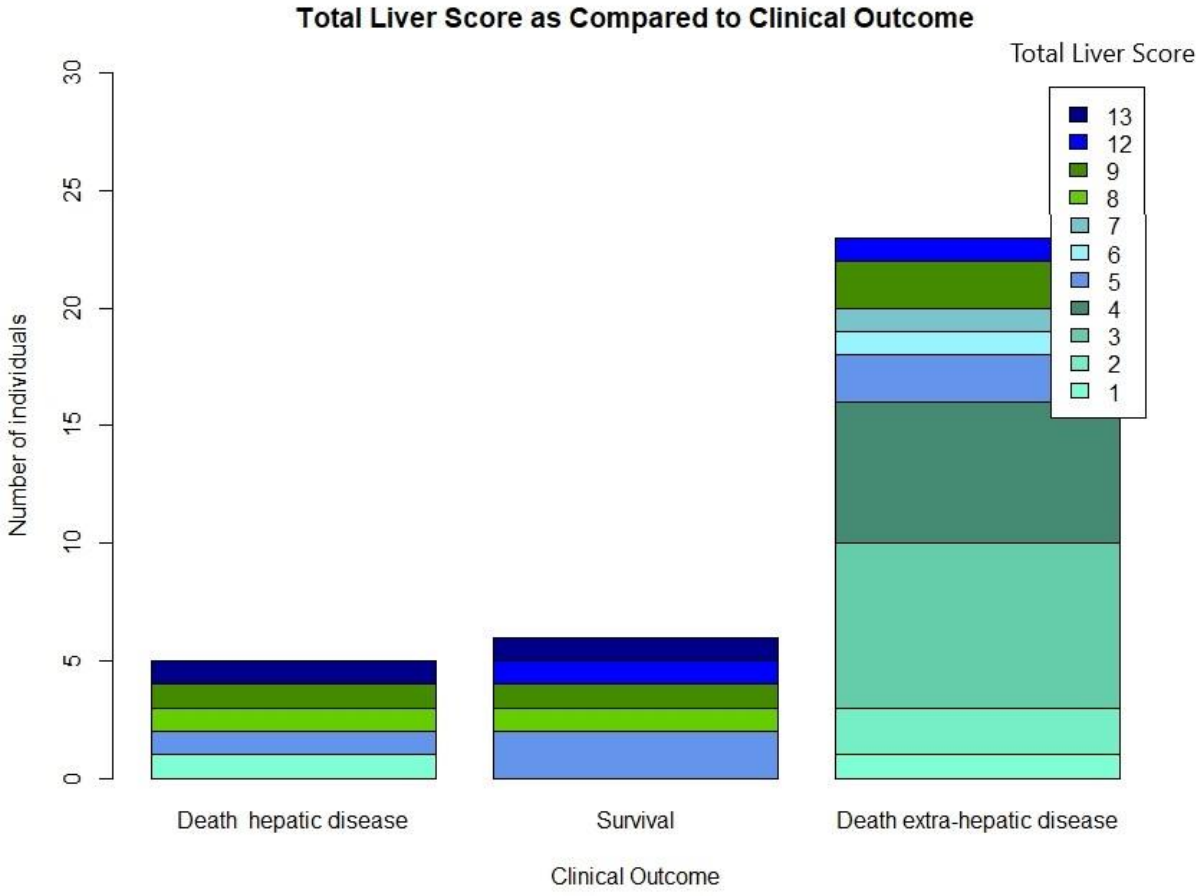

Figure 3.5 depicts total liver score as compared to the clinical outcome in cases where this was known. In this case survival equates to "survival without repeat clinical signs" (Chapter 2 Section 2.1.3) as no horses within this cohort had repeat clinical signs from their initial clinical investigations.

Distributions of all other liver scoring parameters can be found in Table 3.2. As irreversibly cytopathology can be caused by three specific changes (amyloidosis, megalocytosis and necrosis) which may reflect aetiology, it is of note that only one sample in the study was found to have amyloidosis (confirmed on Congo Red staining), which was given a score of 2. Two horses were found to have megalocytosis with a score of 2 and no horses were scored 2 for necrosis. Three tissue samples were found to have both mild megalocytosis and mild necrosis; all other tissue samples showed either one type or no signs of irreversible cytopathology. The three samples with two types of irreversible cytopathology were given an overall irreversible cytopathology

score of 1 as the total amount of irreversible cytopathology present did not affect 25% of the lobule (Durham et al., 2003c)

When considering the breakdown of hepatic and extrahepatic cases for the highest scores within each parameter, horses with a clinical diagnosis of hepatic disease made up a greater percentage of the highest scores. However, when looking at moderate levels of damage, horses with extrahepatic disease often made up the greater percentage of cases (Table 3.3). This finding may reflect the effects of some extrahepatic disease on the liver (i.e. gastrointestinal disease or cardiac disease), or may reflect subclinical hepatic disease.

		Score 2	**Score 4**
Portal Fibrosis			
	Hepatic cases	4	6
	Extra-hepatic cases	5	2
	Unknown case history	0	2
Haemosiderin accumulation			**Score 2**
	Hepatic cases		10
	Extra-hepatic cases		5
	Unknown case history		1
Inflammatory infiltrate		**Score 1**	**Score 2**
	Hepatic cases	12	8
	Extra-hepatic cases	13	3
	Unknown case history	3	2
Biliary hyperplasia		**Score 2**	**Score 4**
	Hepatic cases	9	10
	Extra-hepatic cases	15	4
	Unknown case history	4	1
Irreversible Cytopathology		**Score 1**	**Score2**
	Hepatic cases	15	3
	Extra-hepatic cases	10	0
	Unknown case history	4	0

Table 3.3 provides the distribution of hepatic and extrahepatic cases within the highest scoring brackets for each liver scoring parameter.

3.2.2.2 Standard liver scoring statistical results

Cohort characteristics were assessed with regards to their relationship to clinical diagnosis of hepatic disease and outcome. Horses who died of extrahepatic disease were less likely to be diagnosed with hepatic disease ($p<0.001$) while those that survived a disease process (hepatic or extrahepatic) for longer than six months were found to be significantly associated with a clinical diagnosis of hepatic disease ($p=0.02$). Middle aged horses (6-14 years of age) were found to be significantly associated with hepatic disease ($p=0.04$). Interestingly, despite being the most common breed within the dataset, Thoroughbreds were less likely to have a clinical diagnosis of hepatic disease ($p= 0.01$) (Table3.4).

Outcome	Explanatory Variables	p Value	Coefficient Estimate	Odds Ratio	95% Confidence Interval	Standard Error	Number of Observations
Clinical diagnosis of hepatic disease	Death due to non-hepatic disease	<0.001	-4.5949	0.01	-7.07 to -2.03	1.288	33
Clinical diagnosis of hepatic disease	Survival without repeat clinical signs	0.02	2.8622	17.5	0.53 to 5.19	1.1892	33
Clinical diagnosis of hepatic disease	Middle aged horses	0.04	1.4351	4.20	0.08 to 2.79	0.6926	39
Clinical diagnosis of hepatic disease	Thoroughbred	0.014	-2.7726	0.06	-4.97 to -0.57	1.1231	42
Death due to non-hepatic disease	Portal fibrosis score 4	0.015	-2.9957	0.05	-4.1 to -1.9	0.5590	34
Survival without repeat clinical signs	Portal fibrosis score 4	0.047	2.1972	9	0.03 to 4.36	1.1055	34

Table 3.4 The significant univariable regressions in assessing impact of liver scoring parameters on clinical outcome. The p value, coefficient estimate, odds ratio, 95% confidence interval, standard error and number of observations included in the regression are provided.

None of the standard liver scoring parameters were found to be significant with regards to predicting clinical hepatic disease (48 observations for each bivariate regression), i.e. no specific parameter nor the total liver score was significantly associated with a diagnosis of clinical hepatic disease.

Five of the 10 horses with a portal fibrosis score of 4 had a known clinical outcome (Figure 3.6). Only one of these horses died due to hepatic disease, while three were alive without repeat clinical signs of liver disease when data collection concluded (2017), and one horse who died due to extra-hepatic disease was found to have a

portal fibrosis score of 4. Conversely, two horses with portal fibrosis scores of 0 died due to hepatic disease.

When assessing predictors of clinical outcome using univariable regression analysis, having a portal fibrosis score of 4 was significantly associated with a reduced risk of death due to extra-hepatic disease and horses (p= 0.002) with this score were borderline significantly more likely to be alive without repeat clinical signs (p=0.05) (Table 3.4). In other words, the highest score for portal fibrosis was not significantly associated with death due to hepatic disease, and instead was significantly associated with survival in this study (odds ratio 9).

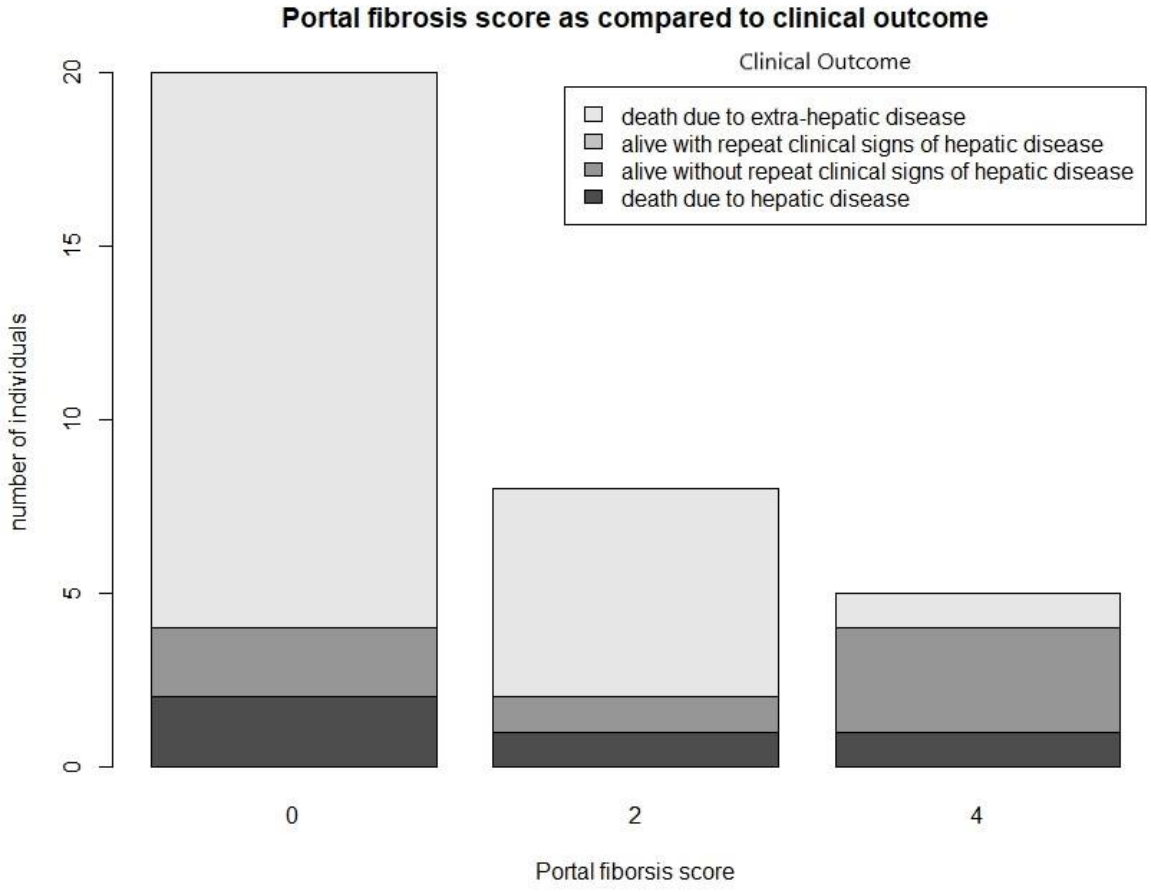

Figure 3.6 The distribution of portal fibrosis scores as compared to known clinical outcomes.

No other significant relationships between clinical outcome and liver grading parameters were found using univariable regression analysis.

Significant univariable ($p<0.05$) regressions were used to inform multivariable regression analysis (modelling) in an attempt to increase statistical robustness by controlling for confounders. When modelled with age, breed and sample origin as controls, a portal fibrosis score of 4 remained significantly associated with survival without repeat clinical signs. Being a Shetland pony was found to be significantly associated with survival. None of the explanatory variables included in the multivariable analysis for death due to extra-hepatic disease were found to be significant. With regards to the model of hepatic disease based on clinical outcomes, age, breed and sample origin, extrahepatic death was still significantly unlikely to be associated with hepatic disease, however, survival was no longer significant (Table 3.5).

Outcome	Explanatory Variables	p Value	Coefficient estimate	Odds ratio	95% Confidence Interval	Standard Error	Number of Observations
Death due to non-hepatic disease	Portal fibrosis score 0	-	-	-		-	-
	Portal fibrosis score 2	0.5	-0.1821	0.83	-0.76 to 0.39	0.2934	25
	Portal fibrosis score 4	0.07	-0.5071	0.60	-1.02 to 0.006	0.2616	25
	Foal	-	-	-	-	-	-
	Young horse	0.7	-0.1821	0.83	-1.12 to 0.76	0.4801	25
	Middle aged horse	0.85	-0.1071	0.90	-1.19 to 0.98	0.5518	25
	Old horse	0.63	0.2571	1.29	-0.78 to 1.30	0.5301	25
	Highland	-	-	-	-	-	-
	TB	0.84	0.0750	1.08	-0.65 to 0.80	0.3695	25
	Shetland	0.48	-0.2071	0.81	-0.76 to 0.35	0.2843	25
Survival without repeat clinical signs	Portal Fibrosis score 0	-	-	-	-	-	-
	Portal fibrosis score 2	0.094	0.43673	1.55	-0.04 to 0.92	0.24434	25
	Portal fibrosis score 4	0.014*	0.60816	1.84	0.18 to 1.04	0.21787	25
	Foal	-	-	-	-	-	-
	Young horse	0.29	0.43673	1.55	-0.35 to 1.22	0.39984	25
	Middle age	0.97	0.01531	1.02	-0.89 to 0.92	0.45954	25
	Old horse	0.75	-0.14388	0.87	-1.01 to 0.72	0.44143	25
	Highland	-	-	-	-	-	-
	TB	0.36	0.29286	1.34	-0.31 to 0.90	0.30767	25
	Shetland	0.023*	0.60102	1.83	0.14 to 1.07	0.23676	25
Clinical diagnosis of hepatic disease	Death due to hepatic disease	-	-	-	-	-	-
	Death due to extrahepatic disease	0.0102*	-1.004	0.37	-1.68 to -0.33	0.3422	25
	Survival without repeat clinical signs	0.4795	-0.2674	0.765	-0.99 to 0.46	0.3687	25
	Foal	-	-	-	-	-	-
	Young horse	1.0	4.080×10^{-16}	1	-0.68 to 0.68	0.3462	25
	Middle aged horse	0.7923	-0.1258	0.88	-1.05 to 0.79	0.4695	25
	Old horse	0.7266	0.1663	1.18	-0.75 to 1.08	0.4667	25
	Highland	-	-	-	-	-	-
	Thoroughbred	0.6555	-0.1416	0.87	-0.75 to 0.47	0.3110	25
	Shetland	0.8130	6.292×10^{-2}	1.07	-0.45 to 0.58	0.2613	25

Table 3.5 Multivariable regressions for prediction of clinical outcome with explanatory variables of that outcome, p value, coefficient estimate and standard error provided. * indicates a significant relationship.

Liver scoring parameters in a subset of horses with known hepatic disease were assessed using univariable regression analysis. No relationships between liver grading parameters and any clinical outcome were found within this group.

3.2.2.3 Extended liver scoring descriptive results

The distribution of sinusoidal and bridging fibrosis presence as measured on H&E staining can be found in Table 3.6.

	Sinusoidal fibrosis score	
	Absent	Present
Number of tissue samples	12	41
	Bridging fibrosis score (H&E)	
	Absent	Present
Number of tissue samples	18	35

Table 3.6. Tissue samples with the presence of bridging and sinusoidal fibrosis

All horses with a portal fibrosis score of 4 were found to have bridging and sinusoidal fibrosis present (Figures 3.7 and 3.8). However, twenty-five horses were found to have sinusoidal fibrosis without portal fibrosis being present, and twenty-two horses were found to have bridging fibrosis on H&E staining without portal fibrosis being present.

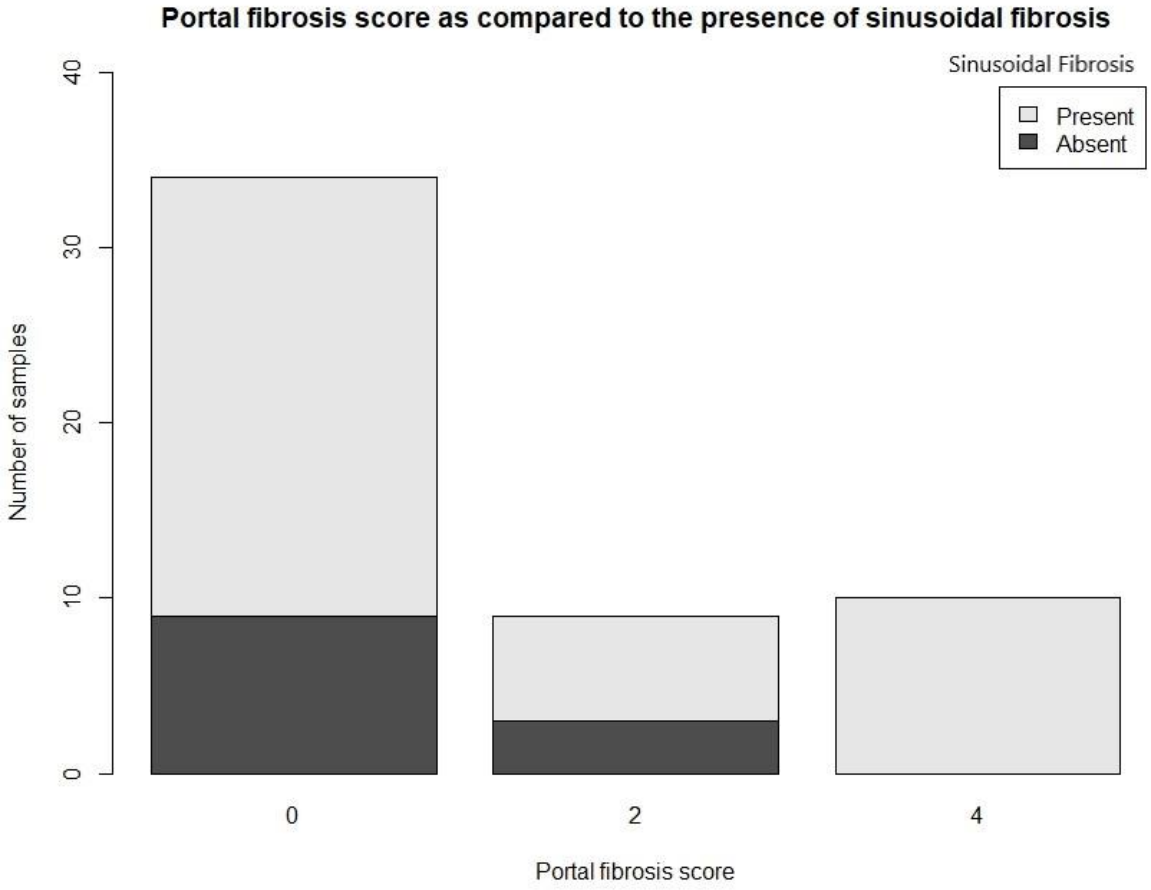

Figure 3.7. The number of tissue samples with and without the presence of sinusoidal fibrosis and their portal fibrosis scores.

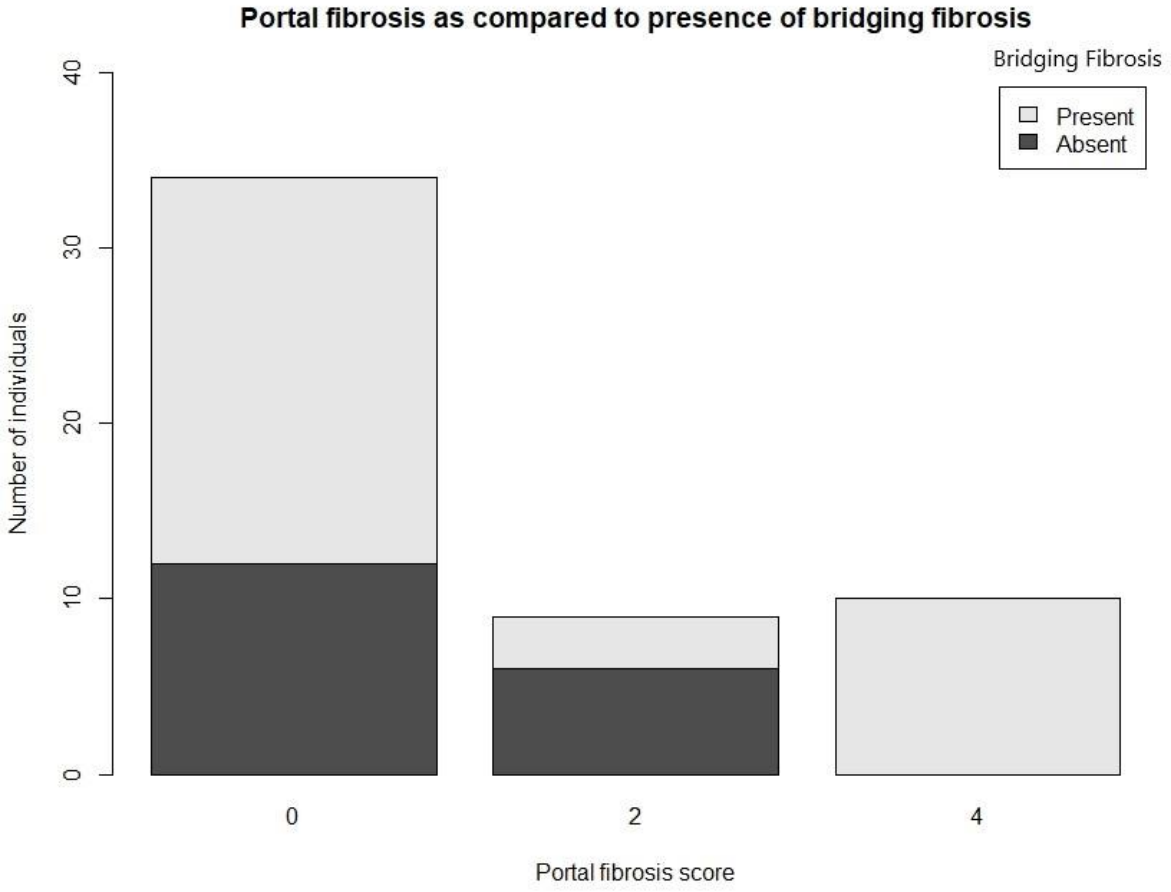

Figure 3.8. The number of tissue samples with and without bridging fibrosis (as seen on H&E) and their portal fibrosis scores.

Interestingly, centrilobular fibrosis was present in thirteen horses which did not have portal fibrosis. One of these horses had a centrilobular fibrosis score of 4. Eight horses with portal fibrosis scores of 2 or 4 did not have centrilobular fibrosis present (Figure. 3.9). Centrilobular fibrosis was not able to be determined for four tissue samples. Table 3.7 provides the distribution of centrilobular fibrosis scores.

Centrilobular fibrosis score	**0**	**1**	**2**	**4**
Number of tissue samples	29	11	6	3

Table 3.7 Number of tissue samples within each centrilobular fibrosis score

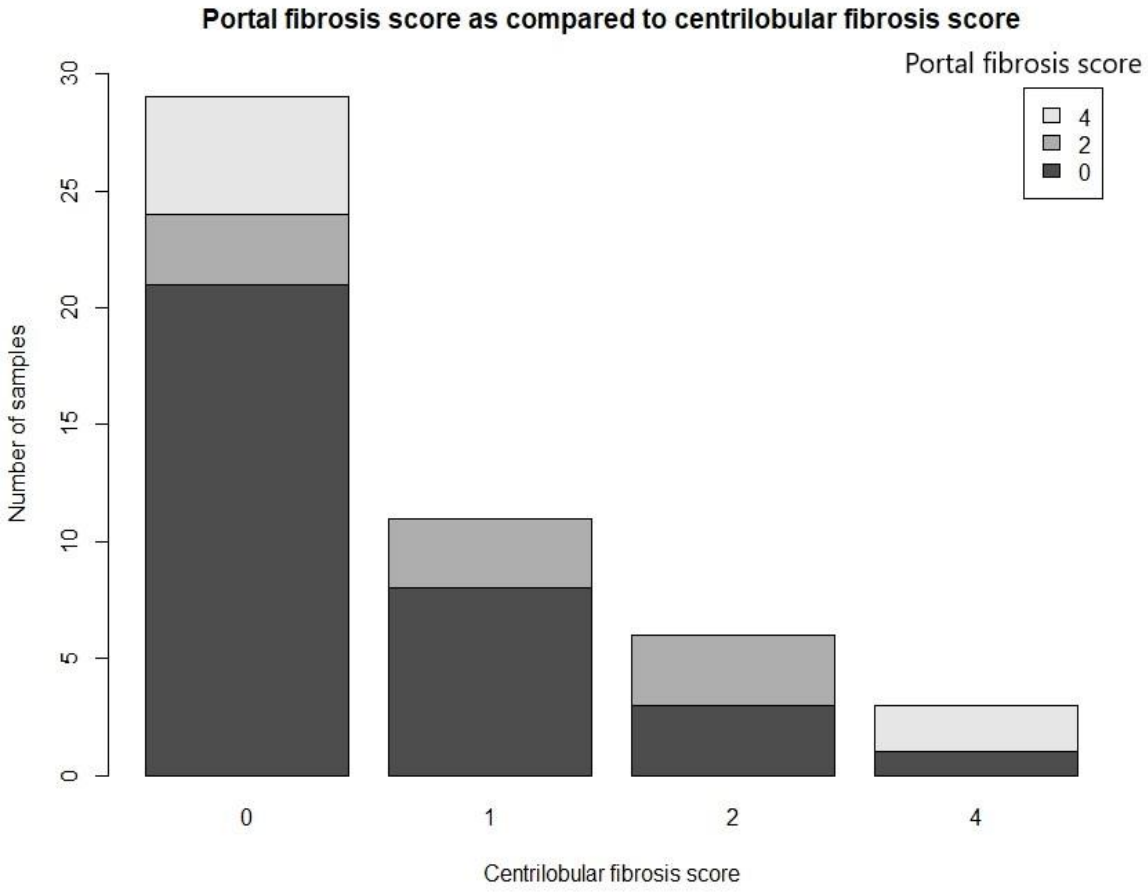

Figure 3.9 Distribution of portal fibrosis score compared with centrilobular fibrosis scores.

Table.3.8 provides the distribution of bridging fibrosis scores as seen with Sirius Red staining. Bridging fibrosis was not able to be assessed on one sample.

Bridging fibrosis score (SR)	**0**	**1**	**2**	**3**
Number of tissue samples	2	26	14	10

Table 3.8 Tissue samples within each bridging fibrosis (SR) score

Bridging fibrosis was seen on H&E with one sample that was not noted on SR staining. Conversely, bridging was not noted on sixteen H&E stained samples, where it was noted on SR staining. However, of these sixteen cases, fifteen of them had a bridging fibrosis score of 1 and only one had a bridging fibrosis score of 2, suggesting that only mild bridging was not detectable on H&E (Figure 3.10).

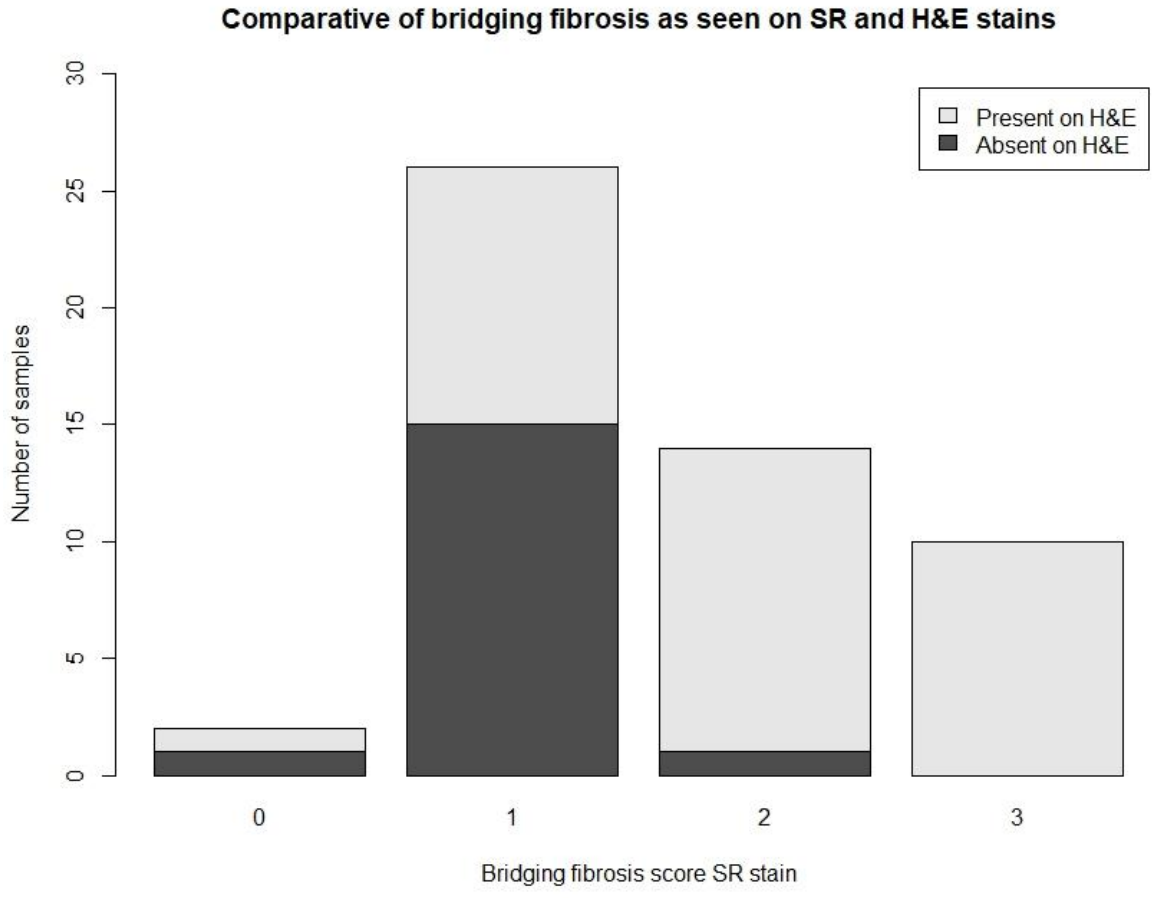

Figure 3.10 Bridging fibrosis as assessed on H&E and SR stains.

3.2.2.4 Extended liver scoring statistical results

Using univariable regression analysis, a centrilobular fibrosis score of 1 was found to be significantly associated with a reduced risk of hepatic disease, i.e. it was less likely to be associated with hepatic disease (Table 3.9). None of the extended parameters of liver grading were found to be significant with regards to predicting clinical outcome. No significant relationships between extended liver grading and clinical outcome were found within the cohort of horses with known hepatic disease.

Outcome	Explanatory Variables	p Value	Coefficient Estimate	Odds Ratio	95% Confidence Interval	Standard Error	Observations
Liver disease	Centrilobular fibrosis score 1	0.01	-2.2	0.11	-2.90 to -1.46	0.88	44

Table 3.9 Significant findings of ordered logit estimations of extended liver grading and clinical outcome. The p values, coefficient estimates, odds ratio, 95% confidence interval, standard error and number of observations included in the regression are provided.

A model of the predictors of hepatic disease was attempted using multivariable regression analysis. Initially, all significant explanatory variables across both the histology and biochemical/haematological portions of the project were included in the model- centrilobular fibrosis, age, breed, sample origin, GGT value, death due to extra-hepatic disease and survival. Owing to incomplete data sets, only 12 horses were included in this regression. Therefore, the model was considered too underpowered to provide credible results.

When GGT values, death due to extra-hepatic disease and survival were removed from the model, the number of observations included in the regression increased which increased confidence in the results. Centrilobular fibrosis was no longer found to be a negative predictor of hepatic disease which aligns with expectation.

Outcome	Explanatory Variables	P Value	Coefficient Estimate	Standard Error	Observations
Liver disease	Centrilobular fibrosis score 1	0.6	-.011301	0.23543	33
	Centrilobular fibrosis score 2	0.8	-0.10274	0.52752	
	Centrilobular fibrosis score 4	0.3	0.57008	0.51959	
	Foal	0.9	-0.05650	0.49208	
	Young horse	0.88388	-0.04624	0.31277	
	Middle aged	0.73950	0.05580	0.16561	
	Old horse	NA	NA	NA	
	TB	0.65146	-0.16214	0.35381	
	Welsh	0.67695	0.12687	0.30028	
	Shetland	0.69881	-0.08809	0.22456	

Table 3.10 The parameters and number of observations included in a multivariable model of liver disease prediction. GGT values, and disease outcomes were excluded from the model. * indicates a significant relationship. NA indicates there was not enough data to be included in the regression.

3.2.2.5 Impact of sample type on liver scoring findings

While the effect of biopsy size on pathological evaluation has been reviewed in human medicine (Bedossa et al., 2003, Hølund et al., 1980, Manning and Afdhal, 2008, Poynard et al., 2004, Schlichting et al., 1983), to the author's knowledge, no such review has been conducted in horses. As both biopsy and post-mortem samples were evaluated for liver grading (both the current and extended systems), it was uncertain if there would be a statistically significant difference in the distribution of scoring between sample types. Univariable regression analysis found only a significant difference between sample type utilised and centrilobular fibrosis score (Table 3. 11 and 3. 12). There was no statistical difference between post-mortem and biopsy material with any of the standard grading parameters.

Outcome	Explanatory Variables	P Value	Coefficient Estimate	Odds Ratio	95% Confidence Interval	Standard Error	Observations
Presence of sinusoidal fibrosis	Biopsy	0.9	0.4879	1.63	-0.80 to 1.78	0.65647	53
Presence of bridging fibrosis (H&E)	Biopsy	0.922	0.05716	1.06	-1.08 to 1.19	0.58017	53
Haemosiderin score	Biopsy	0.6118	0.3054	1.36	-0.87 to 1.49	0.6018	53

Table 3.11 Univariable logit regressions assessing the effect of tissue sample type on liver scoring (standard and extended). No significant relationships were found.

Outcome	Explanatory Variables	T Value	Coefficient Estimate	95% Confidence Interval	Standard Error	Observations
Total liver score	Biopsy	-1.595	0.7876	-0.889 to 1.756	0.4939	53
Portal fibrosis score	Biopsy	0.5274	0.2955	-0.738 to 1.33	0.5274	53
Centrilobular fibrosis score	Biopsy	-3.707*	-4.126	-6.31 to -1.95	1.113	49
Bridging fibrosis score (SR stain)	Biopsy	1.838	0.9995	-0.07 to 2.07	0.5439	52
Irreversible cytopathology score	Biopsy	0.694	0.3777	-0.69 to 1.45	0.5442	53
Portal inflammation score	Biopsy	0.9955	0.5279	-0.46 to 1.51	0.5030	53
Bild duct proliferation score	Biopsy	1.037	0.5515	-0.49 to 1.594	0.5318	53

Table 3.12 Univariable ordered regressions assessing the effect of tissue sample type on liver scoring (standard and extended). * indicates significant relationship.

3.3 Discussion

3.3.1 Liver scoring and clinical application

Histopathology and liver scoring have been held as the gold standard for diagnosis and prognostication with regards to hepatic disease in the horse since 2003, when the liver

scoring algorithm was devised (Durham et al., 2003c). As compared to human medicine where the aetiological agent/pathological process and division between grading and staging helps in determining the scoring algorithm used as well as disease progression, equine medicine has only one scoring system. This difference may reflect the fact that often the aetiological agent is undetermined in equine cases, making determination of a disease specific scoring system difficult. Given the reliance on the scoring system to offer owners and clinicians a prognosis, it is interesting to note that no review of the equine liver scoring system has been carried out since its creation. Also of note, the utility of the various scoring systems in individual patient case management in human medicine has been found to be lacking (Goodman, 2007). This study has attempted to review the current scoring system as well as investigate further pathological characteristics, particularly patterns of fibrosis, with regards to equine liver disease.

3.3.2 Review of the traditional liver scoring algorithm

This study found that no significant association between any of the parameters assessed by the current liver scoring system, including total liver score, and the clinical diagnosis of liver disease were present within this cohort. The original paper by Durham et al (2003) included seventy-three cases of suspected hepatic disease and twelve horses that were euthanased due to orthopaedic injury (Durham et al., 2003c). The current study included a total of fifty-three cases, twenty-six of which were being investigated for hepatic disease, twenty-two which were being investigated for extra-hepatic disease and five with an unknown clinical history. The univariable regressions used for exploring the relationships between the scoring parameters and hepatic disease included forty-eight observations.

As fibrosis is considered the best prognostic indicator of clinical outcome (Durham et al., 2003c, Malhi and Gores, 2008), it was expected that high portal fibrosis scores would be associated with clinical signs of hepatic disease and death due to hepatic disease. Six of the ten horses with a portal fibrosis score of 4 had hepatic disease, while of the remaining four cases, two had extra-hepatic disease, and two had unknown clinical histories. While this suggests a trend that horses with a portal fibrosis score of 4 are more likely to have hepatic disease, the difference between the two groups was not statistically significant. Furthermore, while a portal fibrosis score

of 4 was a negative predictor (i.e. protective) with regards to death due to extra-hepatic disease, it was a positively associated with survival.

While the small number of horses with a maximum portal fibrosis score (score=4) which had known clinical outcomes (5 out of 10 cases) may have impacted this association, the aetiology of disease, the spectrum of pathological versus clinical findings for specific diseases, and/or the reversibility of the fibrosis present (Hernandez-Gea and Friedman, 2011) may also be implicated in these findings.

None of the other parameters of the traditional liver scoring algorithm were found to be significant in predicting clinical outcome when assessed as an entire cohort (hepatic and extra-hepatic cases) or as a subset of horses being investigated for hepatic disease. The lack of pathognomonic signs associated with liver disease, as well as the compensatory capability of the liver, may lead to under-reporting/recognition with regards to clinical diagnosis of hepatic disease. The overlap between the pathology noted in the livers of extra-hepatic and hepatic cases, as many extra-hepatic diseases can lead to a hepatic response, may reflect this clinical problem and may have impacted statistical analysis. The aetiologies of liver disease included in this study and the potential for reversible tissue changes (i.e. reversible fibrosis) may also have played a role in the lack of significance between liver scoring and prediction of liver disease and clinical outcome. Furthermore, the small number of horses included in this study with known clinical outcomes decreases the statistical power of the results.

Two of the hepatic cases submitted for this study were found to have iron toxicity after biopsy (cases 36 and 37, Appendix 1). Both horses were from the same premises, both were Shetland ponies, one was a gelding, the other was a mare, and they were twelve and sixteen years of age respectively. The gelding presented with severe clinical signs including dullness, inappetence, that progressed to neurological signs. The mare had no clinical signs and was biopsied as part of monitoring for the premises Once a diagnosis of iron toxicity was made for the gelding. The gelding was found to have a portal fibrosis score of 0 and a total liver score of 5, while the mare was found to have a portal fibrosis score of 4 and a total liver score of 13. Both horses responded well to treatment and survived beyond six months.

These two cases highlight the variance between clinical and histopathological findings when looking at a specific disease. Differences such as those described between clinical presentation and pathological findings of these two cases may impact statistical assessment of the current or indeed any scoring system with regards to ability to detect and prognosticate on hepatic disease. While this study did not include a biopsy from these two horses after completion of treatment, it would be interesting to compare the scoring of the clinical and post treatment biopsies, and in particular assess any differences in the fibrotic patterns as well as severity of fibrosis seen.

3.3.3 Extended liver scoring and consideration of diagnostic and prognostic utility

With regards to the extended liver scoring system, all horses with a portal fibrosis score of 4 had both sinusoidal and bridging fibrosis present, however, not all had centrilobular fibrosis present. Conversely, many horses with a portal fibrosis score of 0 had sinusoidal, bridging and/or centrilobular fibrosis. Of these non-portal forms of fibrosis, only centrilobular fibrosis was found to have any significant association with predicting hepatic disease; a score of 1 was found to be negatively associated with hepatic disease (i.e. it was not likely to be seen in cases of hepatic disease). None of the extended fibrosis parameters showed significance with regards to clinical outcome. However, the additional forms of fibrosis assessed were not statistically weighted as are the scores of all parameters in the traditional scoring algorithm. Instead, the subjective degree of fibrosis present or simply the presence or absence of a specific type of fibrosis was recorded. Furthermore, the type of fibrosis noted may reflect disease aetiology. Aetiology may impact reversibility of fibrosis as well as disease outcome. These links are difficult to quantify as the majority of hepatic cases in this study had an unknown aetiology. Further work investigating this relationship may be warranted.

Interestingly, there was not a significant difference in assessing bridging fibrosis on H&E and SR staining. While it was more common that SR staining highlighted bridging fibrosis, bridging fibrosis noted on H&E was not always seen on SR. Fifteen of the samples with bridging fibrosis seen on SR staining, but not H&E, had a score of 1 which correlates with thin fibrous strands extending from the portal region, but not

connecting. One horse had a score of 2 which equates with the majority of portal areas being connected by thin bridges, which may be difficult to detect on H&E. Given the variation in "normal" liver histology, the significance of a score of 1 on SR stain is uncertain with regards to pathology and may reflect part of normal lobule structure. Furthermore, with regards to statistical comparison, the scoring systems for bridging did not share the same archetype. The H&E scoring system was dichotomous—if bridging was present, it received a score of 1 and if it was absent, it received a score of 0. This is in comparison to the SR scoring system whereby bridging was assessed by severity with scores running from 0-3 yielding four categories. The lack of congruity between systems (dichotomous vs categorical) may have skewed statistical analysis.

3.3.4 Tissue sample type and impact on liver scoring

Biopsy samples were not found to be significantly different to post-mortem tissue sections with regards to the standard liver scoring system and only impacted centrilobular fibrosis scoring within the extended liver scoring algorithm. This is interesting as post-mortem sections provide a far greater area for assessment compared to biopsy sections. As of present, ideal biopsy size has not been evaluated for the equine liver, but this finding suggests that as long as the tissue sample can be assessed for all aspects of the liver scoring algorithm, increasing the tissue size does not impact the scoring process.

3.3.5 Further considerations for liver scoring

The original study only included cases from a small region in England (Durham et al., 2003c). Inferences drawn from the results of the original study (seventy-three hepatic cases, twelve orthopaedic cases included (Durham et al., 2003c)) may therefore lack the sensitivity and specificity to be applied to a more global population of horses as findings may reflect a regional prevalence of specific hepatic disease. While this study included cases from England, Scotland and Ireland, conclusions about the variability in disease prevalence/disease characteristics from these areas cannot be drawn due to sampling bias. Samples submitted from equine hospitals were more frequently considered primary hepatic cases, while those submitted from the laboratory in Ireland or the horse meat factory were more likely to be extra-hepatic cases.

Discussions with pathologists highlighted the difficulty in assessing the degree of portal fibrosis present in the absence of a concrete, defined “normal” equine liver (P. Johnston, A. Rupp, personal communication, 2017, University of Glasgow). It is difficult to define what constitutes a “normal” amount of fibrous tissue in the portal tracts, making the traditional scoring algorithm, which advises that a score of 2 is associated with portal tracts that are increased two-fold and a score of 4 is increased four-fold, open to subjective interpretation. Trying to define “normal” amounts of fibrous tissue within the portal tracts may be even more difficult if large areas of a tissue sample are affected and no “normal” tissue is present for comparison. Furthermore, assessment of “normal” may be impeded when considering there may be variations in hepatic architecture may reflect breed differences or management (including diet) differences between individuals. Interestingly, when one post-mortem sample that had severe hepatic disease was shown to three different anatomic pathologists, the resulting fibrosis score was different for each pathologist. It was scored 0 by the pathologist who assessed the samples for this project, and was scored a 2 and a 4 by other two pathologists in the pathology department at the University of Glasgow. This variability in interpretation of the scoring system will impact total scores and therefore prognostication based on this system

The sample size used in this study was small and the variation in the coefficient estimates from zero in non-significant, and at times significant regressions suggests that underlying relationships in the data may reflect statistical noise as opposed to true relationships, i.e. the smaller the sample size and the larger the absolute value of the coefficient estimates, the larger the overestimate of significance and size of effect. Caution should be used to not over-interpret the results, as significance may in fact be artifact.

Many of the factors that proved significant were found to have coefficient estimates in the opposite direction of the hypotheses (i.e. centrilobular fibrosis is negatively associated with liver disease, a portal fibrosis score of 4 is positively associated with survival, etc.) which may reflect a sample collection bias not controlled for, sample size, the overlap in pathology of hepatic and extra-hepatic disease, or may suggest a true deviance from the original findings of Durham et al. (Durham et al., 2003c).

The data suggests that a singular histopathological assessment of the liver may not be prognostic. The data from the extended liver scoring along with weak significance of portal fibrosis in predicting clinical outcome is not in concordance with previous studies that found that fibrosis is greatest prognostic indicator (Durham et al., 2003c, Malhi and Gores, 2008). There may be a requirement for scoring systems based on aetiology to be developed for equine liver disease (Neuman et al., 2016), or that assessment of grade (disease activity as denoted by inflammation etc.) and stage (degree of fibrosis present) should be separated as it is in scoring systems like Ishak (Goodman, 2007). Such a development may prove clinically useful when aetiology is known in informing treatment plans and prognostication. Despite the small sample size, this study suggests prognostication based solely on current scoring system should be undertaken with caution. Consideration of aetiology, clinical signs and other diagnostic findings should inform clinical decision making in conjunction with biopsy findings.

Chapter 4 Image analysis and comparison to manual histopathological assessment of equine liver samples

4.1 Introduction

4.1.1 Image analysis: utilisation in human medicine

Image analysis is a highly accurate technique which has been used to quantify pathological change in histological samples. Slides are scanned and images are digitised. These digitised images can be assessed using software which is able to detect various histopathological/image characteristics such as colour pixel intensity, nuclei, number of cells, and total area affected. By adjusting the colour pixel thresholds with reference to areas of interest, total pixel counts can be obtained which provide a quantitative assessment of the degree of certain pathological changes (Mohammed et al., 2012) as opposed to the somewhat subjective assessment trained anatomic pathologists provide.

The technique has been used in various biopsy assessments in human medicine. With regards to the mitosis associated nuclear protein, Ki-67, (McCormick et al., 1993, Scott et al., 1991), assessment of breast cancer biopsy samples showed good agreement between image analysis and visual assessment (Mohammed et al., 2012). Assessment of hepatic fibrosis in biopsies from patients with hepatitis C found good agreement between digitally analysed images and high Knodell scores. However biopsies with low scores were not found to have good agreement with digital analysis (O'Brien et al., 2000). Other studies have shown not only good agreement between image analysis results and hepatic grading systems in human medicine, but also an association between image analysis results and clinical outcome (Calvaruso et al., 2009, Huang et al., 2014, Pilette et al., 1998).

4.1.2 Aims of the chapter

Agreement between both current and extended equine liver scoring algorithms and image analysis results were assessed along with the agreement between image analysis results, clinical diagnosis of hepatic disease and clinical outcome. Effect of

the use of biopsy versus post-mortem material for image analysis results was also considered.

4.2 Image analysis results

Owing to the timing of sample collection as well as the need to batch stain tissue, not all cases underwent the staining protocols for image analysis. The numbers of samples stained with SR, PPB, Coll I, Coll III and SMA are shown in Table 4.1. Means and standard deviations of percent areas affected (the computer measured amount of tissue that relates to a particular pixel colour threshold for a specific staining type divided by the total computer measured tissue area) for each stain analysed are listed in Table 4.2.

Stain	Number of samples
SR	41
PPB	41
Coll I	31
Coll III	32
SMA	31

Table 4.1 Numbers of samples submitted for image analysis for each stain type. SR= Sirius Red, PPB= Perl's Prussian Blue, Coll I= Collagen I, Coll III= Collagen III, SMA= Smooth muscle actin.

	Percentage area affected SR	Percentage area affected Coll I	Percentage area affected Coll III	Percentage area affected SMA	Percentage area affected PPB
Mean	5.32	1.33	2.41	6.144	2.749
Standard deviation	7.76	1.583	2.797	7.323	5.777

Table 4.2 Mean value of the percentage area affected and standard deviations for image analysis data for horses included in this study. As tissue size varied between samples percentage area affected was compared across samples. SR= Sirius Red, PPB= Perl's Prussian Blue, Coll I= Collagen I, Coll III= Collagen III, SMA= Smooth muscle actin.

Regression analysis was used to assess relationships between image analysis data and the liver scoring rubrics, including the extended fibrosis assessment. Special stains were used to detect specific fibrosis components of the fibrotic response (SR, collagen I and collagen III), activated hepatic stellate cells (SMA), which could act as a proxy for fibrosis and haemosiderin (PPB). Sirius Red and SMA staining were found to be significantly associated with the manual scoring of portal, centrilobular and bridging fibrosis as seen on SR staining. Collagens I and III were significant predictors of the manual scores for bridging fibrosis as seen on SR staining and collagen III was also a significant predictor of centrilobular fibrosis manual scoring (Table 4.3). These results suggest that the image analysis of these stain types is in agreement with the pathologist's grading.

None of the special stains were significantly associated with sinusoidal fibrosis or bridging fibrosis as see on H&E staining. The lack of agreement between the image analysis results and bridging fibrosis as seen on H&E is interesting as there was no statistical difference between detection of bridging fibrosis on H&E and SR stains. This again may reflect the comparison between a binary scale of grading (absent or present on H&E) with the multi-category scale derived for SR staining. PPB staining appears to be a strong positive predictor for haemosiderin score (Table 4.4).

All image analysis data were found to be positive predictors of total liver score (Table 4.5). Sirius Red, collagen III and SMA were found to have statistically significant differences between biopsy and post-mortem samples being assessed using image analysis (Table 4.6), with biopsy being less likely to be associated with higher image analysis results (percent area affected). No relationships between image analysis results and prediction of hepatic disease or clinical outcome were found.

Outcome	Explanatory Variables	t Value	Coefficient Estimate	Standard Error	Observations
Portal fibrosis score	SR image analysis	2.61	0.12	0.04	41
Centrilobular fibrosis score	SR image analysis	3.128	0.2526	0.08074	37
Bridging fibrosis score (SR stain)	SR image analysis	2.638	0.1337	0.05067	40
Bridging fibrosis score (SR stain)	Coll I image analysis	2.671	1.042	0.39	30
Bridging fibrosis score (SR stain)	Coll III image analysis	2.126	0.3509	0.1651	31
Centrilobular fibrosis score	Coll III image analysis	2.303	0.9255	0.04019	28
Portal fibrosis score	SMA image analysis	2.051	0.13	0.0634	31
Centrilobular fibrosis score	SMA image analysis	2.898	0.666	0.2298	27
Bridging fibrosis score (SR stain)	SMA image analysis	2.257	0.1674	0.07417	30

Table 4.3 Significant univariable ordered regressions. Regressions included both necropsy and biopsy sample material.

Outcome	Explanatory Variables	p Value	Coefficient Estimate	Odds Ratio	95% Confidence Interval	Standard Error	Observations
Haemosiderin score	PPB image analysis	0.02	0.7097	1.02	0.09 to 1.33	0.31	41

Table 4.4 Results of the univariable logit regression model for PPB acting as a predictor of haemosiderin score. Both biopsy and section material were utilised.

Outcome	Explanatory Variables	t Value	Coefficient Estimate	Standard Error	Observations
Total liver score	SR image analysis	2.02	0.07	0.04	41
Total liver score	Coll I image analysis	2.289	0.477	0.2084	31
Total liver score	Coll III image analysis	2.023	0.2366	0.117	32
Total liver score	SMA image analysis	2.264	0.1046	0.04623	31
Total liver score	PPB image analysis	3.465	0.3731	0.1077	41

Table. 4.5 Results of significant ordered univariable regressions examining the relationship between total liver score and image analysis results of all staining techniques utilised. Both section and biopsy material were utilised.

Outcome	Explanatory Variables	t Value	Coefficient Estimate	Standard Error	Observations
SR image analysis	Biopsy	-3.416	-7.634	2.235	41
Coll III image analysis	Biopsy	-3.296	-3.909	1.186	32
SMA image analysis	Biopsy	-4.428	-12.441	2.810	31

Table 4.6 Results of significant univariable linear regressions (using maximum likelihood estimation) examining the relationship between tissue material type and image analysis results. Biopsy acted as the explanatory variable while PME section was the referent.

As tissue sample type appeared to affect image analysis for SR, collagen III and SMA stains, regressions re-assessing relationships with fibrosis carried out on only biopsy material were performed. Sirius Red image analysis remained a predictor of both portal fibrosis and bridging fibrosis as seen on SR staining (Table 4.7).

Outcome	Explanatory Variables	t Value	Coefficient Estimate	Standard Error	Observations
Portal fibrosis score	SR image analysis	2.386	1.462	0.613	26
Bridging fibrosis score (SR stain)	SR image analysis	2.19	0.923	0.4214	25

Table 4.7 Significant univariable regressions when controlling for the effect of tissue sample type on relationships.

Regressions were also repeated on the subset of horses with known hepatic disease, and no relationships were found between image analysis results and liver scoring parameters within this subset.

4.3 Discussion

4.3.1 Image analysis: relation to the scoring system and clinical applications

Image analysis has been considered as a methodology to reduce intra- and inter-observer bias when reviewing histology. By providing a quantitative result for a particular stain type/characteristic, a definitive assessment can be provided as opposed to a subjective impression and consistency across assessors can be achieved. The methodology appears to have significant agreement with grading systems used in human medicine (Calvaruso et al., 2009, Huang et al., 2014, Mohammed et al., 2012, O'Brien et al., 2000, Pilette et al., 1998). Image analysis has never been assessed for equine liver scoring, nor have the immunohistochemical stains used in this paper been used on equine tissue.

Sirius red and SMA staining were found to be significantly associated with the scoring of portal, centrilobular, and bridging fibrosis as assessed on SR staining. While collagen I and III staining were significantly associated with bridging fibrosis as seen on SR stain, and collagen III was found to be significantly associated with centrilobular fibrosis scoring, which delineated absence or presence of this type of fibrosis. However, when controlling for tissue sample type, and assessing the use of biopsy samples only, only SR image analysis results were in agreement with portal fibrosis

and bridging fibrosis (SR staining) scores. All image analysis of special stains, including PPB, had a significant positive association with total liver score.

Staining for collagens I and III were chosen as these forms of collagen are associated with non-reversible fibrosis. Theoretically, SR staining should stain all types of fibrotic tissue. It was hypothesised that increased collagen I and III staining would be strongly associated with horses that died due to hepatic disease, while SR staining would have a weaker association. No special staining techniques were associated with clinical outcome or clinical diagnosis of hepatic disease. These findings demonstrate all methods analysed, histological assessment in association with liver scoring (current and extended forms) and image analysis, yield similar results with regards to diagnosis and prognosis of hepatic disease. Unfortunately, image analysis may be hindered by the same problems found with liver scoring (varied aetiology leading to different pathological presentation, reversible fibrosis, etc.), and was not able to predict clinical manifestations of hepatic disease, nor was it able to offer prognostic information. Furthermore, the lack of clearly defined parameters for normal amounts of fibrous tissue in equine liver may decrease clarity in assessing if there is an increased percent of stain uptake.

The lack of relationship between image analysis results on SR, SMA, collagen I and collagen III stained sections and sinusoidal fibrosis may reflect the colour pixel threshold or the predominance of this type of fibrosis as noted by the pathologist (n=43 for presence of sinusoidal fibrosis), while the lack of relationship between these stains and bridging fibrosis as seen on H&E staining may reflect the difficulty in seeing mild bridging on this stain (n=35 for presence of bridging fibrosis on H&E) or the chosen colour pixel threshold. Determination of the colour pixel threshold is a subjective assessment, and one threshold was determined for each stain type. While the threshold was set to provide the most specific result possible, variability in slide staining may have skewed results, i.e. some slides may have stained darker or lighter than others, thus having a degree of variation as to threshold intensities that are considered positive. Slides were batch-stained where possible to keep stain intensity consistent, however, immunohistochemistry required slides to be stained individually. Furthermore, variation in sampling (size, location, number of intact portal tracts) as well as variation in the distribution of fibrosis and alterations in fibrotic patterns that

may inform a pathologist's score may represent difficulties with which image analysis cannot contend (O'Brien et al., 2000).

Tissue sample type impacted image analysis results for SR, SMA and collagen III staining. Given that the majority of horses being investigated for hepatic disease had biopsies taken as part of clinical investigation, regressions on biopsy only material were re-run to assess the utility in a clinical setting. Only SR image analysis and portal fibrosis and bridging fibrosis (SR staining) scores remained in agreement. Bedossa et al. (2003) found that biopsy length has a significant impact on assessment of fibrosis in cirrhotic patients using image analysis. Their study found that despite the accuracy of image analysis, coefficients of variation (CV) in biopsies between 15 and 20 mm in length are so high that the results would be discarded. It was not until biopsy length reached 40 mm that the CVs generated by image analysis were clinically acceptable (Bedossa et al., 2003). Biopsy length in this study was not measured, however, to allow for variation in tissue size, percent area affected was used as a measurement of image analysis results.

Interestingly image analysis results had a positive association with total liver score. While total liver scores are meant to carry prognostic value, they were not found to be associated with clinical outcome in this study, with potential reasons for this discussed in Chapter 3 Section 4. However, further assessment of a greater number of cases where clinical outcome is known and compared with known aetiologies of liver disease may lead to a clinical role for image analysis.

Perls Prussian Blue staining was associated with haemosiderin grades. Only haemosiderin that has accumulated within hepatocytes is considered significant in the current scoring algorithm. Perls Prussian Blue stain, however, does not discern between hepatocellular and Kupffer cell accumulation of haemosiderin. Image analysis results are therefore reflective of total haemosiderin within the tissue sample. More modern image analysis software (e.g. Halo image analysis platforms which have AI technology that learns the types of tissue that is of interest) may be able to discern hepatocellular haemosiderin versus haemosiderin accumulation in other areas of the liver. Further assessment of this parameter is warranted to discern if the significance between total haemosiderin accumulation as opposed to solely hepatocellular haemosiderin and haemosiderin score is maintained.

The lack of agreement between clinical outcome and clinical diagnosis of hepatic disease compared to image analysis results suggests that this technique may not provide further information to the clinician or owner with regards to equine liver disease at present. Larger studies with more complete datasets, including aetiology and outcome, are required to further assess the utility of image analysis in a clinical setting. Furthermore, if scoring systems unique to aetiology are developed, image analysis may play a greater clinical role.

Chapter 5 Discussion

5.1 Project overview

Initial diagnosis of hepatic disease in the horse requires a multi-faceted approach. Clinical presentation, biochemical markers of hepatic injury and function, and clinical imaging modalities such as ultrasound are necessary for diagnosis. Confirmation of hepatic involvement in a disease process—either primary hepatic disease, or injury to the liver owing to extra-hepatic disease, prognostication and aetiological information requires biopsy results. A tool used to aid prognosis has been a liver scoring system which was developed in 2003 (Durham et al., 2003c).

This study had a two-armed approach. The first looked at traditional manual histopathological assessment of equine hepatic samples and aimed to assess.1) the utility of the current liver scoring system in determining a clinical diagnosis of hepatic disease, 2) the prognostic capability of the current liver scoring system, 3) the utility of an extended liver scoring system on determining a clinical diagnosis of hepatic disease 4) the prognostic utility of the proposed extended scoring system. The second arm looked a at quantitative histological method for assessing equine hepatic tissue using image analysis. This computerised portion of the study sought to assess 5) the relationship between both the current and extended liver scoring systems and image analysis using staining techniques to highlight features of fibrosis and haemosiderin deposition, and finally 6) the impact of post-mortem tissue samples versus biopsy samples on both grading systems and image analysis results.

The study population consisted of fifty-three cases, of which twenty-six were being investigated for hepatic disease, twenty-two were being investigated for extra-hepatic disease, and five with no known reason for investigation.

5. 2 Liver scoring: limitations and discrepancies

Cases included in the study had a wide range of liver scores. The distribution of total liver scores ranged from 1-13 (possible score range = 0 to 14), with thirty-nine cases scoring between 1 and 6 and fourteen cases scoring 7 or more. Horses that had been diagnosed with hepatic disease had a mean total liver score of 6.2 (standard deviation

= 3.3), while horses being investigated for extra hepatic disease a mean total liver score of 4.6 (standard deviation = 2.7). The scoring difference between these groups, however, did not equate to a statistical difference—horses with higher total liver scores were not more likely to be associated with a clinical diagnosis of hepatic disease, and nor were they more likely to die as a result of hepatic disease. Furthermore, no aspect of the current liver scoring system was found to be statistically significant with regards to clinical diagnosis of hepatic disease or outcome. This is contrary to the original publication which found that horses with total liver scores of 7-14 were fifty times less likely to survive hepatic disease for 6 months as compared to those horses that had a score of 0, and horses with scores of 2-6 were 12 times less likely to survive 6 months as compared to horses with a total liver score of 0 (Durham et al., 2003c).

Several factors may be at play when considering this discrepancy. This study included a larger population of horses without primary hepatic disease (n= 22) as compared to the original study (n=12), with a wider array of reasons for clinical investigation for comparison to cases of primary hepatic disease. The original study aimed to design a scoring system that could offer prognostic value as opposed to discern hepatic disease from extra-hepatic disease, and in so doing, statistically weighted pathological changes noted in hepatic cases in accordance with case outcome (Durham et al., 2003c). Thus, the system focused less on discerning variation in normal liver and did not account for hepatic changes present in extra-hepatic disease, but instead assesses the gravity of change in liver that was already considered abnormal. As previously stated, extra-hepatic disease can impact the liver, and thus the overlap between lesions noted with primary hepatic disease and extra-hepatic disease may be considerable. While it might be assumed that primary hepatic disease would lead to more severe or a greater accumulation of lesions in the liver, consideration for when in the spectrum of disease a biopsy is taken is also required.

With regards to the development of scoring system, a small number of controls restricts the perception of “normal” liver. As there can be variation within a normal population, horses that are clinically disease free need to be compared with those that have both hepatic and extra-hepatic disease to determine the degree of variation that is considered physiological as opposed to pathological. Lack of a defined “normal” becomes difficult in the interpretation of the present scoring system

particularly with regards to fibrosis as it uses phrases such as portal fibrosis was considered “moderate” when “… a representative tract was 3 times normal size” (Durham et al., 2003c). Pathologists infrequently look at healthy tissues. With regards to the horse, diet and management can vary considerably between countries, regions, yards, and even individuals. Also, consideration of the horse’s primary use may be warranted when considering what is normal architecture for the liver. Does a racehorse, who has an increased metabolic demand, have the same microscopic features of the reticulin meshwork of the liver as a native breed pleasure horse, who primarily hacks once or twice a week and lives out in field that has minimal grass management? A horse’s “job” was not considered when compiling data for this project but may have an implication for hepatic histology. These differences in management may impact the physiological representation of “normal” when undergoing histopathological assessment. Some of what we attribute as normal may be pathological and vice versa.

Twenty-two horses were found to have bridging fibrosis, as seen on H&E, which did not have portal fibrosis present. This was interesting as bridging fibrosis is thought to be a result of chronic severe hepatic insult and connects portal tract to portal tract or central veins (Brown et al., 2017). Therefore, its presence would not normally be considered in the absence of portal fibrosis. In other words, it is difficult to conceive of a hepatic insult that could be large enough to connect multiple lobules via fibrosis (bridging fibrosis), however, not cause enough damage to any individual lobule to yield portal fibrosis. The results of the original scoring system found samples with portal fibrosis scores of 4 often had bridging fibrosis present, however bridging was not mentioned for lower fibrosis scores (Durham et al., 2003c).

These results may reflect the lack of a defined “normal” amount of fibrous tissue in the equine liver. A pathologist may consider using what may appear to be “normal” tissue within a sample that is predominantly abnormal. This methodology could be misleading with regards to the amount of fibrous tissue that is truly normal within a portal tract, and by extension, lead to the mismatch between these bridging and portal fibrosis scores. It may also be worth considering drying artifact and sample dehydration in preparation of tissue causing “enlarged” or more obvious reticulin meshwork.

When considering the question of geographical impact on equine liver disease more globally, in a species where many of the aetiologies of hepatic disease remain unknown, does looking at hepatic disease over a narrow geographic range decrease the number of aetiologies seen? The Durham scoring system was derived from cases seen in a small area of Southeast England. If infectious causes of hepatitis were present, they may have had similar aetiologies, and similarly, if hepatic disease was caused by ingestion of toxins, grazing may be more similar in a narrow geographic range. If this indeed was the case, the statistical weighting of the histopathological changes seen may have been biased to reflect the outcomes of a smaller number of disease processes. This may in turn effect the prognostic capability of the scoring system when used to describe another aetiology. While this study had sample submissions from several different locations within the United Kingdom, there were not enough varied locations to infer any impact on disease trends or trends in scoring. In order to tease out the impact of specific diseases on specific changes to hepatic tissue, definitive aetiologies of hepatic cases would need to be known. Unfortunately, aetiology was not known in many of the cases included in this study, making this assessment unfeasible.

Within human medicine, scoring systems tend to make a distinction between grading (disease activity which equates to inflammation) and staging (which equates to fibrosis) (Goodman, 2007). This differentiation allows for disease progression and chronicity to be more clearly ascertained, and where relevant, to assess treatment protocols or efficacy during clinical trials (Goodman, 2007). As fibrosis is considered the outcome of longstanding, consistent injury (Brown et al., 2017) assessing the relative fluctuation in inflammation may inform therapeutic management over a shorter course of time, while assessment of degree of fibrosis and fluctuation in fibrosis will reflect the longer term clinical picture. This benchmarking process, established through assessment of multiple biopsies, appears less available in equine medicine. In the author's experience, despite being a safe procedure, owners may be reticent to have their horse undergo a liver biopsy. This finding may reflect both cost and the owner's concept both of risk and utility. Biopsies taken during the course of treatment (i.e. follow-up biopsy) may elucidate not only the efficacy of treatment, but also the degree to which certain types of fibrosis is reversible. In this study, only one case had a follow-up biopsy, which, unfortunately was too small and damaged for

thorough assessment. However, the lack of statistical association between outcome and liver score may reflect in some part to the reversibility of fibrosis and regenerative nature of the liver.

Despite the lack of agreement between diagnosis and outcome with the current liver scoring and extended liver scoring systems, biopsy remains a highly useful clinical tool. It provides a confirmation of hepatic injury, may provide the aetiology of that injury, and even in the absence of scoring, it can still provide an indication of the extent and gravity of damage to the liver. In clinical cases of hepatic disease, biopsy acts as a tool to benchmark disease and often provides evidence for clinical decisions to clients. However, it should be noted that biopsy results may not always reflect the clinical picture. In the cases of iron toxicity, the pony who was clinically unwell had a total liver score of 5 while the pony who showed no clinical signs had a total liver score of 13. Both ponies received treatment and made a clinical recovery. Cases such as these highlight that in a biological system, the clinician frequently needs to be guided by the patient's clinical signs and response to treatment as opposed to resorting to complete reliance on a number.

5.3 Predictors of hepatic disease and outcome

As expected, death due to non-hepatic disease was a negative indicator for having hepatic disease, but interestingly survival without repeat clinical signs was found to be a predictive indicator for hepatic disease in this cohort with bivariate analysis. This may reflect the aetiology and/or number of cases for which outcome data was available. Cases that were submitted from Glasgow University Veterinary School (GUVS) that were being euthanased and undergoing post-mortem were frequently from a rescue equine charity. Charity cases may have had restricted investigations owing to financial restrictions, and under-reporting of hepatic disease may have occurred in these cases. In direct opposition to this, referral cases from Liphook Equine Hospital, Rossdales Veterinary Surgeons, and Weipers Equine Hospital may have had a greater budget for investigations and treatment, which can bias not only the outcome for these horses but also the clinical findings. As euthanasia and natural death were not considered independently, outcome data may be skewed with regards

to disease severity. Finally, aetiological diagnoses were infrequent in hepatic cases, so impact of different agents on survival was not possible.

Interestingly, several cases from the of extra-hepatic disease subgroup were found to have high total liver and fibrosis scores. It is uncertain if these horses did not present with clinical signs of liver disease, and hence support under reporting/diagnosis of the condition, or if their other clinical signs were more serious and thus full clinical investigations were not performed. Three were diagnosed with extra-hepatic disease and two had unknown clinical history. These cases were collected from GUVS. As the extent of clinical investigations was unknown for these cases, under-reporting of hepatic disease within this subset of cases is possible.

With regards to predictors of a diagnosis of hepatic disease, individual bivariate regressions found that survival, death due to extra-hepatic disease, middle aged horses, and being a Thoroughbred were significant. However, when a model that looked at clinical outcomes, age, breed, and sample origin was designed, only death due to extra-hepatic disease remained significant—it was found to be less likely to be associated with hepatic disease. When assessing the clinical significance of age and breed on diagnosing hepatic disease, the relationships more likely reflect the distribution of cases submitted as opposed to a direct relationship between these parameters and development of hepatic disease. Of the forty-four cases whose breeding was known, nine were Thoroughbreds (20.5%), six were Shetland ponies (13.6%), and four were Highland ponies (9%). All other breeds included were found in lower proportions. Forty-one of the cases with known breeding had known clinical history, and of these, twenty-three were being investigated for hepatic disease, and eighteen of these were of English origin.

5.4 Image analysis: current findings and possible future uses

As stated previously, image analysis provides a quantitative result for a previously qualitative assessment with regards to histological specimen review. By providing a numeric result, intra- and interobserver bias is reduced (Calvaruso et al., 2009, Huang et al., 2014, Mohammed et al., 2012, O'Brien et al., 2000, Pilette et al., 1998). Within this study, agreement between some of the fibrosis patterns as scored by an anatomic

pathologist and percentage area affected as determined by image analysis was found. A summary of the results is shown in table 5.1

Stain type	Fibrotic pattern
Sirius Red	Portal fibrosis *, centrilobular fibrosis, bridging fibrosis (SR)*
Smooth muscle actin	Portal fibrosis, centrilobular fibrosis, bridging fibrosis (SR)
Collagen I	Bridging fibrosis (SR)
Collagen III	Bridging fibrosis (SR), centrilobular fibrosis

Table 5.1 provides the significant relationships found between image analysis results and current (portal fibrosis), and extended (centrilobular fibrosis, bridging fibrosis (SR)) fibrosis scoring parameters as assessed by an anatomic pathologist. The * delineates those patterns with the specific stain type that remained significant when controlling for tissue sample type (biopsy).

Interestingly, image analysis results from all stains that looked for fibrosis (SR, collagen I, collagen III) or were a proxy for activated hepatic stellate cells (SMA) were not found to be in agreement with either sinusoidal fibrosis patterns nor bridging fibrosis as seen on H&E staining, both of which were scored as present (score of 1) or absent (score 0). This dichotomisation of the fibrotic pattern may not in fact be an appropriate way to consider a process that is a continuous variable. The statistical analysis conducted may have lacked statistical power to detect a relationship or confirm that there is no relationship. Additionally, there may be a lack of consideration of the variation of the amount of fibrosis present in each specific fibrosis pattern (i.e. all of the fibrosis scoring parameters were too ridged in assessment of the amount of fibrosis present and require more categories). Non-linear relationships may have been concealed as a result of dichotomising sinusoidal and bridging (H&E) fibrosis patterns.

Within the findings of this study, image analysis does not appear to add any benefit to histological analysis of equine hepatic tissue with regards to clinical decision making—there was no relationship found between image analysis results, hepatic disease or outcome. It does appear to be largely in agreement with pathologist led grading of the tissue. As only one pathologist graded the tissue samples in this project, and these were scored only once by this pathologist, it is not possible to assess the impact on inter- or intra-observer variation and equine liver scoring. Nor can conclusions be drawn with regards to a reduction in intra-or interobserver variation by using image analysis. However, this modality may prove useful in academic and research settings to provide a quantitative result for statistical analysis as the changes described in the present scoring system may be better considered over a continuum as opposed to finite categories. In other words, all tissue can only respond to damage in a set number of ways—these are types of changes that dictated the scoring rubric (fibrosis, inflammation, irreversible change, etc.). However, the degree of change present happens over a spectrum that may not lend itself to discrete categorisation (i.e., numbering systems for each category of change). Thus, a modality that can account for subtle variability may, in time, be better placed to assess histologic samples, aid in creating a more robust or fine-tuned scoring system and inform prognosis.

5.5 Final thoughts and future research

While the numbers of cases included in this study were small, the findings call into question the clinical utility of an equine hepatic scoring system. Both the current scoring system and the proposed extended scoring system were not found to be associated with either a clinical diagnosis of hepatic disease, nor were they found to be associated with clinical outcome. Possible reasons for this are outlined above. The results of this study instead favour the adage, “treat the horse, not the number”.

While human medicine has developed multiple scoring systems that take into account aetiology, the underlying finding has been that these systems are not useful with regards to individual case handling, and they should not inform treatment protocols. Instead, these systems allow for large study statistics to be performed (Goodman, 2007). With regards to the current scoring system for equine medicine, the same

finding may be true. For example, clinically, hypertriglycerideaemia in horses is known to be lethal if not treated in a timely fashion. The effect on the liver is reversible, with the main changes being fat accumulation within hepatocytes. As this finding would be scored a 0 and few other hepatic changes may be found, this disease which carries a guarded prognosis, would carry a low liver score. The cases of iron toxicity in this study (cases 36 and 37, Appendix 1) also provide a good example of how the scoring system, when taken in isolation does not fit the clinical picture and knowledge of aetiology is what guided treatment.

These cases and clinical scenarios may underline the fact that while categorisation is useful for large scale comparison, the interpretation of these categories based on a single time point is imperfect. In trying to develop a more robust scoring system, even if systems were individualised on an aetiological basis, there would be a narrowing of interpretation to one body system as opposed to taking into consideration a more holistic clinical interpretation. As such, the ability of a scoring system to prognosticate, would likely be undermined.

Instead of focusing on a scoring system that looks at a single sample, it may be more clinically useful to investigate sequential biopsies taken over the course of hepatic cases to outline resolution of disease and look for potential therapeutic targets. If, like human hepatic tissue, equine hepatic tissue has reversible fibrotic change, this may feature as a good therapeutic target. Imaging modalities and staining techniques that could elucidate the amount of potentially reversible fibrosis present could be clinically relevant. Further research into aetiology of equine hepatic disease is also warranted.

Appendices

Appendix 1 Case and sample features including clinical histories

Case Number	Sample type	Clinical signs/diagnosis	Liver vs. Extra-hepatic disease cases (EHD)	Clinical outcome
1	**B**	Liver enzymes markedly elevated; bile acids normal. WBC count 5x10^9/l. Possible Inflammatory liver disease	Liver	NA
2	**B**	Two episodes of fever. Increased liver enzymes.	Liver	Alive
3	**B**	Increased liver enzymes.	Liver	NA
4	**B**	Presented for acute lameness and a septic bicipital bursa. No history of trauma and suspected haematogenous spread. Responded well to treatment, with lavage and antibiotics. Pyrexic in early January. No evidence of joint sepsis/osteomyelitis. Full investigations for pyrexia of unknown origin: thoracic and abdominal ultrasounds, ECG, blood cultures, radiographs, bone scan and white cell scan. No obvious foci of infection. A blood sample revealed normal liver enzymes. The liver has always had hyperechoic areas - 'Christmas tree'/starry sky - granuloma-type appearance. This was initially thought to be incidental. GGT is now increased (approximately 250 iu/l) and bile acids 11umol/l. Treated with cobactam/potassium iodide and two bone scans in between the samples.	EHD	Alive
5	**B**	Herd – poor condition and increased liver enzymes – 3 animals being biopsied.	Liver	NA
6	**B**	A history of liver disease over a few years. Photosensitisation and weight loss. History of ragwort exposure.	Liver	Alive

7	**B**	Ill thrift of several months duration	EHD	Dead due to extra-hepatic disease
8	**B**	Herd – poor condition and increased liver enzymes – 3 animals being biopsied.	Liver	NA
9	**B**	Herd – poor condition and increased liver enzymes – 3 animals being biopsied.	Liver	NA
10	**B**	NA	NA	NA
11	**N**	Two ponies were found dead last weekend. This pony was found showing signs of colic this morning and some circling. The pony deteriorated rapidly and died.	Liver	Dead due to hepatic disease
12	**B**	Previous liver disease in 2006, which responded to treatment. Currently quiet/dull with increased liver parameters on routine blood analysis.	Liver	Dead due to extra-hepatic disease
13	**N**	Fracture of 3rd phalanx on right hind limb, suspensory disease, skeletal abnormalities of cervical and lumbar vertebrae	EHD	Dead due to extra-hepatic disease
14	**N**	Fracture of 4-6th lumbar vertebrae	EHD	Dead due to extra-hepatic disease
15	**N**	Bilateral gastrocnemius rupture with oedma of biceps femoris and semimembranosus	EHD	Dead due to extra-hepatic disease
16	**N**	Obstructive choke with oesphageal ulceration and stricture, secondary aspiration pneumonia, and start of scour	EHD	Dead due to extra-hepatic disease
17	**N**	Head trauma-oedema of head, bilat pulmonary oedma, airway obstruction	EHD	Dead due to extra-hepatic disease
18	**N**	Fractures of left proximal radius, left wing of illium, and transverse processes of sacrum and 5-6th lumbar vertebrae	EHD	Dead due to extra-hepatic disease
19	**N**	Fasciola hepatica, fatty liver, and enterocolitis	Liver	Dead due to hepatic disease

20	**B**	Weight loss, lethargy, high liver enzymes	Liver	NA
21	**B**	Weight loss, liver disease	Liver	NA
22	**N**	NA	NA	NA
23	**N**	Heart murmur, pharyngeal collapse	EHD	Dead due to extra-hepatic disease
24	**N**	Lameness	EHD	Dead due to extra-hepatic disease
25	**N**	Sarcoids, stiffness	EHD	Dead due to extra-hepatic disease
26	**N**	Weight loss	EHD	Dead due to extra-hepatic disease
27	**N**	Lameness, behavior	EHD	Dead due to extra-hepatic disease
28	**B**	NA	NA	NA
29	**B**	Outbreak hepatopathy	Liver	NA
30	**B**	Outbreak hepatopathy	Liver	NA
31	**B**	Hepatopathy	Liver	NA
32	**B**	Outbreak hepatopathy	Liver	NA
33	**B**	Elevated liver enzymes	Liver	NA
34	**B**	Hepatopathy	Liver	NA
35	**B**	Hepatopathy	Liver	NA
36	**B**	Inappetence, lethargy, hyperlidaemia, azotaemia, cholangiohepatitis, and iron toxicity	Liver	Alive
37	**B**	Companion pony to pony with iron toxicity; also found to have iron toxicity despite lack of clinical signs	Liver	Alive
38	**B**	Chronic weight loss, increased liver enzymes, diagnosed with echinococcus	Liver	Dead due to extra-hepatic disease
39	**N**	Sudden onset inappetence,	Liver	Dead due to

		dullness and incoordination		hepatic disease
40	**B**	Hepatic encephalopathy, increased liver enzymes, weight loss, diagnosed with cholelithiasis	Liver	Dead due to hepatic disease
41	**B**	Ataxia, increased liver enzymes	Liver	NA
42	**N**	NA	NA	Dead due to hepatic disease
43	**N**	NA	NA	NA
44	**N**	Ruptured caecum	EHD	Dead due to extra-hepatic disease
45	**N**	Typhlocolitis and severe hepatic lipidosis with evidence of larval migration	EHD	Dead due to extra-hepatic disease
46	**N**	Factory horse	EHD	Dead due to extra-hepatic disease
47	**N**	Factory horse	EHD	Dead due to extra-hepatic disease
48	**B**	Inappetence, dull, elevated liver enzymes	Liver	Alive
49	**N**	Laminitis	EHD	Dead due to extra-hepatic disease
50	**N**	Cataracts	EHD	Dead due to extra-hepatic disease
51	**N**	Dental disease	EHD	Dead due to extra-hepatic disease
52	**N**	Lameness	EHD	Dead due to extra-hepatic disease
53	**N**	Dental disease, laminitis	EHD	Dead due to extra-hepatic disease

This table provides tissue sample type, diagnosis/history/clinical signs and clinical outcome for the cases included in this study. B= biopsy sample, N= necropsy sample, NA= information was not available, EHD= extra-hepatic disease.

Appendix 2 Biochemical analytes

Analyte	Alb	A:G ratio	ALKP	AST	Ca	CK	Creatinine	GGT	Glob	Phosphate	Total bilirubin	Total protein	Trig	Chol	Urea	Na	Cl	K	Na:K ratio	SBA
Units	g/L	NA	IU/L	IU/L	mmol/L	IU/L	µmol/L	IU/L	g/L	mmol/L	µmol/L	g/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	NA	µmol/L

This table provides the list of routine biochemical analytes and their units of measure. Alb= albumin, A:G ratio= albumin:globulin ratio, ALKP= alkaline phosphatase, AST= aspartate aminotransferase, Ca= calcium (non-ionised), CK= creatinine kinase, GGT= γ-glutamyl transpeptidase, Glob= globulins, Trig= triglycerides, Chol= cholesterol, Na= sodium, Cl= chloride, K= potassium, NA:K ratio= sodium:potassium ratio, SBA= serum bile acids, NA= without units.

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