University of Veterinary Medicine Budapest
Department and Clinic of Equine Medicine

# Subclinical Hepatopathies in Herds of Horses in Ireland

By:
Orlaith O' Scanaill

Supervisors:
Dr. Zoltán Bakos, DVM, PhD, Associate Professor, Department and Clinic of Equine Medicine, University of Veterinary Medicine, Budapest, Hungary

Dr. Vivienne Duggan, MVB, PhD, Associate Professor in Equine Clinical Studies, University College Dublin School of Veterinary Medicine, Ireland

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## Abstract

Liver disease in horses is often associated with a guarded to poor prognosis. The liver is extremely regenerative and can tolerate up to 70% depletion without showing any clinical signs. This, while beneficial, can present obstacles to diagnosis and can lead to the recognition of hepatopathy being at an extremely late progression, negatively impacting prognosis. The aim of this study was to outline and evaluate the probable causes of subclinical liver disease in herds of horses in Ireland, and the methods for their diagnosis. This evaluation was carried out via a literature review as well as the evaluation of a case of subclinical hepatopathy in a herd of 14 livery and competing horses evidenced by elevated gamma-glutamyl transferase (GGT) enzyme concentrations in the serum.

A lovak májbetegségeinek kórjóslata gyakran kétes vagy rossz. A máj regenerációs képessége rendkívül jó, és az állat akár 70%-os működéskiesést is elvisel anélkül, hogy klinikai tüneteket mutatna. Bár ez a tulajdonság előnyös, de akadályozhatja a korai diagnózist, ami a hepatopathia progrediálásával annak rendkívül késői felismeréséhez vezethet, jelentősen rontva ezzel a prognózist. A jelen tanulmány célja az volt, hogy felvázolja és értékelje a szubklinikai májbetegség valószínű okait, valamint a kórjelzésükre szolgáló különféle diagnosztikai módszereket írországi lóállományokban. A munkát szakirodalmi áttekintésen és egy szubklinikai hepatopathia esetének értékelésén keresztül végeztük el egy 14 hobbi- és sportlóból álló állományban, amelyek szubklinikai májkárosodásban szenvedtek, amit a megnövekedett gamma-glutamil transzferáz (GGT) aktivitás bizonyított.

## List of Abbreviations

AAAs - Aromatic Amino Acids

ALKP - Alkaline Phosphatase

AST - Aspartate Transaminase

BA - Bile Acids

BCAAs - Branched-Chain Amino Acids

BH - Bacillary Haemoglobinuria

BSP - Sulphobromophthalein

EHCV - Equine Hepacivirus

EqPgV - Equine Pegivirus

EqPV-H - Equine Parvovirus Hepatitis

GGT - Gamma-Glutamyl Transferase

GLDH - Glutamate Dehydrogenase

ICO - Indocyanine Green

IEC - Irish Equine Centre

INH - Infectious Necrotic Hepatitis

LC-MS - Liquid Chromatography Mass Spectrometry

LDH - Lactate Dehydrogenase

MTL - Maximal Tolerated Level

PAs - Pyrrolizidine Alkaloids

PT - Prothrombin Time

SDH - Sorbitol Dehydrogenase

TD - Theiler's Disease

TDAV - Theiler's Disease Associated Pegivirus

## 1. Introduction

The liver of the horse is a multifunctional, complex organ that is involved in many different biological processes in the body [1, 2]. It accounts for approximately 1-2% of the adult horse's entire body weight, making it the largest internal organ of the body [1, 2]. The liver plays varying roles in digestion, immunity, and circulatory filtration [3]. These hepato-dependent biofunctions may suffer severe consequences when faced with insult to the liver [3].

The liver is the main organ responsible for the build-up, breakdown, and distribution of vital nutrients for other tissues in the body [1, 3]. In general, nutrients are taken in via the gastrointestinal tract, where they are absorbed by the bloodstream and travel to the liver via the portal circulation [2]. The liver breaks down these nutrients, repackages them, and transports them to the different tissues to be utilised [3]. [4]. Although other organs and organ systems (for e.g., respiratory, intestinal, musculoskeletal etc.) tend to be prioritised in equine clinical diagnoses due to their more frequent ailments, the clinical health of the liver remains critical to the healthy functioning of the body.

The liver is also responsible for metabolising various chemicals and substances that have been ingested by the horse [3]. This allows for their conversion into more effective forms that can be utilised by the body [3]. For example, phenylbutazone is a widely used non-steroidal anti-inflammatory agent in equine veterinary medicine [4, 5]. It is used as an orally or systemically administered analgesic, anti-inflammatory, and antipyretic agent [4, 5]. It is administered in the form of phenylbutazone which is taken to the liver where it is metabolised to oxyphenbutazone and gamma-hydroxy-oxyphenbutazone [5, 6]. This allows it to have a longer lasting effect in the body [6]. This is beneficial considering phenylbutazone has been proven to cause right dorsal colitis in horses when administered at high dosages frequently [5, 7]. By prolonging its effect, it allows us to administer the agent less frequently and therefore, lowers the chances of the drug causing harm to the animal.

Despite this advantageous characteristic, the liver is incapable of distinguishing between beneficial and harmful substances. By acting as the horse's first line of defence, the liver is susceptible to the absorption of, and initial exposure to, toxic chemicals that

have been taken into the body [5]. Ultimately, the liver is heavily affected by the horse's diet, supplements, and treatments [8].

When speaking of hepatopathy in herds rather than in individuals, most cases develop through the intake of toxic materials from plants, mycotoxins, chemicals, or medications that these horses have access or exposure to [9]. Nutritional contributors to liver disease can also be due to a lack thereof i.e. they can suffer hepatically when a dramatic decrease in their normal diet takes place, forcing the body to express energy from other sources such as fat tissue [10]. This leads to an increase in triglyceride levels in the blood and therefore in the liver,  in other words, causing hyperlipidaemia [10].

Liver deterioration of infectious origin has also been reported in horse herds be it bacterial, viral, or parasitic [2]. This can lead to severe hepatitis and hepatic dysfunction [2]. Infection may not initially stem from the liver directly and can begin in the gastrointestinal tract. An infection or inflammation here can lead to the overproduction of endotoxins [5] or, in the case of urease-producing bacterial infections, ammonia. These endotoxins or ammonia then travel to the liver, where the once efficient detoxification processes become overwhelmed. This can result in the appearance of endotoxins or ammonia in the blood i.e. endotoxemia or hyperammonaemia [11], which can have further consequences in the body.

The versatility of the liver depends on the impressive ability of its cells to repair and regenerate, as well as work more strenuously during the regeneration of neighbouring hepatocytes [2, 9]. This industrious trait is what is responsible for the majority of hepatopathies in horses being subclinical rather than clinical [2, 12]. Clinical signs will only begin to appear once roughly 60-70% of the liver cells are damaged or malfunctioning [2, 12, 13]. The disadvantage to this is that veterinary care and diagnosis of hepatopathies in horses can be incredibly challenging [9, 13]. Most cases go unrecognised, undiagnosed, and untreated until clinical signs begin to appear at a much later stage of disease and dysfunction [13], often negatively impacting prognosis.

The main clinical signs associated with liver disease are weight loss, anorexia, depression, gastrointestinal upset, hyperbilirubinemia (icterus or jaundice) and various

skin disorders [13]. Subclinical insult or injury to this vital organ will therefore likely lead to the suboptimal performance of the equine athlete.

Generally, the first method of diagnosis would be to carry out blood sampling, focusing on liver, muscle, and kidney function - better known as biochemical analysis [14]. This biochemistry blood result can tell the clinician whether certain enzymes are elevated or depressed in the blood serum. These enzymes can be liver-specific or non-specific [8, 13, 15]. Due to the variance in location of these enzymes, their elevation in the serum can indicate to the clinician whether the insult of the liver is at a hepatocellular level, at biliary level, or both [9, 15]. Some are released during hepatocellular damage including sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GLDH), aspartate transaminase (AST), and lactate dehydrogenase (LDH) [13, 15]. Others are released in biliary epithelial damage including gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALKP) [13, 15].

However, this is the first step of many in the diagnostics of equine hepatopathy as these enzymes may indicate hepatocellular injury without giving a full picture on the dysfunction of the organ [9]. In cases of chronic hepatitis, these enzymes may not become elevated at all due to the lack of living cells that would allow this spike to occur [15]. Further methods of diagnosis include liver function tests, ultrasonography, and hepatic biopsy [14]. Furthermore, these elaborate tests can give us a slightly clearer picture into the functionality of the liver [1, 13].

Thankfully, liver disease in a string of horses, rather than in individuals, is one of the rarer cases observed by equine veterinary practitioners on the island of Ireland. However, when practitioners are faced with these cases, diagnosis and treatment is significantly challenging [9, 12]. The further liver disease in a horse deteriorates, the worse the prognosis. Therefore, hepatopathy should always be considered by veterinarians as a possible differential diagnosis in herds and should ideally be investigated.

## 2. Objectives

The aim of this study is to identify and review the diverse contributors to subclinical liver disease occurring in herds of horses. The liver is an extremely diverse organ, having a vast number of responsibilities within all organ systems in the horse's body [2, 3]. Its dysfunction has detrimental consequences on those organ systems. Similarly, the dysfunction of those organ systems can also have a deleterious effect on the liver [3, 5]. Therefore, it is difficult to both pinpoint the exact cause of liver disease [9] and also to determine whether the liver injury is a primary or secondary cause of other issues facing the horse. To achieve this aim, this research tackles the following objectives:

Objective 1: Investigate naturally occurring substances in the horses environment that may be harmful to the liver. Ireland is very rich in pasture and many horses are kept outdoors to graze during the summer months. It is without doubt that horse owners face serious threats when unaware of the possible dangers of impure grassland.

Objective 2: Identify diagnostic tools that can be used in cases of subclinical hepatopathies in herds of horses, and review their prognostic potential. When it comes to the challenge of diagnosing subclinical liver disease in a herd of horses, there are various tools that can be utilised. These can be useful for diagnosis and prognosis, as well as aiding in the distinguishment of the possible contributors to the disease. The clinical tools evaluated in this study includes liver function and clearance tests, feed and forage sampling, ultrasonography, and hepatic biopsy. We aimed to gain a better insight into the prioritisation of such diagnostic methods in varying cases of hepatopathies in horses.

Objective 3: Outline the available treatment options and management plans for such cases of hepatopathy and evaluate their viability from the perspective of owners. It is without question that horse owners face a serious obstacle when a herd of horses has been diagnosed with liver disease. Not only is there the initial challenge of the recognition of the issue with a lack of clinical signs, but horse owners then face having to eliminate the possible cause as well as having to treat and manage multiple horses.

To fulfil these aims and objectives, we researched various literature sources, as well as evaluated a specific case of subclinical hepatopathy observed in a herd of 14 livery and competing horses in Dublin, Ireland. These horses experienced multiple periods of fluctuation in serum GGT concentrations, with some reaching dangerously high elevations. Various diagnostic tools were utilised in this case including blood biochemical analysis, forage testing, water testing, and liver biopsy. We evaluated the results gathered from the attending veterinarian and aimed to provide a clearer conclusion as to the root cause of hepatic insult in these horses.

# 3. Literature Review

## 3.1 Liver Enzymes

The enzymes present in the liver can be either hepato-specific or non-specific [13]. The liver-specific enzymes include gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), and sorbitol dehydrogenase (SDH) [13]. The enzymes that are non-specific to the liver include aspartate transaminase (AST), alkaline phosphatase (ALKP), and lactate dehydrogenase (LDH) [13]. These enzymes are what we evaluate in haematological biochemistry analysis [14, 16]. When these enzyme concentrations in the serum are abnormal, they can individually indicate potential health issues within the body. These enzymes are located intracellularly within their individual tissues of the body [2]. Their fluctuation in the blood serum indicates cellular membrane disturbance, through injury or an increase in permeability [2, 5]. Each enzyme being distributed in different amounts in various anatomical tissues, allows us not only to interpret whether cellular upset has taken place, but also which organ that cellular disturbance is likely to have occurred, as well as the extent of the damage [17]. The levels of both the specific and non-specific hepatic enzymes can narrow down our list of differential diagnoses when dealing with liver disease [17].

*Table 1. Anatomical distribution, stability, and half-life of liver enzymes in the body of the horse [17, 18]*

| | **Liver** | **Kidney** | **Pancreas** | **GIT** | **Muscle** | **Stability** | **Half-life** |
|---|---|---|---|---|---|---|---|
| *GGT* | ✓ | ✓ | ✓ | | | High | 3 days |
| *GLDH* | ✓ | | | | | Moderate | 12-24 hrs |
| *SDH* | ✓ | | | | | Low | <12 hrs |
| *AST* | ✓ | | | | ✓ | High | 7-8 days |
| *ALKP* | ✓ | ✓ | | ✓ | | High | 3 days |
| *LDH* | ✓ | ✓ | ✓ | ✓ | | Moderate | |

Serum elevations of non-specific hepatic enzymes such as AST and ALKP, indicate a more chronic issue within the organ, whereas an increase in liver-specific enzymes in the serum, such as GGT, SDH, and GLUT, indicate a more acute problem associated with the liver [15].

### *3.1.1 Gamma-Glutamyl Transferase (GGT)*

GGT is a liver-specific enzyme, despite its distribution in the pancreas, lungs, mammary gland, and kidney - specifically the proximal convoluted tubule [15, 17]. Its levels are markedly low in these organs in comparison to the liver [15]. In renal damage, GGT is thought to display an increase in urine rather than in the serum [14, 19].

This enzyme is located in the membranes of both hepatic and biliary cells and therefore, when increased, can indicate hepatobiliary damage [15, 19]. Elevated serum GGT concentration has been specifically linked to the ingestion of pyrrolizidine alkaloid producing plants [2, 19]. For example, ragwort (*Jacobaea vulgaris*), which is found very frequently in Ireland, is a pyrrolizidine alkaloid producing plant that can severely affect the liver of horses [20]. In neonatal foals, a significant increase in serum GGT concentration can be due to high amounts being present in the colostrum [15]. Measurements can be up to three times the reference range in foals and an observation of elevation should not be interpreted as liver disease [15].

A dramatic increase in the serum concentration of this enzyme indicates a poor prognosis however, moderate elevations may be present in very minor cases of liver damage, if any at all [14, 19]. Essentially, caution should applied upon diagnosis through the observation of this enzyme in the serum. In acute cases of cholestasis, elevations in other parameters may give us an indication or the prediction of an elevation in serum GGT concentration [19]. These include increased bile acids and direct bilirubin [19]. When elevated serum GGT is due to hepatocellular damage, it may indicate a more acute problem when compared to elevated AST and ALT. However, an elevation in serum GGT indicates more chronic issues when associated with the proliferation of the biliary epithelial cells [19].

Elevated serum GGT has also been associated with over-training of the equine athlete, specifically racehorses [19]. This is yet to be undisputedly proven and it is usually in combination with an increase in creatine kinase (CK) which is present in both the kidney and muscle tissues [19, 21].

### *3.1.2 Sorbitol Dehydrogenase (SDH)*

This enzyme is extremely liver-specific, with almost all abnormal serum concentrations being associated with problems on a hepatocellular level [15, 17, 19]. This makes SDH parameters in biochemical analysis extremely informative when speaking of liver disease [17]. The disadvantage faced by the testing of this particular enzyme in haematological serum is its stability - this enzyme has a low stability in the serum, meaning that it cannot withstand time and storage conditions well once the sample has been taken [17]. Measurements should be interpreted as soon as possible as with time, the level of the enzyme will give falsely depressed results [17, 22]. Its half-life is estimated to be less than 12 hours in the horse [15]. This being said, if we do see elevations in SDH in the serum, it is almost certain that the insult to the liver is an acute rather than chronic issue [19].

Although its elevation can strongly indicate liver disease, it is difficult to distinguish whether this is a primary or secondary issue, as an elevation of SDH can be observed in cases of gastroenteritis causing hepatic insult [23]. Much like GGT, elevations in serum SDH can be linked to cases of hepatotoxic plant ingestion, or mycotoxin poisoning [19].

### *3.1.3 Glutamate Dehydrogenase (GLDH)*

Much like SDH, this enzyme is also liver-specific [13]. This mitochondrially situated enzyme is more stable in the serum than SDH, with a half-life of 12-24 hours [15, 17]. Therefore, this is a more reliable result when elevated in the serum. However, as seen in *Table 1.*, it remains less stable than GGT, AST, and ALKP [15, 17]. Its leakage into the systemic circulation indicates an acute, and more profound liver disease [15]. The limitation to the measurement of this enzyme in the serum is its level in foals - they tend to be increased in lactating foals in the absence of any kind of liver disease [15]. Because of this, caution should applied upon interpretation of its elevation in the blood.

### *3.1.4 Aspartate Transaminase (AST)*

AST is non-specific to the liver and its elevation may indicate insult to either the liver, skeletal muscles, or cardiac muscle [17]. In comparison to that of SDH, AST is much more stable in the serum, with samples having a half-life of over a week in horses

[*Table 1*]. Despite this enzyme's serum elevation being present in almost all cases of hepatopathy, this tends to be only mild [15, 17]. Its elevation alone does not allow for a definitive diagnosis of liver dysfunction, as it is found in higher concentrations in skeletal muscle. Other differential diagnoses associated with the serum elevation of this enzyme are haemolysis through its presence in erythrocytes; or lipaemia, with its elevation occurring in both cases [15, 19]. Due to the serum elevation of this enzyme becoming obvious with skeletal muscle injury, a minor increase may be associated with animals receiving intramuscular administration of a substance [19]. Again, extreme caution is to be applied when interpreting serum AST concentrations, with thorough investigation into differential diagnoses required.

When investigating hepatic insult, it is useful to interpret both AST and SDH concurrently [19]. AST will elevate in the serum at a slower rate than SDH in case of liver disease whereas AST will have a much more prolonged elevation in comparison to SDH [19]. Evaluating both enzyme concentrations can ensure a diagnosis at a wider range of progression points.

### *3.1.5 Alkaline Phosphatase (ALKP)*

ALKP is present in the bone and liver where it is known as tissue nonspecific ALKP; and the gastrointestinal tract where its isoenzyme is specifically known and measured as intestinal ALKP [19]. The kidney in the horse is capable of expressing both of these ALKP isoenzymes [19]. In the liver, it is present in the biliary epithelial cells [18, 19]. Therefore, its elevation in the serum can indicate both bone metabolism and chronic hepatopathies in the form of biliary epithelial damage [18, 19]. Injury to the hepatocytes alone are not directly associated with serum elevations of this enzyme [18, 19]. Elevations of this enzyme in the serum are rarely associated with renal or gastrointestinal issues as the kidney does not generally release this enzyme into the blood, and the half-life intestinal ALKP is quite short [19]. Caution must again be applied with interpretation as the non-hepatic ALKP values extracted from the bones must be considered also.

Again, foals and young horses are a limitation with the measurement of this enzyme in the serum of horses. ALKP can have serum elevations up to three times the reference range which are associated with bone growth rather than biliary damage [15].

#### *3.1.6 Lactate Dehydrogenase (LDH)*

LDH is present in all tissues in the body of the horse [17] therefore, its elevation in blood biochemical analysis does not grant us with much information regarding the issue at hand. Because of this, we should request an isoenzyme profile of LDH from the laboratories. Isoenzymes are those of identical role and function, but with differing structure and tissue distribution [17]. The lactate dehydrogenase isoenzyme present in the liver, indicating liver dysfunction when elevated in the serum, is LDH-5 [24].

### 3.2 Poisonous Agents in the Liver

Toxicosis is the induction of disease or pathological condition brought on via a toxin or poison [25]. The majority of these toxins require metabolism and breakdown in order to have an effect on the body [2]. Because of this, many toxins taken in by the horse tend to significantly affect the liver, where its breakdown primarily takes place after travelling via the portal circulation [2, 26]. There are numerous agents that are hepatotoxic to horses. When speaking of hepatopathies that affect horses on a herd level, toxic agents present in the environment in which the herd are being kept play a huge role. These range from plants and forage, to mycotoxins, and even medications used for the treatment of other underlying issues.

#### *3.2.1 Hepatotoxic Plants*

Horses are herbivores and their evolutionary makeup is designed for them to graze almost 24 hours of the day. Although horses tend to avoid the majority of toxic plants, there are circumstances that increase the probability of their ingestion. This includes the scenarios of horses being kept on low amounts of pasture, being fed forage cut from fields containing these plants, and the fact that some of these plants can be favoured by the horse.

#### *3.2.1.1 Pyrrolizidine-alkaloids*

Pyrrolizidine alkaloids are metabolites produced by multiple species of plants to aid in their survival against grazing herbivorous mammals . These metabolites are extremely hepatotoxic to mammals, and horses are of no exception [27]. The most commonly found pyrrolizidine alkaloid plants in Ireland are of the genus *Senecio*, namely *Senecio vulgaris* - commonly known as 'Common Groundsel', and *Senecio jacobaea* - otherwise known as 'Ragwort'.

These plants are very resistant and seem to survive in the winters while thriving in the summers that the climate of Ireland has to offer [2]. These plants, specifically Ragwort, are one of the most well-known causes of liver cirrhosis in herds of horses [28]. Because of this, 'ragwort poisoning' tends to be one of the most commonly reported contributions to liver disease in herds of horses in Ireland. Despite this, recent studies suggest that this type of toxicity is not as common as first perceived [9, 28]. It is unclear why it is such a popular diagnosis among horse owners and veterinarians, perhaps it is due to its common growth throughout Ireland however, horses tend thankfully to avoid its bitter taste [2].

It has been found that measuring the serum GGT concentration of horses can be an efficient diagnostic tool in cases of pyrrolizidine toxicosis, specifically seneciosis [29]. The reference range for serum GGT measurement in the horse is approximately 5-20 IU/L [16]. In horses suffering from subclinical liver damage induced by pyrrolizidine alkaloid toxicosis, serum GGT concentrations above 50 IU/L may be observed [2, 29]. If horses continue to ingest these metabolites, be it through grazing or via impure forage, this liver damage may not remain subclinical for very long. Horses may begin to show signs of jaundice, alongside severe weight loss and depression. This may even progress neurologically with horses showing signs of hepatic leukoencephalopathy and even death [2].

The best treatment for these deadly yellow menaces is prevention. Clearing paddocks of all traces of such hepatotoxic plants prior to grazing or growing is of utmost importance when it comes to horses [2]. Pulling the plants by hand can be done, preferably after rainfall so the ground is soft, and roots can be easily removed with the plant [30]. It is

important to dispose of the plant appropriately as seeds can still spread to neighbouring forage and also hold toxicity [2, 30].

#### *3.2.1.2 Alsike clover*

Alsike clover, or *Trifolium hybridum*, is a flowering plant of the family *Fabaceae* [2]. It is commonly found in Ireland between the months of June and September - prime grazing time for horses and livestock [31]. This plant has been associated with hepatic disease in horses, specifically biliary fibrosis, through both pasture and forage nutrition [2]. *Trifolium hybridum* poisoning is more commonly due to its presence in hay, as horses tend to avoid the plant in pasture [2].

Alsike Clover poisoning, if continued, can manifest clinically. This can be seen in the form of type III (or hepatogenous) photosensitisation however, it is not yet confirmed whether these cases of photosensitization due to alsike clover poisoning are accompanied by hepatic insult [32]. This being said, horses being fed Alsike Clover tend to tolerate the toxicosis subclinical for a long period, some reports suggesting continuous feeding for up to a year before displaying clinical signs of hepatic insult [2, 32].

It is important to remove these plants from pasture being used for grazing and is of much more importance to refrain from growing any forage from pasture that contains such plants [31]. Certain broad-leaf herbicides are available for clover weed control that are also safe for horses.

### *3.2.2 Hepatotoxic Mycotoxins*

Several species of fungi can be found growing on different types of grain and forage that our horses consume. Mycotoxins are the secondary metabolites produced by these fungi [33, 34]. Initially, these metabolites were used as antibacterial, antiviral, and antifungal agents. They can, however, also be quite toxic to horses, causing devastating effects in some cases [9]. When horses develop hepatopathy, mycotoxins should arguably be considered higher on our list of differential diagnoses when compared with hepatotoxic plants [9]. A quarter of the world's crops are estimated to be affected by mycotoxins, posing a great health threat to humans and animals [9].

As mentioned previously, the liver is the first line of defence against poisonous agents, acting as their first metabolic and breakdown site [2, 26]. Due to this, mycotoxins are often associated with hepatopathy in horses [9]. Due to their monogastric nature, horses tend to be more heavily affected than ruminants [9]. It is important to note that there are several mycotoxins that can negatively affect horses' livers, most notably Aflatoxins, Fumonisins, Zearalenone, and Deoxynivalenol (DON) [34]. When these mycotoxins are associated with the diet of the horse, the problems in the liver typically resolve following the correct disposal and replacement of the affected feed or forage [9]. It is possible to find multiple species of mycotoxins in a single sample of feed or forage however, it is rare. In such cases, Aflatoxins and Fumonisins may be seen together while Zearalenone and DON can also be seen in combination [34].

The growth of these mycotoxins can occur at any stage from their growth as a crop in the field through to their processing and storage as feed or forage [35]. Their prevalence is catalysed by temperature, humidity, and pH. Warmer temperatures and higher moisture content is associated with an increased fungal growth and therefore, a higher production of these dangerous metabolites [35]. The maximal tolerated levels (MTL) of such mycotoxins are regulated by EU Directive 2002/32/EC [36].

A study was carried out in the United Kingdom by Durham et al where forage was sampled from 29 premises with horses suffering from liver disease and 12 control premises, for over 50 different species of mycotoxins [9]. The aim was to determine whether the hepatopathies experienced by these horses could be categorically linked to the presence of mycotoxins in the forage. Results showed that mycotoxins were present in 23 of the 29 premises affected hepatically however, they were also present in 10 of the 12 control premises sampled [9]. Through this, the study highlighted the sheer frequency of such mycotoxins in forage in the United Kingdom. With the similarity in climate and forage growth, it can be said that the abundance of mycotoxins in Ireland can be similar to that seen in this study. It was also concluded that specific species of mycotoxins known to have deleterious effects on the liver of horses, were found exclusively on the premises suffering from liver disease [9]. This shows that there may be an association between these forage mycotoxins and the depletion of the liver of the horses [9].

#### *3.2.2.1 Aflatoxins*

Aflatoxins are mycotoxins produced by various fungi, most significantly *Aspergillus flavus* and *Aspergillus parasiticus* [2, 37]. Aflatoxin production associated with the growth of *Aspergillus flavus* typically exacerbates in conditions of 30°C and a relative humidity of 85-95% [38]. When storing forage and feed, warm barns with poor ventilation and a high moisture content should be avoided, especially in the summer months [38]. It should be noted that the growth of Aspergillus species is not always associated with aflatoxin growth or hepatotoxic effects in horses fed such contaminated feed and forage.

The age of the horse can have an impact on the outcome of the toxicosis with younger horses being more susceptible to damage and clinical manifestation, including death [2, 37]. Much like with the ingestion of pyrrolizidine alkaloids, these mycotoxins inhibit the regenerative effect of the hepatocytes [2], as well as the synthesis of enzymes needed for metabolism in the body [38]. Centrilobular necrosis can be observed, with cells in these regions being replaced by inflammatory cells [2, 37].

Aflatoxins are composed of different groups, the four main groups being B1, B2, G1 and G2 [35, 37]. When speaking of cases of hepatopathy in horses, Aflatoxin B1 (AFB1) is of major significance [2, 37]. Its mechanism of action is via a cytochrome-P450 enzyme reaction which allows for the production of its most potent metabolite - 8,9-epoxide [2, 37]. This metabolite can covalently bond with portions of DNA to form DNA adducts, which can initiate the process of cancerous cell growth and further activation of AFB1 [2, 37]. The breakdown of this epoxide can lead to the production of dihydro-diol which is what causes the death of hepatocytes observed in such cases of aflatoxicosis [2, 37]. The specific CYP450 enzymes CYP3A4 and CYP2A6 are located hepatically and are involved in the metabolism of xenobiotics in the body. It is suggested that these enzymes can speed up the activation of the 8,9-epoxide metabolite of AFB1 [37].

The growth of AFB1 can be strongly linked to maize, wheat, and oats. In a study conducted in 2016 where 185 samples of wheat were tested, 50% of samples were contaminated with various species of fungi, 31% of which were of the *Aspergillus*

species [39]. 48 samples (26%) were shown to contain AFB1, with AFB2, AFG1 and AFG2 being present less so. However, only less than 3% of samples were shown to be over the maximal tolerated levels in accordance with EU Directive 2002/32/EC [35, 39]. According to this legislation, feed materials should not exceed 20 ppb of aflatoxin in order to be considered safe for consumption.

Similar to the majority of other mycotoxins, the most direct mode of treatment would be to remove the source of aflatoxin from the horse's diet; however, the effects of aflatoxins have also been shown to reduce with the administration of calcium aluminosilicates [34].

#### *3.2.2.2 Fumonisins*

Fumonisin is a mycotoxin commonly found in maize and its by-products. It is a secondary metabolite to fungal species *Fusarium verticillioides* and *Fusarium proliferatum* [2, 40, 41]. Fumonisins are linked to contrasting clinical manifestations in differing species [42]. In horses, it is linked to equine leukoencephalomalacia, due to their similarity in structure to sphingosine [2, 40, 41]. This is a sphingolipid found in the brain and is responsible for signal induction and cell recognition [43]. Because of this similarity, fumonisins are known to competitively inhibit the sphingosine N-acyltransferase enzyme that is responsible for the breakdown of sphingosine [43]. This leads to an increase in the levels of sphingosine in the animal [40] and a decrease in sphingolipids [2]. This depletion in sphingolipids can be the causative factor of hepatic damage that is shared by all mammal species suffering with such a toxicity [2].

Fumonisins can be broken down into four major groupings - FA, FB, FC, and FP [44]. Various relatives of such groupings exist including FA1, FA2, FB1, FB2, FB3 and FB4 [44, 45]. FB1 is of most significance in terms of toxicosis in horses. It can be found in grains other than maize such as wheat, barley, and soybean [44]. It comprises over 70% of the total FB genus [44]. Much like Aflatoxins, Fumonisins favour conditions of high temperature and moisture content [44]. However, heating grains to 160°C has been shown to reduce the levels of fumonisins [41, 44].

There is no direct treatment for such toxicity described however, horses suffering from subclinical hepatopathy associated with this toxicosis, like most mycotoxins, tend to do well once the feed or forage source of Fumonisin has been removed [2]. Horses displaying neurological signs tend to have a more guarded prognosis [40]. In accordance with EU Directive 2002/32/EC, the MTL of Fumonisins in animal feed is 5 ppm [36].

#### *3.2.2.3 Zearalenone and Deoxynivalenol*

Zearalenone (ZEN) and Deoxynivalenol (DON) are of huge significance when considering hepatopathies of horses as they appear in various plant sources including hay, feed, and even straw used in the stable [34]. This is exacerbated especially when stored in warm, humid conditions. They are produced by the Fusarium fungal species, namely *Fusarium graminearum* [33, 34, 46]. A study in Germany concluded traces of Zearalenone in 67% of rye grasses sampled [47] while another study in the United Kingdom found Zearalenone to be present in over 20% of samples, with an absence of any other mycotoxin contaminant [9].

Zearalenone has a similar structure to 17β-oestradiol, meaning that it can bind to the oestrogen receptors in the animal to cause estrogenic activity as well as hepatic toxicosis [33, 34]. Because of this, Zearalenone is known as a mycotoxin as well as a phytoestrogen or mycoestrogen [33, 34]. Due to its oestrogen-like structure, this mycotoxin is sometimes considered more beneficial than toxic when speaking of its useful anabolic activity in cattle and sheep [33]. It has also been used to suppress the symptoms experienced by women in stages of post-menopause, with the risk to human health being minor [33]. This suggests that hepatic toxicosis with Zearalenone may be distinguished from other mycotoxicosis through the presence of reproductive disorders.

Much like Zearalenone being known as a mycoestrogen, Deoxynivalenol in humans is known as a vomitoxin as well as a mycotoxin due to its emetic effects when consumed [46]. In horses, these effects are seen in the form of colic symptoms rather than vomiting [48]. It is suggested that this is due to the inflammation induced by the movement of the mycotoxins across the mucosa of the gastrointestinal tract which may affect protein synthesis [48]. Colic induced by other means may also contribute to

harmful effects of ingested DON as its metabolism to its less toxic de-epoxy-DON will be diminished [48]. The liver, like with all mycotoxin ingestion, will be affected primarily by DON however, there may also be secondary damage to this organ due to the inflammatory effect it has on the gastrointestinal tract. This is down to the liver being the first site for clearance of endotoxins in the case of malabsorption and function of an inflamed gut lining [48]. Taking this into account, when experiencing a case of horses suffering hepatically due to an ingestion of Deoxynivalenol, it can be hypothesised that it may be both accompanied and exacerbated by symptoms of colic.

### 3.3 Hepatitis

Hepatitis is a generalised term used for the inflammation of the liver, due to either infectious or non-infectious origin [2]. Hepatitis can be observed both diffused throughout the organ or found in more focalised regions [2]. Cholangitis refers to the inflammation of the biliary tract alone however, cholangiohepatitis can be used to describe inflammation of the biliary tract that has extended to the surrounding liver parenchyma [2]. Hepatitis is an umbrella term used to cover varying types of inflammation in the liver that differ according to their nature i.e., acute or chronic, location e.g. biliary epithelium or hepatocytes; and cause i.e., infectious or non-infectious [2].

Hepatitis can be induced by an abundance of causes. These contributors may be of a non-infectious origin such as pyrrolizidine alkaloid toxicosis however, other non-infectious insults to the liver tend to be of a degenerative nature rather than inflammatory [2]. This may suggest that the majority of cases of hepatitis tend be of an infectious origin however, it can be difficult to determine the exact cause of disease and, to the author's knowledge, statistical evidence on the most common causes of hepatitis in the horse has not yet been determined [49].

Hepatitis of infectious origin should be strongly considered as a possible contributor to hepatopathy in herds of horses, especially those residing on a shared grassland. These infections can be of a bacterial, parasitic, or viral nature and may also present concurrently.

### *3.3.1 Bacterial Infection in the Liver*

When it comes to the infection of the horse, those of a bacterial nature are common [2]. With the exception of peracute infection, it is rare for bacterial infections that cause any type of continuous or recurring bacteraemia, not to travel to the liver via the bloodstream to cause a deleterious effect [2]. This, in turn, suggests that almost all bacterial infections that result in bacteraemia for a significant amount of time can potentially lead to bacterial hepatitis in the horse if left untreated [2]. Bacterial infection may also travel to the liver following any form of damage or inflammatory process in the organ. If bacteria are already present in the horse's system when hepatitis occurs, it can travel to the liver and cause focal damage [2].

This being said, those bacterial infections that may eventually negatively affect the liver are rarely subclinical in the horse [2]. They tend to be diagnosed prior to the progression of hepatic disease and are subsequently treated with antimicrobial drugs. In other words, the progression to clinical bacterial hepatitis in the horse is uncommon. However, there is a certain amount of bacterial infection in the horse in which the liver is considered one of the primary target organs. The largest of these groups are Clostridial infections, but Salmonella infections may also be observed [2].

#### *3.3.1.1 Tyzzer's Disease (Clostridium piliforme)*

Despite the uncommon occurrence of bacterial hepatitis reported in horses, Tyzzer's Disease has been suggested to be the most common cause [50]. It is brought about via an infection with *Clostridium piliforme* and is generally seen in foals less than six weeks of age [2, 50, 51]. Mature horses are said to be resistant to this bacterium however, they can pass it into the environment via their faeces, which in turn can be ingested by young foals in which coprophagy can be commonly observed [50].

The pathogenesis of this disease it the initial ingestion of the spores by the foal, followed by the colonisation in the colon before entering the bloodstream to finally reach the liver via the portal circulation [2, 50, 51]. This is predisposed in immunodeficient animals and therefore, may occur following previous infections or immunosuppressive therapy [2, 50].

When speaking of subclinical hepatopathies observed in herds of mature horses, Tyzzer's Disease tends not to be the common contributor, with it affecting young foals (< 5 weeks) and resulting in sudden fatalities [2, 50].

#### *3.3.1.2 Infectious Necrotic Hepatitis (Clostridium novyi B)*

*Clostridium novyi* type *B* can cause infectious necrotic hepatitis (INH) in animals, otherwise known as Black Disease. However, despite its common manifestation among sheep and cattle, its occurrence among horses is reportedly less so [52].

*Cl. Novyi B* is a spore-forming bacterium present in the soil [52]. Once these spores are ingested by the horse, they are phagocytosed and persist in the macrophages, specifically in the liver, spleen, and bone marrow [52]. In other words, these spores can remain dormant until the optimal conditions are presented. They require an anaerobic environment in order to produce the alpha exotoxins needed to emit a necrotising effect on the liver cells [52]. These anaerobic conditions are usually brought about via prior migrating parasitic infections, namely Liver Fluke or *Fasciola hepatica* [2, 50, 51]. The final result of this is the infectious necrotic hepatitis of the horse. Because of this, INH tends to be seen in regions in which hepatic parasitic infections are endemic [2, 51].

#### *3.3.1.3 Bacillary Haemoglobinuria (Clostridium haemolyticum)*

Much like in INH, bacillary haemoglobinuria (BH) brought about by an infection of *Clostridium haemolyticum*, tends to be more commonly seen in cattle and sheep in comparison to horses [2, 51]. This bacterium is very similar to that of *Cl. novyi B,* and it has been suggested that they are derived from the same species [50, 51]. Again, exotoxins are produced and released by this bacterium following anaerobic conditions likely to have resulted from migrating parasitic infections in the liver. However, in this case beta toxins are responsible for the necrotic hepatitis and haemolysis that occurs [2, 51]. The addition of the necrosis of erythrocytes leading to haemoglobinuria [51], allows for an additional opportunity for diagnosis of this infection e.g. urine sampling.

### *3.3.2 Parasitic Infection in the Liver*

There are many different parasites that can present in the liver of horses through various methods of migration throughout the body [2]. These parasites are usually of helminth

form, the most common of which in horses is *Fasciola hepatica*, or Liver Fluke [2]. Other helminths can also be seen to affect the liver including *Dicrocoelium dendriticum* or Lancet Liver Fluke, *Capillaria hepatica* or Hepatic Capillariasis, and the migrating larvae of *Strongyle* species [2].

#### *3.3.2.1 Liver Fluke (Fasciola hepatica)*

*Fasciola hepatica* is also known as the common Liver Fluke. It is a trematode that mainly affects the biliary ducts of sheep and cattle, however, can also be observed in horses [2, 53]. Only one fluke is needed to cause an infestation in the horse by laying up to 20,000 eggs each day, rapidly accelerating their survival [2]. Their survival time in the host is astonishingly long and can last over a decade [2]. Their presence in the biliary duct may lead to cholangitis, which is a lack of bile flow from the liver [2].

*Fasciola hepatica* can be found in all regions around the world and typically favour the moist and temperate conditions in Ireland [54]. The most common intermediate host for liver fluke on the island of Ireland is *Galba truncatula*, which seems to be the case across the majority of countries in Europe [54]. Up until the 1960s, liver fluke was not thought to be common in Ireland and the UK but soon began to emerge in sheep and cattle mainly, before affecting donkeys and horses [53]. A study conducted in Ireland on a handful of sheep concluded a 62% presence of liver fluke within the flock, this is one of the highest percentages of liver fluke in a flock of sheep ever recorded in Europe [54, 55].

This being said, there is no doubt *Fasciola hepatica* has a strong presence on the island of Ireland and poses a threat to horses, both through their presence and survival in the climate of the country and via infection from livestock. Unfortunately, the diagnosis of this infection is challenging, with faecal egg count having little sensitivity in their detection [49, 56]. A previous study was carried out in 2016 where the “prevalence of liver fluke infection in Irish horses,” was researched [49]. It concluded that liver fluke was present in 19 of the 200 horses randomly sampled in an abattoir (9.5%) [49].

As mentioned previously, liver fluke is a common migrating parasite that can cause damage to the hepatocytes and lead to anaerobic conditions in the liver, which may be

favoured by certain hepatic *Clostridial* infections [2, 50, 51]. Despite the failure of a definite prevalence of infection with liver fluke in horses being determined [49], the presence of this trematode in terms of livestock in Ireland and the UK suggests this infection should be considered as a likely differential diagnosis in terms of hepatitis.

#### *3.3.2.2 Lancet Liver Fluke (Dicrocoelium dendriticum)*

The Lancet Liver Fluke or *Dicrocoelium dendriticum* has been seen in some countries with similar distribution of *Fasciola hepatica* however, this trematode favours less moist conditions [2]. It is mainly seen in ruminants, with its presence in horses less common [57]. Its mode of insult, similarly to *F. hepatica*, is in the biliary duct to cause cholangiohepatitis of less severity to the common liver fluke [2].

#### *3.3.2.3 Hepatic Capillariasis (Capillaria hepatica)*

*Capillaria hepatica* is one of few nematodes or roundworms that can be found in the liver in its mature adult stage [2]. The mature worm initiates hepatitis in the liver, in which the eggs are laid in clusters throughout the parenchyma of the organ. The risk of spread is low with the eggs remaining in the liver until the death ingestion of the animal by a predator [2]. The ingested unembryonated eggs are then passed into the environment via the faeces of the predator and the eggs can finally germinate. The larvae are then ingested by further hosts [2, 58]. The presence of *Capillaria hepatica* has not been reported widely in horses or livestock and is usually found in rodents [58]. Therefore, despite its hepato-damaging pathogenesis, is an unlikely contributor to hepatopathies in herds of horses in Ireland.

### *3.3.3 Viral Infection in the Liver*

Similar to bacterial infections, there are various viral infections that can have deleterious effects on the liver following persistent or recurring stages of viraemia however, there are certain viral agents that infiltrate the liver as a primary target organ [2] and those are what have been researched in this review.

#### *3.3.3.1 Serum Hepatitis (Theiler's Disease)*

Serum Hepatitis or Theiler's Disease (TD) was initially outlined by Sir A. Theiler in the early 1900s in a herd of horses after being administered a vaccination and antisera

against African Horse Sickness virus [2, 59]. Serum Hepatitis occurs in horses several weeks following the administration of an equine-origin biological product [59, 60]. Since the initial description of this disease in 1918, the condition has been associated with commonly administered biological products of equine origin including botulism and tetanus antitoxin, plasma, *Streptococcus equi* antiserum, and stem cells [60].

Much like other causes of hepatic insufficiency, the exact aetiopathogenesis of this disease has not yet been determined [59]. However, following next generation sequencing, four viral probable contributors to the disease have been determined. They include Equine Parvovirus (EqPV), Equine Hepacivirus (EHCV), Theiler's Disease-associated Pegivirus (TDAV), and Equine Pegivirus (EqPgV) [60]. In the first description of TDAV being associated with TD in 2013, it was observed that the horses were also serologically positive for EqPV [60].

Of the 4 viral infections, EqPV and EHCV have been shown to be more hepatotropic, meaning that they have more of an efficiency to the liver with it posing as the viruses' primary target organ [60–62]. Since the discovery of the virus, EqPV has been associated with an astounding majority of TD cases reported, making it the most probable cause to the phenomenon [60–62]. Equine serum products for commercial use in Europe, the US, Canada, and New Zealand were analysed. The results showed almost a 78% seropositivity and a 61% PCR positivity for EqPV [60]. This further suggests the strong association between EqPV-H and TD.

Despite their prevalence in TD, an infection of EqPV-H or EHCV may also occur independent of the administration of equine-origin biological products for e.g. via oral transmission from seropositive horses or as an arbovirus from arthropod vectors [60, 62, 63]. It is these routes of infection, independent of the administration of equine-origin biological products, that is of significance when investigating subclinical hepatopathies in herds of horses as TD is often associated with mortality, while seropositivity of EqPV-H independent of TD can be observed on a subclinical level in the horse, majority of which have hepatic biochemical parameters within the normal reference ranges [61].

#### *3.3.3.2 Equine Parvovirus*

Equine Parvovirus Hepatitis (EqPV-H) was initially identified following the death of a horse due to Theiler's disease in 2018 [60–62]. Since then, it has been described on various occasions in other cases of Theiler's Disease which suggests it being a likely contributor to the disease [60, 61]. In a study where 10 horses were experimentally inoculated with a strain of EqPV-H [62], 8 of those horses developed hepatitis of some kind (80%). This form of hepatitis was represented by lymphocytic infiltration and the necrosis of hepatocytes histologically, and the elevation of at least 2 hepatic enzymes above the normal reference range [62].

Despite the epidemiology of EqPV-H being in its preliminary stages the last six years, our understanding of the virus is growing rapidly. Research suggests that its occurrence and prevalence worldwide is much more common than initially thought [60]. Its lack of description and recognition in Ireland may be due to its subclinical manifestation in the horse. This highlights the importance of the consideration of EqPV-H in our list of differential diagnosis in cases of subclinical hepatopathies in horses, and its diagnosis prior to further progression of the disease.

#### *3.3.3.3 Equine Hepacivirus*

Equine Hepacivirus (EHCV) is a Flavivirus that has only been described as prevalent in horses in the last decade however, its presence has been observed worldwide in Europe, Asia, South America, and New Zealand [59, 64, 65]. The determination of seroconversion and viraemia varies between horses with the prevalence of viraemia in seropositive horses ranging from 2.1% to 40.8% [59]. This suggests that the seroclearance of the virus can vary between individuals. This is further highlighted by the persistence of viraemia reported in some infected horses for up to twelve years [59]. The presence of seroconversion of EHCV has been demonstrated with transient elevations in serum GGT concentrations [59].

The prevalence, aetiology, and pathogenesis of EHCV has been described even less-so than EqPV-H, making it difficult to distinguish whether it should be of concern for veterinarians facing cases of subclinical hepatopathies in horses in Ireland.

## 3.4 Diagnostic Methods Used in Liver Disease

As mentioned in the introductory section, liver disease fails to emit clinical signs in the horse unless up to 70% of damage to the organ has taken place. This rules clinical signs out as being a reliable diagnostic parameter, with clinical cases being associated with very late progression and guarded to poor prognosis. It is without doubt that the majority of hepatopathies being subclinical, are diagnosed solely based on haematological testing, ultrasonography, and hepatic biopsy.

### *3.4.1 Liver Function and Clearance Tests*

The measurement of various substances in the circulation of the horse can tell us many things about the health state and functionality of the liver. The liver is responsible for synthesising, excreting, and metabolising many different biological substances in order for the body to function appropriately. By measuring the levels of these substances in the blood, we can evaluate the efficiency of the liver to carry out these functions.

#### *3.4.1.1 Enzymology*

As mentioned previously, there are many hepatic enzymes present in the hepatocytes of the liver. If these hepatocytes are to undergo damage of any sort, certain enzymes can be released into the circulation. These elevations may be picked up through haematological testing and allow for a clearer picture on the location of damage faced by the liver. For example, elevations in serum AST particularly, would indicate damage at a hepatocellular level, while elevations in serum ALKP and GGT would indicate the damage is in the biliary epithelium, namely cholestasis[13, 15, 66]. The measurement of these enzymes falls under ‘Liver Function Tests’ but despite the name, the appearance of these enzymes fails to give us an idea of the overall functionality of the liver but rather only indicate hepatic insult of some sort, with the severity of elevation indicating the progression of the damage. Functionality is highlighted more thoroughly through the measurement of bilirubin, prothrombin time, and albumin levels [67].

As indicated in *Table 1.*, elevations in these liver enzymes should not rule out problems independent of the liver as these enzymes can be found in various tissues in the body. This explains why an elevation in ALKP alone does not immediately indicate liver damage. Its measurement in the serum should always be accompanied by that of GGT

which, in elevations of ALKP caused by subclinical liver disease, is usually also elevated in the serum. The appearance of hepatic enzyme elevation in the bloodstream may take a while and therefore, usually only occurs in cases of chronic liver disease, especially the appearance of GGT. This, when combined with the lack of specificity to the liver, makes these liver function tests less reliable when carried out individually. They should ideally be taken in conjugation with other liver function or clearance tests.

#### *3.4.1.2 Bile Acids*

Bile acids (BA) are proteins synthesised by the liver cells through the breakdown of cholesterol, before being excreted from the liver in bile [2, 17]. Small amounts, like bilirubin, are recirculated back to the cells of the liver to be reprocessed again [68]. In cases of liver disease, these bile acids would be increased in the serum due to either a lack of bile acid re-uptake by the liver from the bloodstream, or a lack of excretion of bile acids in the bile, usually due to cholestasis [68]. In other words, serum BA concentrations would be elevated due to the lack of hepatic clearance. The measurement of serum BA therefore falls under the term 'Liver Clearance Test'. The difference between the elevation of hepatic enzymes and of bile acids is that the enzymatic elevation is due to hepatocyte damage releasing the enzymes into the portal circulation whereas the elevation of BA is due to the lack of clearance [69, 70]

#### *3.4.1.3 Bilirubin*

The biological process of bilirubin begins with the breakdown of haem particles to biliverdin, and then to unconjugated bilirubin (indirect bilirubin). This form of bilirubin is not water-soluble and in order for it to enter the bloodstream, it is bound to the protein albumin. This form of unconjugated bilirubin is incapable of being processed by the kidneys. It is transported back to the liver and taken up by hepatocytes to bind glucuronic acid. This is how conjugated bilirubin, or direct bilirubin, is produced. Direct bilirubin then travels to the blood. From there, it is secreted in the bile to be excreted as faeces, or it travels to the kidneys to be excreted as urine. However, small amounts are recirculated back to the liver to be reprocessed [68, 71].

Hyperbilirubinemia can be in the form of indirect (unconjugated) bilirubin, or direct (conjugated) bilirubin in the bloodstream [68, 71]. In order to measure the indirect

bilirubin, we must measure the total bilirubin using the Diazo method, using alcohol. The alcohol allows the detection of the non-water-soluble indirect bilirubin. We then calculate the indirect bilirubin level by subtracting the direct bilirubin from the total bilirubin [68, 71]. The direct bilirubin is measured using the van den Bergh reaction with an aqueous solution. Elevated levels of indirect bilirubin can be associated with decreased uptake of bilirubin by the hepatocytes in the liver once haemolytic anaemia has been ruled out. Elevated concentrations of direct bilirubin are due to a lack of excretion of bilirubin in the bile, usually attributed to a lack of bile flow [68, 71]. In other words, in cases of hepatocellular hepatopathies in horses, elevated levels of total bilirubin may be seen, while in cases of cholestasis in horses, elevated levels of direct bilirubin may be observed [68, 71].

It is important that, when evaluating the levels of bilirubin in the horse, other possible causes of said elevations would be ruled out. Bilirubin levels in the horse may increase in cases of starvation, pneumonia, and gastrointestinal impaction. Direct bilirubin levels are less sensitive to these non-hepatic influences. As opposed to other biomarker abnormalities mentioned, elevations in bilirubin levels in the serum are usually attributed to acute issues in the liver of horses with less dramatic increases being observed in cases of chronic liver disease [72].

#### *3.4.1.4 Albumin*

Albumin is a protein synthesised by the liver and enters the bloodstream bound to unconjugated bilirubin [71]. A decrease in albumin levels may indicate hepatic dysfunction. The downsides to solely measuring albumin levels in order to diagnose liver disease is that albumin has quite a long half-life in the bloodstream [71]. This means that the decline in albumin may take longer to show on biochemistry blood results. In other words, decreased serum albumin levels will not be observed in cases of acute liver disease compared to that of chronic liver disease. Albumin levels in the serum are not 100% reliable and should be measured alongside other liver disease biomarkers, such as GGT, which is usually elevated in cases of chronic liver disease; direct bilirubin, and prothrombin time [71].

#### *3.4.1.5 Prothrombin Time*

The liver is also responsible for producing certain metabolic proteins needed for the synthesis of red blood cells and haemoglobin [71]. These include clotting or coagulation factors, and amino acids. Hepatic insult may lead to a decline in their appropriate production by the liver, leading to issues such as anaemia and haemoglobinuria [73]. In order to access this, measuring the prothrombin time (PT) can be extremely useful. This is done by measuring the time it takes for a clot to form in a blood sample i.e., measuring the presence of coagulation factors in the blood [73]. This is more useful in cases of acute liver disease in comparison to albumin, as these have a shorter half-life in the serum. The elongation of PT can also be due to a lack of vitamin K, which is needed for coagulation factor synthesis [73]. Once starvation and malabsorption in the horse has been ruled out, this lack of vitamin K can be down to cholestasis being present in the liver, which prevents the absorption of this fat-soluble vitamin [71, 73].

#### *3.4.1.6 Endogenous Compounds*

The functionality of the liver can also be tested through the clearance of certain endogenous compounds administered intravenously and known to be excreted into the bloodstream solely by the liver. In the past, the substance Sulphobromophthalein (BSP) has been used however, its administration has been linked to fatalities in horses and is no longer recommended [72]. When used, it was administered intravenously and sampled 10 minutes later. Its retention in the horse should be little to none [73].

Indocyanine Green (ICO) has been used in various studies of different species to determine the functionality of the liver via its clearance. ICO is a tricarboxylic acid that is excreted entirely by the liver. When injected intravenously, we are able to measure its excretion and therefore, the liver's functionality [69, 74].

#### *3.4.1.7 Urea and Ammonia*

Theoretically, low levels of urea in the serum along with hyperammonaemia should accompany cases of hepatic insufficiency in horses. This is down to the liver being responsible for the breakdown of ammonia to urea before it is excreted in the bloodstream to the kidneys [14]. However, studies have suggested a lack of association between serum urea and ammonia concentrations with liver disease in horses [75]. Not

all cases of hepatopathy in horses may be accompanied with a decreased serum urea however, its presence may indicate a more guarded prognosis and more advanced progression [14]. A presence of hyperammonaemia is usually accompanied by hepatic encephalopathy due to the permeability of the blood brain barrier to ammonia in the plasma [76]. In other words, when increased levels of ammonia in the bloodstream is associated with hepatic insufficiency, it is rarely subclinical.

In another study, in cases where a low serum urea was observed in horses suffering from liver disease, low serum creatinine was also occasionally observed [77]. This may indicate that the low levels of urea in the bloodstream may be attributed to the polyurea experienced due to polydipsia induced by liver disease in some horses [78, 79].

#### *3.4.1.8 Amino Acids*

Certain aromatic amino acids (AAAs) are broken down or metabolised in the liver after being transported from the bloodstream. These include tyrosine, tryptophan, and phenylalanine. In cases of hepatic insufficiency, the serum levels of AAAs increase due to a lack of clearance and thus, a lack of metabolism, by the liver [14, 80].

In opposition to this increase, a decrease of branch-chain amino acids (BCAAs) such as leucine, isoleucine, and valine; may be observed, secondary to the muscular breakdown associated with hepatic insufficiency [14, 80]. Unfortunately, the haematological testing of amino acid levels in the horse is of limited availability and therefore, rarely utilised as a diagnostic tool in cases of hepatopathy, despite its usefulness and specificity [14]

### *3.4.2 Forage and Feed Sampling*

As outlined in section 3.2, there can be naturally occurring hepatotoxins present in both forage and grains. The testing of such substances is available and can be extremely useful as a diagnostic tool in subclinical hepatopathies in horses. Hepatotoxins being present in forage, feed, or grassland make them more likely in affecting herds of horses than just individuals.

#### *3.4.2.1 Sampling for Hepatotoxic Plants*

When it comes to the ingestion of hepatotoxic plants in the horse, they tend to be unpalatable and therefore, avoided. However, their ingestion is more probable if the plants are dead and dried in the grassland, or the horse has been fed with forage grown and harvested from a field containing such plants. As outlined in section 3.2.1.1, the best treatment for this type of toxicosis is the initial prevention. This can easily be carried out by visually analysing fields used for both grazing and forage growth, to ensure the prevention of their presence in the horse's diet. Visual analysing of fields can also be used as a diagnostic tool in horses displaying signs of subclinical liver disease, such as elevations in hepatic enzymes.

Because hepatotoxic plants can be of harm to humans as well as animals, methods of identification of such plants in food, forage, and grain have been established. In terms of pyrrolizidine alkaloids, new restrictions have been put in place in the European Union to limit the maximum levels of these alkaloids in certain foods. This is outlined in Commission Regulation (EU) 2023/915 [81].

In a study conducted in 2021, liquid chromatography mass spectrometry (LC-MS) and its usefulness in the identification of toxic alkaloids in forage grass, was researched [82]. This was carried out using 134 samples of forage grass, testing for 15 species of toxic alkaloids, pyrrolizidine alkaloids included. It was concluded that the method researched, based on QuEChERs protocol and LC-MS was accurate and efficient in the identification of these substances [82].

In a volume of the 'Journal of Natural Products' published in 1987, two possible field tests have been outlined that allow for the testing of, mainly, unsaturated PAs and their nitrogen-oxides [83]. However, they may be slightly outdated and are not equipped for the quantitative testing of PAs but rather their occurrence only. They also fail to test for saturated PAs in the sample [83].

#### *3.4.2.2 Sampling for Hepatotoxic Mycotoxins*

While visual analysis of the field is much more practical in comparison to the testing of contaminants in terms of hepatotoxic plants, when it comes to hepatotoxic mycotoxins,

testing forage or feed samples can be more straightforward [84]. The methods of identification of mycotoxins in a sample were researched in a study conducted in 2019, where the practicality of sample methods was evaluated based on their performance, speed, and cost [84].

It is without doubt that the sampling of forage and feed for the presence of mycotoxins is an excellent tool for both diagnosis of hepatopathies faced by herds of horses, as well as a prevention tool.

### *3.4.3 Ultrasonography*

Ultrasonographic imaging is widely used in cases of hepatopathies in horses, as the liver lies behind the diaphragm and is too far cranial to be examined via transabdominal or rectal palpation in the horse [85]. Horses suffering from subclinical liver disease, may be recognised incidentally via blood biochemical analysis. Ultrasound imaging is a useful follow-up tool to evaluate the physical state of the liver [79].

The liver should look similar in texture to the spleen on the ultrasound image, but slightly denser and less echogenic. Any abnormal mass including an abscess, haematoma, tumour, cyst, or cholelithiasis in the liver, can be seen and distinguished quite clearly using an ultrasound scanner. Inflammation in the liver may be demonstrated as a mottled image with round rather than sharp lobular edges [86].

A study was published in The Journal of the Australian Veterinary Association Ltd. in 2016 where the “ultrasonographically visible hepatic locations in clinically normal horses” [85] was researched. It concluded that the liver can be visualised using ultrasound imaging from the right side in 97% of cases, from the left side in 71% of cases, and from both sides in 67% of cases. This study also concluded that the liver can be localised between the 5th and 16th intercostal spaces from the right side, and between the 4th and 11th intercostal spaces from the left [85].

Ultrasound imaging can be carried out alone or as an aid to other diagnostic tools such as hepatic biopsy, acting as a guide for biopsy needle insertion [79]. In a study conducted in 2014, on the “ultrasonographic visualisation of the liver in sites

recommended for blind percutaneous liver biopsy in horses" [87], it concluded that less than 40% of the horses examined ultrasonographically displayed liver tissue in all intercostal spaces recommended for blind percutaneous liver biopsy. Ultrasound guidance is also useful in the removal of the biopsy needle from the liver, to ensure there is no haemorrhaging in the organ [87]. This outlines the importance and value of ultrasound guidance in hepatic biopsy sampling.

Ultrasound imaging is a non-invasive diagnostic method and is rarely affected by hair in horses with an average length coat [86]. It has become more economically efficient in recent years, much more portable, and its results are instantaneous [86]. Unfortunately, not all cases of hepatic disease, especially those of a subclinical nature, will show physical abnormalities on ultrasound. In other words, the sensitivity of this diagnostic method is low. That being said, there is an 86% specificity of ultrasound imaging of the liver, suggesting that those that do display abnormalities on ultrasound, tend to be suffering from hepatic insult of some kind [85].

There is no doubt that ultrasound imaging in cases of hepatopathy should be strongly considered by veterinarians as a diagnostic tool; however, clinical examination of the liver using this technique should be observed carefully, ensuring the lack of abnormalities does not rule out dysfunction of the organ [79].

### *3.4.4 Liver Biopsy*

When it comes to hepatopathies in horses, hepatic biopsy is regarded as the most informative among antemortem diagnostic parameters used. It is thought to be the most sensitive and specific [87]; however, it is also one of the most invasive and requires a skilled hand to be carried out. As previously mentioned, it is highly recommended for hepatic biopsies to be carried-out under ultrasound guidance [79, 87].

#### *3.4.4.1 Localisation*

In a study published in 1997 where the "liver biopsy techniques for adult horses and neonatal foals to assess copper status" [88] was researched, liver biopsies were carried out on 20 of 24 thoroughbred mares. In this study, it was determined that the optimal insertion point of the 14-gauge Tru-Cut biopsy needle used, would be between the 12th

and 14th intercostal spaces on the right side and within a triangle extending from the tuber coxae to the proximo-caudal point of the olecranon, to the scapulohumeral joint and back to the tuber coxae [88]. It was found that this target area for needle insertion was inappropriate for 4 of 24 mares evaluated (17%) due to the presence of intestinal tissue ultrasonographically, rather than liver tissue. In 2 of the 20 mares that were liver biopsied, intestinal content rather than liver parenchyma was aspirated (8%) [88]. This leaves this anatomical localisation for biopsy needle insertion with a 75% success rate of suitable hepatic biopsy samples obtained in this study.

Upon visually outlining the approximate area, the ultrasound is placed on the horse. This allows for individual differences between horses to be observed. In order to determine the exact optimal area for needle insertion, two characteristics on the ultrasound should be evaluated [85]. The first is to observe the area of the liver which has the most depth in tissue to avoid the needle perforating through to the other side but is close enough to the abdominal wall to ensure the needle can reach it. The second point to consider is the presence of hepatic blood vessels [85].. It is best to avoid blood vessels to ensure the aspiration of liver parenchyma rather than blood, and also because in some cases of hepatopathy, the synthesis of coagulation factors may have diminished which can lead to haemorrhage if blood vessels are perforated.

#### *3.4.4.2 Complications*

The main risk in terms of hepatic biopsy, is haemorrhage. Hepatopathies are often seen in conjunction with coagulopathies, due to the lack of coagulation factors being synthesised by a dysfunctional liver [89]. Because of this, it is recommended to evaluate the coagulation status of the horse prior to the procedure [89].

It is also essential for the insertion site to be aseptically prepared as it is possible for infection to occur in the pleural or peritoneal cavities once the biopsy needle has been inserted [12, 17].

Another complication that may occur is the perforation of the large colon. Not only would this give a suboptimal tissue sample, but it may also lead to the leakage of colon content into the abdominal cavity, leading to possible peritonitis and colic [17]. This can

be avoided by ensuring the procedure is ultrasound guided and is not carried out if intestines are present between the abdominal wall and liver on ultrasound [79].

#### *3.4.4.3 Diagnostic Potential*

Hepatic biopsy can be extremely useful for diagnosing specific problems in the liver. For example, in cases of pyrrolizidine alkaloid toxicosis in a herd of horses, centrilobular necrosis is characteristic along with portal fibrosis. It also has good prognostic potential by evaluating the extent of fibrosis and necrosis of the organ and therefore, informing us on the progression of the disease. Because of this, hepatic biopsy has been titled the 'gold standard' in diagnostic value [90] as it is capable of recognising the presence of liver deterioration, highlight a specific diagnosis, determine a probable prognosis, and therefore allow guidance for optimal therapeutic and management plans.

# 4. Materials & Methods

## 4.1 Literature Review

In order to begin the literature review section of this thesis, I worked closely with my supervisors to discuss the topics associated with hepatopathies that affect herds of horses on a subclinical level in Ireland specifically. The topics that we decided to research were based on the guidance from my supervisors, books accessed in the library of the University of Veterinary Medicine Budapest, animal disease surveillance reports published by the Department of Agriculture, Food & Marine in Ireland, and equine infectious disease surveillance reports published in the United Kingdom. From there, we were able to decide which topics we would like to research and create a layout for the literature review that would best suit the evolution of this research in the thesis.

To begin, we became familiarised with various online websites and scientific publication databases such as Google Scholar, PubMed, ResearchGate, and ScienceDirect. We were able to access numerous published writings including review papers, studies, articles from journals, and extracts from books. I was granted this access through the library of my university. There is no doubt that upon searching these databases, there was a plethora of publications regarding hepatopathies and liver disease in horses – the causes and contributors, how those causes lead to the dysfunction of the organ, the diagnostic methods that can be utilised, and the prognosis of the disease.

To identify specific cases in Ireland, we searched these databases as well as reviewing disease surveillance reports in Ireland from the last 5 years. It was quickly realised that there was a lack of reported cases of hepatopathies in horses in Ireland. It is unsure whether this is due to hepatopathies being low in number in recent years, if the majority of them being subclinical means they tend to go unrecognised or, if they are so little understood that veterinarians fail to diagnose them. By researching publications on hepatopathies in horses worldwide, we were able to broaden our access to writings which were of valid information in this area.

Upon reviewing the various publications on numerous topics related to our title, we were then able to piece together information that best correlated with our chosen headings and subheadings in our literature review. In order to reference these

publications correctly, we used the Zotero computer application. I worked closely with my supervisors at various times throughout the study to ensure the writing and relevance of the literature review section of this thesis was adequate. This was done via email, Zoom Meetings and through the use of shared Google Docs.

## 4.2 Case Study

A case study review was conducted of a herd of horses situated in Dublin who experienced subclinical hepatopathies between 2019 and 2022. This case was made up of a herd of 14 livery and competing horses of various breeds, sex, age, and athleticism, who were experiencing dramatic fluctuations in serum gamma-glutamyl transferase concentrations that varied from within normal reference range to extremely elevated. Upon establishing the normal reference range for serum GGT concentration in the horse, a key was developed for which results would be grouped into. Those groups included mildly elevated, moderately elevated, greatly elevated, and extremely elevated. The attending veterinarian had concluded that the cause for the fluctuations in serum GGT concentrations was the presence of aspergillus fungi in some of the haylage being fed to the horses.

### *4.2.1 Diagnostic Data Collection*

We worked closely with the attending veterinarian of this case who was able to provide us with all the scientific and diagnostic data they acquired throughout the three years. This diagnostic data included serum GGT measurements, feed sampling, forage sampling, water sampling, and images of plants from a field used for grazing of horses and haylage production on the premises. All blood samples were brought to the practise of the veterinarian to be placed into the blood biochemistry analysing machine, in order to detect the GGT concentrations in the serum. All feed, forage, and water samples were sent to the Irish Equine Centre (IEC) to be processed and reported back to the veterinarian. We gathered all reports and results from all diagnostic tests carried out, from the veterinarian.

#### *4.2.1.1 Serum GGT Measurements*

We gathered results from the multiple serum GGT measurements taken from all 14 horses. We were able to place these results into tables using Microsoft Word, and

graphs using Graph Prism 10 and Microsoft Excel. This allowed us to readily interpret the fluctuations in all horses simultaneously, and how they correlate with each other. For the ease of this paper, the 14 horses were labelled from A to N for the course of the study. We were able to place the dates of blood sampling of each horse into a table, as well as their serum GGT concentrations on each date. We then placed these results into bar chart graphs and linear graphs to visualise the fluctuations of each individual horse, as well as the correlations in comparison to each other in order to determine whether there was a trend in fluctuations.

#### *4.2.1.2 Feed & Forage Sampling*

The feed and forage samples gathered by the veterinarian included samples from the haylage stores, haylage in the stables of the horses, bedding in the stables of the horses, dry feed cubes, wet pulp feed, feed mix from one feed-bin (Bin 1), and feed cubes from another feed-bin (Bin 2). These samples underwent environmental monitoring which tested for traces of pathogenic and non-pathogenic fungi, for the presence of mycotoxins produced by such fungi. The mycotoxins tested included Aflatoxin, Ochratoxin, Fumonisin, Deoxynivalenol, and Zearalenone.

#### *4.2.1.3 Water Sampling*

The water samples were taken from three separate water sources being used on the premises. These included water from the mains pipe, water from the mains tap, and water from the well on the premises. These samples underwent microbiology testing via membrane filtration. This allowed for the testing of the total *Escherichia coli* content in the sample, the total number of coliform bacteria in the sample, the detection of *Escherichia coli* at 44°C, the detection of enterococci bacteria, and the total bacterial counts at 22°C and at 37°C.

The water samples also underwent environmental monitoring, in which various parameters within the water samples were tested. This included the testing of the pH of the water, the hardness of the water, the nitrite and nitrate levels, and the alkalinity. The mineral contents of these samples were also detected. These included aluminium, arsenic, calcium, cobalt, copper, iron, lead, potassium, magnesium, manganese, selenium, sodium, and zinc.

#### *4.2.1.4 Visual Field Analysis*

The veterinarian also provided us with images of certain plants found in the fields used for both grazing of horses and haylage production. It is unsure whether all, or any, of the horses who displayed elevations in serum GGT values were grazed on the field analysed at any time during the case. We evaluated these images and identified them using PlantNet in order to verify whether hepatotoxic plants were present.

### *4.2.2 Presentation of the Data*

#### *4.2.2.1 Serum GGT Measurements*

Upon gathering the data, we were then able to place the results of the serum GGT concentrations into tables and graphs. As mentioned previously, these tables were created using Microsoft Word, and the graphs were created using Graph Prism 10.

The tables created demonstrate the following:

| | |
|---|---|
| *Table 2.* | Dates at which blood samples were taken from horses A-N |
| *Table 3.* | Serum GGT concentrations of horses 'A' and 'B' in 2019 |
| *Table 4.* | Serum GGT concentrations of horses 'A' and 'B' in 2020 & 2021 |
| *Table 5.* | Serum GGT concentrations of horses 'A' and 'C-N' from 2020 to 2022 |
| *Table 6.* | Key to *Table 5.* |

The graphs created demonstrate the following:

| | |
|---|---|
| *Graph 1.* | Bar chart of serum GGT concentrations of horses 'A' and 'B' in 2020 & 2021 |
| *Graph 2.* | Line graph of serum GGT concentrations of horses 'A' and 'C-N' from 2021 to 2022 |

The creation of both tables and graphs to represent and demonstrate the results gathered, made it easier to interpret the results as well as clearly highlighted fluctuations in individual horses and correlations between fluctuations within the herd of 14 horses.

#### *4.2.2.2 Water Sampling*

The interpretation of the results obtained from the water samples gathered, were divided into the environmental monitoring results and the microbiology results. Again, tables were used to demonstrate the results obtained. These tables are listed opposite.

| | |
|---|---|
| *Table 7.* | Environmental monitoring results from water samples taken 11.10.2021 |
| *Table 8.* | Microbiology test results from water samples taken 11.10.2021 |

#### *4.2.2.3 Feed & Forage Sampling*

After gathering the reports with results of the samples collected, from the veterinarian, we were able to create 2 tables in which the results of the feed and forage taken on two separate dates are demonstrated. These tables are listed below.

| | |
|---|---|
| *Table 9.* | Environmental monitoring results from feed & forage samples taken 26.11.2021 |

#### *4.2.2.4 Visual Field Analysis*

Upon the identification of plants pictured by the veterinarian, we demonstrated the results on a singular table. The table was titled as follows:

| | |
|---|---|
| *Table 11.* | Plants identified from a field used for grazing and haylage production on site |

The representation of the diagnostic data gathered from the veterinarian using tables and graphs, allowed us to interpret the results much more readily, as well as highlighted the situation within the herd in this case.

### *4.2.3 Interpretation of the Results*

In order to interpret the results of each of the diagnostic tests carried out, we distinguished whether the results were abnormal. To do this, we obtained the normal reference range of serum GGT concentration in the horse, the concentration at which non-pathogenic fungi can cause harm, the maximal tolerated levels (MTLs) of mycotoxins in feed and forages, the MTL of bacteria in water samples, and finally the suggested maximal limit of minerals and metals in water. We also researched whether the plants identified posed a threat to the liver of horses. These concepts were researched using books, publications, and EU legislation.

# 5. Case Study

In this study we evaluated a case of 14 horses from a yard in Dublin. All horses, at some point, suffered from an elevation in serum GGT concentration. This was first observed by the attending veterinarian in June 2019, where blood samples were taken of a particular horse (horse 'A') to evaluate their overall health. Incidentally, an elevated serum GGT concentration was observed and an investigation into these horses began. The number of horses being sampled gradually increased throughout the case, with the final horse being sampled in June 2022. All participants of this case including the veterinarian, the veterinary practise, horse owners, and premises owner have been kept anonymous.

## 5.1 Diagnostic Methods Utilised & Their Results

Upon the recognition of an elevated GGT concentration in horse 'A' in June 2019 despite a lack of clinical signs, many of the diagnostic methods outlined in the literature review section of this paper were utilised by the attending veterinarian. They included the further measurement of serum GGT concentrations in 14 horses including horse 'A', feed and forage sampling, water sampling, and visual field analysis.

### *5.1.1 Serum GGT Measurements*

A total of 212 blood samples were taken from 14 horses across the three years of this case. The dates each horse was sampled is demonstrated in *Table 2.* In this table red lines represent a period of time between two blood biochemistry samples ranging from nine to twelve months. The yellow highlighted areas on the table represent the dates between which the water samples on the premises were acquired. The areas highlighted orange in the table represent the dates between which the feed and forage samples of the premises were acquired. For the ease of this thesis, the horses have been named A to N according to the alphabet. The horses can be found along the top of the table, while the dates can be found along the side of the table.

*Table 2. Dates of serum GGT concentrations acquired from each horse by the veterinarian*

| HORSE: | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13.06.19 | ✓ | | | | | | | | | | | | | |
| 15.07.19 | ✓ | | | | | | | | | | | | | |
| 08.08.19 | ✓ | | | | | | | | | | | | | |
| 10.09.19 | ✓ | ✓ | | | | | | | | | | | | |
| 12.10.19 | ✓ | ✓ | | | | | | | | | | | | |
| 09.10.20 | ✓ | ✓ | | | | | | | | | | | | |
| 04.12.20 | ✓ | ✓ | | | | | | | | | | | | |
| 28.12.20 | ✓ | ✓ | | | | | | | | | | | | |
| 15.01.21 | ✓ | ✓ | | | | | | | | | | | | |
| 30.01.21 | ✓ | ✓ | | | | | | | | | | | | |
| 07.10.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | | | | |
| 28.10.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | | | | |
| 24.11.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 03.12.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 09.12.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 16.12.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 20.12.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 24.12.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 31.12.21 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 12.01.22 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 25.01.22 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 22.02.22 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 26.04.22 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 11.05.22 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 19.05.22 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 20.06.22 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

A blood biochemistry sample was taken from horse ‘A’ on 13.06.2019. The horse underwent a physical clinical examination by the attending veterinarian. The horse was deemed clinically sound, and the premises was lightly visually inspected for the presence of any contaminants that may pose a threat to the liver for example, visually dirty water or rotting feed or forage. The blood biochemical analysis was repeated four to five weeks following this to reveal a further elevation in serum GGT concentration. A third sample was taken three weeks later to reveal again, that the serum GGT concentration was exponentially increasing. It was decided on 10.09.2019 that a second horse on the premises, horse ‘B’, was also to undergo blood biochemical analysis. The serum GGT concentration of horse ‘B’ was also elevated. A final sample was taken on 12.10.2019 of both horse ‘A’ and horse ‘B’. This sample revealed a decrease in serum GGT concentration in both horses to only mildly elevated levels. These elevations are represented in *Table 3.* below.

*Table 3. Serum GGT measurements (IU/L) of horses ‘A’ and ‘B’ in 2019*

| | ***13.06.2019*** | ***15.07.2019*** | ***08.08.2019*** | ***10.09.2019*** | ***12.10.2019*** |
|---|---|---|---|---|---|
| ***Horse A*** | 120 | 180 | 220 | 380 | 100 |
| ***Horse B*** | - | - | - | 151 | 110 |

The reference range of serum GGT concentration in the horse was established to be 0-87 IU/L according to the biochemistry machine utilised by the veterinarian. As demonstrated in *Table 3*., the serum GGT values in horse ‘A’ reached a moderate elevation of 380 IU/L before declining back down to a mildly elevated 100 IU/L. The serum GGT elevation of 151 IU/L in horse ‘B’ also declined to 110 IU/L. It was concluded by the veterinarian, that the source contributing to the elevated GGT concentrations in the serum had unknowingly been removed by the owner which may have allowed for the values to decline simultaneously.

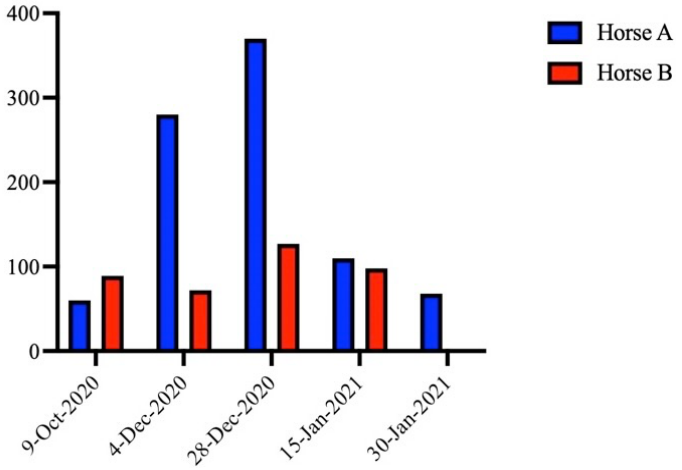
A period of 363 days elapsed before resuming blood sampling of horses ‘A’ and ‘B’. Samples were taken from both horses on 09.10.2020 and serum GGT concentrations did not appear to be elevated of a considerable amount. However, following a span of 56 days, the measurements in both horses were repeated and revealed a moderate elevation in horse ‘A’ of 280 IU/L, while there was a decline in horse ‘B’ of 72 IU/L. Twenty-four days later, the measurements were taken again to reveal an elevation in horse ‘A’

almost to the peak seen in the previous year at 370 IU/L, and an increased concentration of 127 IU/L in horse ‘B’. Measurements were repeated fifteen days later to reveal a decline once again to 110 IU/L in horse ‘A’ and to 98 IU/L in horse ‘B’. A further fifteen days showed a decline in GGT concentration of horse ‘A’ to within normal reference range. These results are demonstrated in *Table 4.* and *Graph 1.* below.

*Table 4. Serum GGT measurements (IU/L) of horses ‘A’ and ‘B’ in 2020 & 2021*

| | ***09.10.2020*** | ***04.12.2020*** | ***28.12.2020*** | ***15.01.2021*** | ***30.01.2021*** |
|---|---|---|---|---|---|
| ***Horse A*** | 60 | 280 | 370 | 110 | 68 |
| ***Horse B*** | 89 | 72 | 127 | 98 | - |

*Graph 1. Bar chart of serum GGT concentrations (IU/L) of horses ‘A’ and ‘B’ in 2020 & 2021*




The correlation in elevations between horses and the fluctuation within individual horses demonstrated in *Graph 1.,* suggests that the source of elevation of this hepatic enzyme is not persistent in the environment of these horses and is also capable of inflicting dramatic fluctuations in gamma-glutamyl transferase release from hepatocytes when the horse is in exposure to it.

At this point of the case, horse ‘B’ was removed from the premises and no further diagnostic testing was carried out on the horse. A period of 250 days passed before blood biochemical analysis resumed. With the removal of horse ‘B’ and the addition of horses ‘C’ to ‘I’, meant that a total of 8 horses were now being measured for serum GGT elevation. Upon their samples, it was observed that 2 of the 8 horses had mildly elevated concentrations while 6 of the 8 horses had moderately to greatly elevated

concentrations. Two weeks later, these measurements were taken again in the same horses to reveal further elevations in all 8 horses. Four weeks following this, the measurements were repeated in these 8 horses and a further 5 horses (horses 'J' to 'N'). Significant increases were observed in the 8 previously sampled horses, while all 5 new horses sampled also demonstrated moderate to extremely elevated concentrations of serum GGT. The results observed are demonstrated below in *Table 5*.

*Table 5. Serum GGT measurements (IU/L) of Horses 'A' and 'C-N' in 2021 & 2022*

| ***HORSE:*** | ***A*** | ***C*** | ***D*** | ***E*** | ***F*** | ***G*** | ***H*** | ***I*** | ***J*** | ***K*** | ***L*** | ***M*** | ***N*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***07.10.2021*** | 345 | 279 | 152 | 302 | 369 | 91 | 311 | 629 | - | - | - | - | - |
| ***28.10.2021*** | 410 | 345 | 310 | 428 | 453 | 238 | 398 | 690 | - | - | - | - | - |
| ***24.11.2021*** | 446 | 489 | 476 | 592 | 578 | 657 | 450 | 732 | 557 | 253 | 210 | 900 | 423 |
| ***03.12.2021*** | 449 | 679 | 659 | 782 | 672 | 765 | 554 | 876 | 595 | 259 | 204 | 952 | 282 |
| ***09.12.2021*** | 627 | 780 | 879 | 892 | 777 | 892 | 672 | 987 | 751 | 215 | 202 | 923 | 280 |
| ***16.12.2021*** | 778 | 879 | 977 | 979 | 832 | 1023 | 772 | 1003 | 827 | 197 | 208 | 901 | 246 |
| ***20.12.2021*** | 283 | 453 | 331 | 673 | 554 | 732 | 547 | 784 | 291 | 172 | 198 | 750 | 230 |
| ***24.12.2021*** | 264 | 347 | 296 | 462 | 398 | 573 | 333 | 541 | 270 | 168 | 192 | 721 | 223 |
| ***31.12.2021*** | 223 | 287 | 210 | 398 | 246 | 439 | 128 | 337 | 240 | 139 | 159 | 599 | 195 |
| ***12.01.2022*** | 125 | 194 | 139 | 237 | 175 | 310 | 88 | 239 | 140 | 120 | 133 | 487 | 135 |
| ***25.01.2022*** | 118 | 145 | 107 | 106 | 97 | 218 | 80 | 104 | 123 | 85 | 97 | 157 | 105 |
| ***22.02.2022*** | 85 | 67 | 78 | 74 | 56 | 176 | 66 | 77 | 97 | 67 | 78 | 110 | 64 |
| ***26.04.2022*** | 344 | 645 | 710 | 390 | 562 | 673 | 585 | 459 | 279 | 677 | 295 | 679 | 453 |
| ***11.05.2022*** | 311 | 543 | 653 | 344 | 447 | 510 | 329 | 383 | 250 | 480 | 265 | 556 | 298 |
| ***19.05.2022*** | 241 | 327 | 489 | 281 | 367 | 278 | 266 | 210 | 220 | 245 | 212 | 310 | 213 |
| ***20.06.2022*** | 100 | 134 | 167 | 102 | 99 | 130 | 92 | 103 | 87 | 94 | 103 | 127 | 67 |

*Table 6. Key to Table 5.*

| | **GGT Concentration (IU/L)** | **Designated Colour on *Table 5.*** |
|---|---|---|
| **Normal Reference Range** | 0-87 | *GREY* |
| **Mildly Elevated** | 88-173 | *YELLOW* |
| **Moderately Elevated** | 174-434 (2x ref. range) | *PALE ORANGE* |
| **Greatly Elevated** | 435-869 (5x ref. range) | *ORANGE* |
| **Extremely Elevated** | 870+ (10x ref. range) | *RED* |

Through the use of this key outlined in *Table 6.*, we are able to interpret the results in *Table 5.* to observe correlating patterns between horses on specific days. For example, between 24.11.2021 and 20.12.2021, we can see elevations in horses reaching greatly

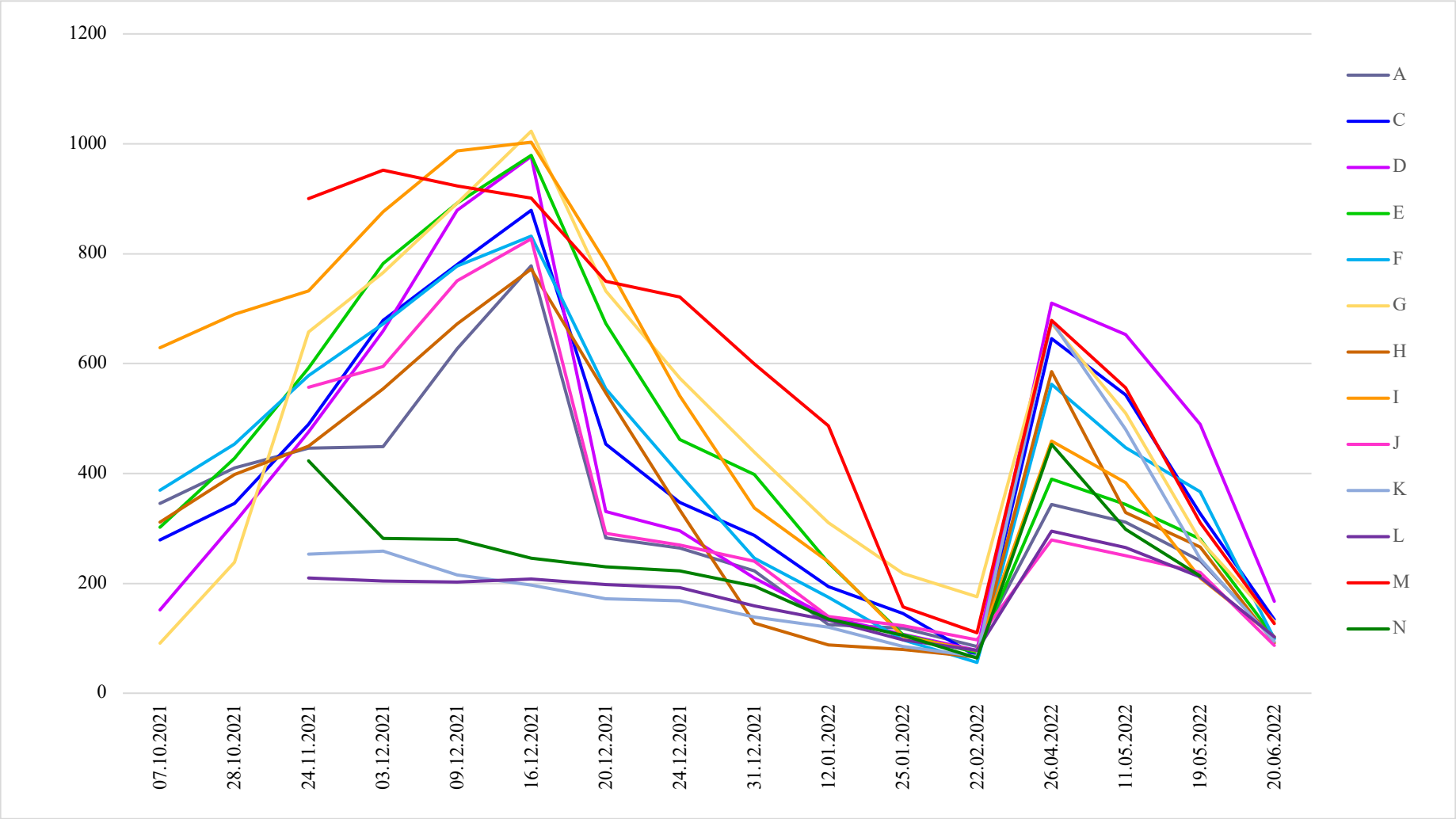
elevated to, in some cases, extremely elevated serum GGT concentration. This suggests that within this time period or shortly prior, the majority of these horses had been exposed to a hepatotoxic (bi-)product of some kind. It also suggests that from the 20.12.2021 to 22.02.2022 where elevations returned to within normal reference range in some horses, that the source of hepatotoxicity had ceased.

We can also establish from *Table 5*. that the date in which the majority of horses were within the extremely elevated grouping was 16.12.2021 and the date in which the majority of horses were within the reference range was 22.02.2022. This indicates that there was a time span of 68 days between serum GGT concentration elevations being of greater than 10 times the highest normal value, and values within the normal reference range. This suggests that the liver of the horse has the capacity to resume to normal functional value in a short space of time upon the removal of this specific source of hepatotoxicity.

These values have been plotted on a line graph seen in *Graph 1.* on the opposite page. From this graph, we can easily interpret when serum GGT concentrations have elevated and when they have declined in each horse. We can also easily correlate elevations and declines in individual horses to each other. This graph highlights the peaks of the majority of horses being reached on the 16.12.2021 and the troughs in the majority of horses (with some in the normal reference range) on 22.02.2022.

Upon observing the return of peaks in serum GGT concentrations in all 13 horses, the haylage source was changed and a rapid decline in measurements can be seen. On the final day of serum GGT measurements of these horses, it was observed that the concentrations were safely declining, with 11 of 13 horses sampled being grouped as mildly elevated, and 2 of the 13 horses having returned to within normal ranges. It was concluded at this point by the attending veterinarian that the source of hepatotoxicity had been exposed and disposed of.

*Graph 2. Serum GGT concentrations (IU/L) of horse 'A' and horses 'C-N' from October 2021 - June 2022*



### *5.1.2 Feed, Forage and Water Sampling*

When serum GGT concentrations began to head towards extremely elevated levels between 28.10.2021 and 24.11.2021, the attending veterinarian decided to collect feed, forage, and water samples from various sources within the premises.

#### *5.1.2.1 Water Samples*

Water samples from three separate sources on the premises were collected on 11.10.2021. These included samples from the well water that, at this point, was the current water source for these horses; the mains pipe, and the mains tap.

Two samples underwent environmental monitoring including a sample from the well water and a sample from the mains pipe. This tested the pH and the hardness of the water with soft being less than 50 mg/L, soft to moderate being 50-100 mg/L, moderate to hard being 150-250 mg/L, hard being >250 mg/l and very hard being >350 mg/L. This also tested the nitrate and nitrite levels, as well as alkalinity of the water. Mineral content was quantified including aluminium, arsenic, calcium, cobalt, copper, iron, lead, potassium, magnesium, manganese, selenium, sodium, and zinc. These results can be seen in *Table 7*.

Three samples underwent microbiology testing including a sample from the well water, a sample from the mains pipe, and a sample from the mains tap. This test involved the screening of the total *Escherichia coli* population in the samples, the total coliform bacteria population in the samples, the *Escherichia coli* population in the sample at 44°C, and the total number of enterococci bacteria in the samples. It also tested for the total bacterial counts at 22°C and at 37°C. The results can be seen in *Table 8*.

The results highlighted in pink in *Table 7.* and *Table 8*. indicate levels above the recommended amount. Both derive from the well water samples. The laboratory deemed the levels of manganese above the EPA recommended maximum limit, and levels of coliform bacteria too numerous to count, therefore deeming the water unfit for human &/or animal consumption.

*Table 7. Environmental monitoring results of water samples taken 11.10.2021*

| | ***Well Water*** | ***Mains Pipe*** |
|---|---|---|
| ***pH*** | 7.2 | 7.2 |
| ***Hardness (mg/L)*** | 228.56 | 183.57 |
| ***Nitrate (mg/L)*** | BLD | 1.350 |
| ***Nitrite (mg/L)*** | <0.001 | <0.001 |
| ***Alkalinity (mg/L)*** | 292.25 | 132.87 |
| ***Aluminium (ug/L)*** | <5 | <5 |
| ***Arsenic (ug/L)*** | <5 | <5 |
| ***Calcium (mg/L)*** | 91.32 | 63.01 |
| ***Cobalt (ug/L)*** | <5 | <5 |
| ***Copper (ug/L)*** | <5 | 90.95 |
| ***Iron (ug/L)*** | 7.98 | 6.73 |
| ***Lead (ug/L)*** | <5 | <5 |
| ***Magnesium (mg/L)*** | 14.49 | 4.98 |
| ***Manganese (ug/L)*** | 217.22 | <5 |
| ***Potassium (mg/L)*** | 1.97 | 2.37 |
| ***Selenium (ug/L)*** | <5 | <5 |
| ***Sodium (ug/L)*** | 31.87 | 13.10 |
| ***Zinc (mg/L)*** | <5 | 161.50 |

**BLD = Below Limit of Detection*

*Table 8. Microbiology test results of water samples taken 11.10.2021*

| | ***Well Water*** | ***Mains Pipe*** | ***Mains Tap*** |
|---|---|---|---|
| ***Total E. coli (cfu/100 mls)*** | 13 | 0 | 0 |
| ***Total Coliform (cfu/100 mls)*** | TNTC | 0 | 0 |
| ***E. coli at 44°C (cfu/100 mls)*** | 0 | 0 | 0 |
| ***Enterococci (cfu/100 mls)*** | 0 | 0 | 0 |
| ***Total Bacteria at 22°C (cfu/1 ml)*** | 60 | 38 | 16 |
| ***Total Bacteria at 37°C (cfu/1 ml)*** | 27 | 14 | 0 |

**TNTC = Too Numerous To Count*

Following these test results, the water source for the horses on the premises was changed from well water to mains water.

#### *5.1.2.2 Feed and Forage Samples*

The feed and forage samples were collected on 26.11.2021 and consisted of samples taken from the haylage stored in the stable yard (Haylage 1), haylage from the stable of one of the horses (Haylage 2), straw bedding from the stable of one of the horses (Straw), dry cube feed sample being fed to some of the horses, wet pulp feed sample being fed to some of the horses, a sample of feed mix from a feed bin on the premises (Bin 1), and a sample of dry cubed feed from another feed bin on the premises (Bin 2). All haylage and feed samples were sent to the Irish Equine Centre (IEC) to undergo 'environmental monitoring'. In this testing, fungal screening and enumeration was carried out that measured the levels of pathogenic fungi, non-pathogenic fungi, and mycotoxins in the samples. These results can be seen in *Table 9.*

In *Table 9.,* the highlighted results are those that would be considered above the maximal tolerated level (MTL), therefore deeming the sample contaminated. It is the view of the laboratory that non-pathogenic fungi are of normal feed and fodder flora. They are not considered to be of consequence unless their count exceeds that of 50,000 cfu/g. None of the samples tested demonstrated this.

From the table, we can see that of the samples tested, only that of the wet pulp feed can be considered 'clean' and that is the view of the laboratory technician. All forage samples, both haylage and straw, are considered to be contaminated with *Aspergillus flavus* or *Aspergillus niger*. Samples taken from both feed bins are also considered to be contaminated with *Aspergillus* fungi. The dry cube feed sample and the sample taken from Bin 2 are considered to contain traces of Deoxynivalenol above the MTL. The straw sample showed traces of Zearalenone above the MTL.

*Table 9. Feed & forage environmental monitoring results from 26.11.2021*

| | | ***Haylage 1*** | ***Haylage 2*** | ***Straw*** | ***Dry Cubes*** | ***Wet Pulp*** | ***Bin 1*** | ***Bin 2*** |
|---|---|---|---|---|---|---|---|---|
| ***Pathogenic Fungi*** | *Aspergillus flavus* | 4200 cfu/g | 3800 cfu/g | 1600 cfu/g | - | - | 4500 cfu/g | 600 cfu/g |
| | *Aspergillus niger* | - | 1200 cfu/g | - | - | - | | 400 cfu/g |
| ***Non-pathogenic Fungi*** | *Rhizopus* | - | - | - | - | - | - | - |
| | *Mucor* | 2100 cfu/g | 4600 cfu/g | - | - | 600 cfu/g | 1100 cfu/g | 1200 cfu/g |
| | *Penicillium* | - | - | 14100 cfu/g | 1100 cfu/g | - | - | - |
| | *Trichoderma* | - | - | 24200 cfu/g | - | - | - | - |
| ***Mycotoxins*** | *Aflatoxin* | 1.6 ppb | 1.85 ppb | BLD | 2.88 ppb | 1.84 ppb | 2.15 ppb | 1.87 ppb |
| | *Ochratoxin* | 2.08 ppb | 2.6 ppb | 2.7 ppb | 9.77 ppb | 3.55 ppb | 6.07 ppb | 9.13 ppb |
| | *Fumonisin* | BLD | BLD | BLD | BLD | 0.398 ppm | BLD | BLD |
| | *Deoxynivalenol* | 0.16 ppm | 0.261 ppm | 0.326 ppm | 0.605 ppm | 0.191 ppm | 0.321 ppm | 0.507 ppm |
| | *Zearalenone* | 31.9 ppb | 35.6 ppb | 290 ppb | 53.1 ppb | BLD | 47.6 ppb | 49.9 ppb |

**BLD = Below Limit of Detection*

After observing that the water, forage, and feed all seemed to be contaminated, it was decided by the attending veterinarian to replace two of the possible contributors to hepatotoxicity and allow for one control. This consisted of changing the water source from the contaminated well water to the clean mains water, as well as changing the source of haylage. The feed ration for each horse varied in amount and type and therefore, for ease of the horse owners, the feed source was chosen as the control and was left unaltered.

After these actions were carried out and serum GGT concentrations continued to be measured, it was observed that the serum GGT began to decline in all horses from 16.12.2021. This suggests that the source of hepatotoxicity was either the well water that had been ceased, or the haylage whose source had been changed. It was concluded by the veterinarian that the various feeds being fed to the horses were not the contributor to rises in serum GGT.

Following the allowance for the serum GGT concentrations to return to within normal reference range in the horses, it was decided by the attending veterinarian to revert the source of haylage back to the original source while remaining on the mains pipe as a source of water for the horses. Dramatic elevations in serum GGT concentrations of all horses were very quickly realised, with levels elevating from within normal reference range to moderately to greatly elevated levels within 61 days. This suggests that the haylage is the source of hepatotoxicity, rather than the well water.

On 26.04.2021 the forage source was reverted back to that considered to be non-contaminated haylage. A sample of this haylage was taken to and sent to the IEC to undergo environmental monitoring, in order to ensure that the haylage was indeed safe for animal consumption. The sample showed no growth of pathogenic fungi, very light growth of non-pathogenic fungi, and no traces of mycotoxins. The haylage sample was deemed clean by the laboratory technician. This solidified to the attending veterinarian that the original haylage source was the contributor to the elevations in serum GGT concentrations.

### *5.1.3 Field Analysis*

The final diagnostic method utilised by the veterinarian was the visual inspection of the field from which the haylage was harvested. Images were captures of various plants and weeds observed upon inspection. We gathered these images and, through the use of PlantNet, were able to identify them. The results obtained from PlantNet are displayed in *Table 10.* These images were taken by the attending veterinarian who carried out the visual field analysis.

*Table 10. PlantNet results from images upon visual field analysis by veterinarian*

| | |
|---|---|
|  |  |
| ***1.*** 57.16% *Ranunculus macrophyllus*<br>7.06% *Ranunculus lanuginosus* | ***2.*** 31.75% *Rumex thyrsoides*<br>23.64% *Rumex acetosa* |
|  |  |
| ***3.*** 26.18% *Rumex obtusifolius*<br>12.83% *Rumex conglomeratus*<br>11.15% *Rumex patientia* | ***4.*** 51.67% *Alopercurus pratensis*<br>7.94% *Phleum pratense*<br>4.35% *Alopercurus geniculatus* |

Upon interpreting the results from PlantNet, we attempted to research whether any of the plants identified in the images were known to be hepatotoxic or lead to an elevation in serum GGT concentration in horses.

The first image has been identified as *Ranunculus macrophyllus* (large-leaved buttercup) and *Ranunculus lanuginosus* (woolly buttercup). Buttercup species has been linked to equine grass sickness in horses in the United Kingdom [91], as well as gastrointestinal ulceration and abortions in thoroughbreds in Kentucky, USA [92]. Buttercup toxicosis has long been known to affect livestock, causing abortions in cattle however, it has not yet been fully determined if these plants have the same effect on horses worldwide [92].

In the second and third images, *Rumex thyrsoides*, *Rumex acetosa, Rumex obtusifolius*, *Rumex conglomeratus*, and *Rumex patientia* have been identified. The *Rumex* species is made up of the dock and sorrel plants. Docks and Sorrels have been linked to anti-inflammatory and antibacterial properties [93] however, they may contain oxalates that bind to calcium and magnesium to cause muscle tremors and weakness in horses [94].

In the fourth image, *Alopecurus pratensis* and *Alopercurus geniculatus* were identified. These are also known as meadow and marsh foxtail. These have not been associated with toxicity in horses. The only issues faced by horses with this plant are physical, with their awns potentially damaging the horse's gums upon chewing. *Phleum pratense*, also known as timothy grass, has been described as an appropriate hay for the maintenance of horses and therefore, does not pose a threat toxicity-wise [95].

### 5.2 Conclusions of Case Study

Elevations in serum GGT concentrations in horses can be linked to many different issues in the body including problems with the hepatobiliary system, the musculoskeletal system, the gastrointestinal system, and the hematopoietic system. When it comes to the musculoskeletal system, the elevation in serum GGT concentration associated with overtraining is not yet fully determined [19]. In this particular case, we had a mixture of both event horses and livery horses & ponies, all were subject to varying exercise routines and levels while suffering with similar

fluctuating elevations in serum GGT concentration. This suggests that the probability of overtraining or strenuous exercise i.e., issues in the musculoskeletal system, being the primary cause is unlikely.

Elevations in serum GGT concentration can also be linked with issues in the gastrointestinal tract e.g. in cases of pancreatitis in the horse however, this is a very rarely seen phenomenon in the horse [96]. It may also be observed in cases of right dorsal displacement as pressure is put on the bile duct of the animal, we see an increase in the serum GGT concentration of roughly 50% of these cases [19]. This being said, issues in the gastrointestinal tract tend to be on a clinical and individual level, rather than subclinical within a herd of horses. For this reason, this was ruled out as the primary cause of the increased serum GGT concentrations in this case.

Increased serum GGT can also be associated with severe cases of anaemia. When researching possible causes of anaemia, several can be linked to herds of horses rather than just individuals. These causes included Equine Infectious Anaemia (EIA), onion toxicity, or red maple leaf toxicity [19]. Anaemia has a higher probability of affecting a herd when compared to previously mentioned contributors however, EIA is not a commonly seen infection in horses in Ireland [97], onions were not ingested by these horses, and maple trees were not present in either the field these horses were kept on or in the field the haylage being fed to the horses had been harvested from. The fact that these horses faced elevations in their serum GGT concentration while presenting as normal under physical clinical examination, significantly lowers the chances of these elevations being linked to any form of anaemia as it is usually associated with other clinical signs. Therefore, it was decided that if any of these horses were to suffer from anaemia, it was more likely to be secondary to the initial liver damage that was more likely to be the primary contributor to the elevated serum GGT, rather than the opposite.

This brings us to the hepatobiliary system. As stated previously, the horse can present clinically normal while experiencing liver disease. The fact that these horses experienced an increase in serum GGT concentration with the absence of any clinical signs, makes it far more likely for the cause to point in the direction of the liver. This

case affecting many horses ruled out a neoplastic or congenital origin. Instead, the possibilities we looked at were contaminated feed, forage, and water.

As displayed in *Table 4.* and *Graph 1*., horse 'A' displays a dramatic elevation in serum GGT concentration in the space of three months from within the normal reference range to a moderately elevated concentration of 370 IU/L. In the two weeks following, this declines to 110 IU/L and finally returns to within the normal reference range another two weeks later. This tells us that the mode of action of the source of hepatotoxicity is the lysis of hepatocytes to allow for a release of GGT from the cells, which in turn would lead to its elevations in the blood serum. This allows us to rule out toxins that tend to attack the biliary epithelium, rather than on a hepatocellular level. This data also suggests that the toxin present is quite potent, with dramatic fluctuations presenting in very short time frames.

It has been concluded from the results presented in the previous section, that samples of water, forage, and feed alike have all demonstrated levels of contamination. However, with the water and forage source changed while the feed remained as a control and elevations began to decline, meant that the feed was unlikely to be the source of toxicity in these horses. Upon deciding to keep the water source as is, and to revert the forage sample back to its original on 22.02.2022, elevations were observed once more with serum GGT concentrations reaching greatly elevated levels from those within the normal reference range, suggests that the water is also not the source of toxicity in these horses, and is more likely to be the forage. This was highlighted more so once this forage was removed once more on 26.04.2022 to initiate a decline once again in the serum GGT concentrations. Therefore, it has been concluded that the forage is the probable contributor to elevations in serum GGT concentrations in these horses.

With the environmental monitoring test results of the haylage and straw samples revealing contamination with pathogenic *Aspergillus* fungi, it is likely that these fungi may have produced hepatotoxic mycotoxins such as Aflatoxin in these horses to have a deleterious effect on hepatocytes and in turn, lead to a rise in serum GGT.

According to the plants and grasses identified from the images in *Table 10*., there has been no link associated between these findings and the elevation of serum GGT

concentrations or subclinical hepatopathies in horses. However, it is clear that the grassland presents in this field that has been used for the growth of haylage fed to these horses is impure. Although these plants identified have been deemed either safe for horses or associated with toxicosis in horses of an extra-hepatic nature, it is unsure whether these plants may pose a threat when ensiled or ingested by the horse in the form of haylage. Therefore, the possibility of these plants being associated with the release of GGT into the serum of these horses cannot be ruled out.

To the author's knowledge, there is a lack of evidence-based studies carried out in order to identify the affects impure or contaminated forage can have on horses. It is unsure whether this subclinical toxicity would have progressed to detrimental consequences if dangerously elevated serum GGT concentrations were to persist however, literature suggests that upon the progression of the lysis of hepatocytes, these horses would eventually face hepatopathies on a clinical rather than subclinical level. Therefore, it is clear that there is a strong requirement for studies to be carried in order to determine the exact cause of these serum GGT concentrations, how to recognise them, and how to prevent them.

## 6. Discussions & Conclusions

The goal of this study was to investigate the different aspects of subclinical hepatopathies observed in herds of horses in Ireland. Once research commenced, it was quickly realised how regenerative and industrious this organ is. This aspect is what allows for the majority of hepatopathies to remain subclinical until over 70% of the organ has been damaged. This is extremely beneficial for the horse as the liver is responsible for a vast amount of functions throughout the body.

Despite this, we also discovered that the majority of hepatopathies tend to go unrecognised, undiagnosed, and consequently untreated. A progression of subclinical hepatopathy to the point of clinical manifestation poses great concern in horses and gives little time for the recognition of the cause, and its cessation. This being said, the industrious trait of this organ suggests that once the root cause of the situation has been removed, and a subsequent amount of cells are still functional, miraculous recoveries can be made in these horses upon the removal of the contributor to disease.

We also concluded that an array of contributors can be found naturally in the environment that can detrimentally face the livers of these animals. These causes can be present in the plants horses graze on in fields, in the environment horses reside in, in the feed and forage horses are given, from contact with other infected horses, and from iatrogenic causes such as in serum hepatitis. These contributors to liver disease being so abundant, makes it difficult to pinpoint exact causes in such cases.

When speaking of hepatopathies in horses on a herd level rather than in individuals, we found that the common denominator tends to be in what the horses are ingesting i.e., in the forage, feed, or grassland being grazed. This is evident in the case studied of elevated serum GGT concentrations due to contaminated forage. This suggests that when signs begin to indicate hepatic dysfunction such as elevations in hepatic enzymes, sample testing of components within the diet of these horses is a good starting point towards a definitive diagnosis.

We realised that with the accompaniment of an abundance of contributors, comes a plethora of diagnostic tools and methods that can be utilised in hepatopathies in horses.

These tools vary in availability, cost-efficiency, invasiveness, and effectiveness. They can complement each other well and aid greatly in the diagnosis of these diseases once the insult has been recognised. In some cases, we concluded that diagnostic tools can also hold strong prognostic potential which is of huge value in terms of treatment and management planning.

Upon researching hepatopathies in horse herd in Ireland, it was quickly realised that little to no cases tend to be reported, according to animal health surveillance reports in recent years. This may be due to the majority of cases scathing unnoticed. In order to avoid this, the author recommended regular haematology and biochemical analysis of horses by owners and veterinarians to gain an insight into the overall health of the animals. It is this aspect that allowed for the recognition of elevated serum GGT concentrations in the case evaluated.

We also concluded that, regardless of numerous publications and studies carried out based on hepatopathy in horse herds, there is still a huge amount of research to be carried out in order to obtain more of an insight into substances that pose a threat to the liver of horses. An example would be a study conducted into the affect certain plants can have when ingested by horses, once they are ensiled. A study such as this would be useful in terms of the case we evaluated as part of this study as it is still unsure whether the release of hepatic enzymes was caused by harmful fungi in the forage, or impure grassland ensiled in the haylage.

## 7. Summary

The goal of this study was to investigate the various aspects of subclinical hepatopathies in herds of horses in Ireland, and bring to light the negative effects associated with the lack of their diagnosis and therefore, the progression of disease.

In this study we compiled a literature review, using various publications, of the possible causes of liver disease and the diagnostic methods that can be utilised for their recognition. The overall results showed an undeniable concern regarding the abundance of contributors to liver insult in the natural environment of horses.

We also evaluated a specific case of subclinical hepatopathy in a herd of horses in Ireland. This allowed us to verify the efficiency of various diagnostic methods that we had researched. It also highlighted the ease at which toxins and contaminants can affect horses. This is a major concern, not only in Ireland but worldwide, as the root of the cause generally lies within the diet or environment of the horses.

In this regard, the adverse toxic effects on health and well-being of these horses if left undiagnosed are impossible to ignore. The most important challenge yet to be faced is to pinpoint exact causes of liver upset in horse herds. This is extremely difficult due to the diversity of this organ, as its insult can affect different horses in many different ways.

As discussed, higher sources of knowledge must seek to correct the current obstacles regarding the outlining of specific causes of liver disease in horses. With the quick-paced, highly aware society that exists in horse ownership today, it is becoming a requirement that more effort is made to incorporate a more informed approach to this phenomenon to remain in sync with present demand. Unfortunately, at present, due to the lack of detailed research, this has not yet been achieved completely.

However, if the correct solutions and testing facilities could be established, then the use of new research would be a massive breakthrough in the search to combat the enigma surrounding specific causative agents in hepatopathies in herds of horses in Ireland.

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