Hepatic concentrations of copper and other metals in dogs with and without chronic hepatitis

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OBJECTIVES: Defects in copper metabolism have been described in several dog breeds, and recently, it has been suggested that changes in other essential trace elements could be involved in the pathogenesis of hepatic disease. This study measured hepatic copper accumulation and its interactions with other essential trace and toxic metals in dogs diagnosed with chronic hepatitis.

METHODS: Liver samples of 20 chronic hepatitis and 20 healthy dogs were collected. Samples were acid digested, and essential metals (cobalt, copper, iron, manganese, molybdenum, selenium and zinc) and toxic metals (arsenic, cadmium, mercury and lead) were analysed by inductively–coupled plasma mass spectrometry.

RESULTS: Copper concentrations were significantly higher in dogs affected by hepatic disease than in controls. Dogs having chronic hepatitis with liver copper concentration greater than 100 mg/kg wet weight showed statistically higher cobalt, manganese and zinc concentrations than dogs having chronic hepatitis with liver copper concentrations less than 100 mg/kg wet weight and controls. Toxic metal concentrations were low – in all cases below the threshold associated with toxicity in dogs.

CLINICAL SIGNIFICANCE: Dogs with chronic hepatitis not only have increased concentrations of copper in the liver but also increased concentrations of cobalt, manganese and zinc; measurement of these elements may perhaps aid in diagnosis of liver disease in dogs.

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INTRODUCTION

Copper (Cu) is an essential element involved in many metabolic processes but, in excess, can become extremely toxic. This is because, at concentrations above physiological needs, the capacity of the liver to bind Cu to metallothioneins (MT) is overloaded. The Cu remains free in the cytosol causing dramatic oxidative damage and cellular death (Mercer 2001).

Within the domestic animals, sheep are the most susceptible species to Cu toxicity due to their low capacity for MT synthesis (Howell & Gooneratne 1987). Nevertheless, episodes of Cu toxicity have been described in many other species, including dogs, in which hepatic Cu accumulation is associated with numerous episodes of hepatic disease, also known as copper-associated chronic hepatitis (CACH). CACH was first identified in Bedlington terriers as an inherited autosomal recessive defect of the COMMD1 gene, which results in reduced biliary excretion of Cu because of hepatic MT sequestration of the metal in hepatic lysosomes (Van De Sluis et al. 2002). During the last decade, an increasing number of pure-breed dogs including Doberman pinscher (Mandigers et al. 2004), West Highland white terrier (Thornburg et al. 1986), Skye terrier (Haywood et al. 1988), Dalmatian (Webb et al. 2002), most recently the Labrador retriever (Hoffmann
et al. 2006, Smedley et al. 2009, Johnston et al. 2013) and other pure-breed dogs (Rifkin & Miller 2014), as well as mixed-breed dogs have been associated with CACH (Watson 2004). For most of these breeds, the pathological mechanisms involved are not well understood. Abnormal hepatic Cu accumulation may be the result of either a primary metabolic defect in Cu metabolism or a secondary event from abnormal hepatic function altering biliary Cu excretion. In primary defects, Cu accumulation is always localized centrilobularly (zone 3). In contrast, in secondary processes Cu is mainly restricted to the periportal parenchyma (zone 1) (Watson 2004, Hoffmann et al. 2006, Johnston et al. 2013, Kilpatrick et al. 2014).

It has been suggested that changes in other essential trace elements could be involved in the pathogenesis of hepatic disease (Webb & Twedt 2008). In addition to Cu, many affected CACH dogs had increased iron (Fe) (Smedley et al. 2009) and/or zinc (Zn) concentrations (Schultheiss et al. 2002, Pressler et al. 2010) although concurrent deficient hepatic Zn can also be observed (Rothuizen & Twedt 2009). Very recently, abnormally high cobalt (Co) (Pressler et al. 2010) and manganese (Mn) blood concentrations have been described in dogs affected by primary hepatitis, which could have a relevance in the development of hepatic encephalopathy in these animals (Kilpatrick et al. 2014). Moreover, the chemical similarity of Cu and other toxic metals to induce and bind to MT (as occurred in environmentally naturally exposed animals; López-Alonso et al. 2002) could also contribute to the pathogenesis of the disease.

This initial descriptive study was conducted to evaluate hepatic Cu accumulation and its interactions with other essential trace [Co, Fe, Mn, molybdenum (Mo), selenium, (Se) and Zn] and toxic metals [arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb)] in dogs with and without chronic hepatitis (CH). MATERIALS AND METHODS

Study population
Liver samples of dogs affected by CH were collected during post-mortem examination. Twenty samples were considered eligible for inclusion in the study after histopathological confirmation when world small animal veterinary association (WSAVA) criteria were fulfilled (Rothuizen et al. 2006). Of these dogs, eight died or were euthanased in an advanced stage of CH; seven had been clinically diagnosed with CH (based on laboratory findings and image) but died were euthanased for other reasons and in the remaining five dogs CH was a post-mortem finding. None of the dogs received a special liver diet restricted in Cu or was treated with Zn or a chelating agent. Samples of 20 healthy livers (histopathologically confirmed) from animals not affected by metabolic diseases were used as controls. Number of dogs per group was decided taking into account the inter-animal variability in trace element concentrations in the liver and availability of dogs for sampling. Reasons for death or euthanasia of the control dogs were trauma, focal neoplasia, rodenticide exposure, etc. Information on age, gender, breed and other data related to the health register of the animals was obtained from the clinical history. The group of dogs with CH (n = 20) comprised of: Dobermann pinscher (3), German shepherd (3), English cocker spaniel (2), boxer (1), bull terrier (1), Siberian husky (1), poodle (1) and crossbreds (8). These comprised 12 females and 8 males, with a mean age 88 months, and a range between 6 and 214 months. The control group (C) were: German shepherd (4), boxer (2), mastiff (2), Pekingese (2), Belgian shepherd (1), English cocker spaniel (1), Siberian husky (1), bloodhound (1), Yorkshire terrier (1) and crossbreds (5). These comprised 12 females and 8 males with a mean age of 113 months, and a range from 26 to 201 months.

Sample collection and chemical analysis
Three deep tissue samples of approximately 2 cm × 2 cm × 2 cm were collected from the left lateral liver lobe in order to simulate sample collection during percutaneous ultrasound-guided needle biopsy (Rothuizen et al. 2006). All samples were taken from near the centre of the lobe. Liver lesions were avoided. One sample was fixed in neutral-buffered 10% formalin at room temperature for histopathology exam. All samples had a minimum of eight portal triads. The other two samples were packed into plastic bags and stored at −20°C for mineral analysis. Approximately 1 g subsamples were digested in 3 mL of concentrated nitric acid and 1 mL 30% w/v hydrogen peroxide in a microwave digestion system (Milestone; Ethos Plus). Digested samples were transferred to polypropylene sample tubes and diluted to 15 mL with ultrapure water. The concentrations of essential trace elements (Co, Cu, Fe, Mn, Mo, Se and Zn) and toxic elements (As, Cd, Hg and Pb) were determined by inductively–coupled plasma mass spectrometry (ICP-MS; VG Elemental PlasmaQuad SOption).

An analytical quality control programme was employed during the study. Blank absorbance values were monitored throughout the survey and were subtracted from the readings before the results were calculated. The limits of detection (LoD) were calculated as three times the standard deviation of the reagent blanks (Table 1). All samples were above LoD. Analytical recoveries were determined from a certified reference material analysed together with the samples (Standard Reference Material® 1577c Bovine Liver; National Institute of Standards & Technology). There was good agreement between the measured and the certified values (Table 1). The precision of the

### Table 1. Analytical quality programme expressed as mean ± standard deviation used in the determination of essential trace and toxic elements

<table>
<thead>
<tr>
<th>Element</th>
<th>Detection limit (µg/L)</th>
<th>Certified levels (mg/kg)</th>
<th>Analysed levels (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>0.3</td>
<td>0.0196±0.0014</td>
<td>0.0201±0.0007</td>
</tr>
<tr>
<td>Cd</td>
<td>0.1</td>
<td>0.0970±0.0014</td>
<td>0.1059±0.0038</td>
</tr>
<tr>
<td>Co</td>
<td>0.2</td>
<td>0.300±0.018</td>
<td>0.318±0.006</td>
</tr>
<tr>
<td>Cu</td>
<td>3.1</td>
<td>2.75±0.046</td>
<td>2.73±0.011</td>
</tr>
<tr>
<td>Fe</td>
<td>12</td>
<td>197.9±65</td>
<td>198.0±4.59</td>
</tr>
<tr>
<td>Hg</td>
<td>0.2</td>
<td>–</td>
<td>0.14±0.012</td>
</tr>
<tr>
<td>Mn</td>
<td>1.2</td>
<td>10.46±0.47</td>
<td>10.48±0.12</td>
</tr>
<tr>
<td>Mo</td>
<td>1.2</td>
<td>3.30±0.13</td>
<td>3.32±0.31</td>
</tr>
<tr>
<td>Pb</td>
<td>0.9</td>
<td>0.0628±0.0001</td>
<td>0.0599±0.0041</td>
</tr>
<tr>
<td>Se</td>
<td>1.2</td>
<td>2.03±0.045</td>
<td>1.989±0.047</td>
</tr>
<tr>
<td>Zn</td>
<td>15</td>
<td>181.1±0</td>
<td>180.4±1.7</td>
</tr>
</tbody>
</table>

As arsenic, Cd cadmium, Co cobalt, Cu copper, Fe iron, Hg mercury, Mn manganese, Mo molybdenum, Pb lead, Se selenium, Zn zinc.
analytical method was evaluated by measuring the absorbance signals in the same digested sample 12 times. To assess the precision of the overall procedure, readings of 12 different digested aliquots were performed. Intra- and inter-assay coefficients of variation were between 1-48 to 4-72 and 3-11 to 10-7%, respectively.

Data were presented in mg/kg wet weight (wt.w.). The dry matter content of the liver (calculated by drying 10 different sub-samples at 80° until reaching a constant weight) was 24.9±3.1 and did not differ between control and affected dogs. Liver tissue Cu concentrations less than 100 mg/kg wt.w. (<400 mg/kg d.w.) were considered physiologically normal (Thornburg 2000, Webb et al. 2002, Hoffmann et al. 2006, Johnston et al. 2013).

**Data analysis**
All statistical analyses were carried out using SPSS for Windows (v. 20.0). Graphics were performed with InvivoStat Graphics. Normal distribution of data sets was checked using the Kolmogorov–Smirnov test. As and Cd concentrations did not follow a normal distribution and so data on toxic elements are given as median and range and differences between groups were tested by non-parametric tests. Differences between Cu concentrations in dogs affected by CH and controls were checked using a t-test. Differences in hepatic concentrations of trace and toxic element in dogs affected by CH, both associated or not to Cu accumulation, and controls were evaluated using a one-way analysis of variance and post hoc honest significant difference Tukey tests. Correlations between Cu and essential trace and toxic element concentrations were tested by Pearson’s correlation analysis with Bonferroni correction, considering significance when P<0.005.

**RESULTS**

Figure 1 shows hepatic Cu concentrations in CH and C groups. Cu concentrations were significantly higher (P<0.001) in dogs affected by hepatic disease compared with the controls. In the CH group, Cu concentrations ranged from 4 to 218 mg/kg wt.w. (median 74 mg/kg); seven dogs showed Cu levels above the normal range (<100 mg/kg wt.w.).; these were five females and two males, three of known susceptible breeds (two Doberman pinschers, one poodle), one German shepherd and three cross-breeds. Within the control group, Cu concentration was 38 mg/kg (median), ranging from 3 to 68 mg/kg; none had Cu above the normal range.

Three categories were identified based on the hepatic Cu content: dogs having CH with liver Cu greater than 100 mg/kg wt.w. (CH-Cu>100); dogs having CH with liver Cu less than 100 mg/kg wt.w. (CH-Cu<100) and control dogs (C). Hepatic Co, Fe, Mn, Mo, Se and Zn concentrations in dogs of the three groups, CH-Cu greater than 100 (n=7), CH-Cu less than 100 (n=13), and control (n=20) dogs, are presented in Fig. 2. Dogs with the higher Cu concentration (CH-Cu>100) showed statistically higher Co (33-3), Mn (2-95) and Zn (67-3) concentrations (P<0.001) than those with lower concentrations (CH-Cu<100) and controls – these latter groups did not differ from each other (Co: 10-1 and 12-9; Mn: 2-15 and 2-19; Zn: 43-7 and 43-6, respectively). A similar pattern (although without any statistically significant difference) was found for Fe and Mo (CH-Cu>100 had more of these elements than CH-Cu<100, which had more than the controls), whereas for Se similar concentrations were found for all three groups.

Statistically significant associations between Cu and Co, Mn and Zn concentrations in the liver are presented in Fig. 3. When considering all animals included in this study, the Cu concentrations were positively correlated with other elements as follows: Co: r=0.542, P<0.001; Mn: r=0.715, P<0.001 and Zn: r=0.546, P<0.001. However, when the analysis was restricted to the CH-Cu greater than 100 group, associations were stronger (in spite of the low number of samples) and showed a steeper correlational slope: Co: r=0.882, P<0.005; Mn: r=0.916, P<0.005 and Zn: r=0.819, P<0.005.

Hepatic toxic metal concentrations in CH-Cu greater than 100, CH-Cu less than 100 and C dogs are presented in Table 2. Toxic metal concentrations were low; in all animals below the threshold associated with toxicity in dogs according to Puls (1994).

**DISCUSSION**

Despite the small sample size, our results indicate that the profile of dogs affected by CH is similar to that in other recent studies (Poldervaart et al. 2009, Bexfield et al. 2012, Kilpatrick et al. 2014). The dogs we diagnosed with CH support the assumption of a higher prevalence of CH in certain breeds, including the Doberman pinscher, American and English cocker spaniels and poodles. Forty per cent (8 of 20) of affected dogs in our study were crossbreeds. Although it is documented that cross-breeds can be affected by CH (Thornburg et al. 1990, Rothuizen & Twedt 2009), little information is available in the literature making comparisons difficult: cross-breed dogs represent a very
FIG 2. Dotplots showing Co, Fe, Mn, Mo, Se and Zn hepatic concentrations in dogs with chronic hepatitis with liver Cu greater than 100 mg/kg wt.w. (CH-Cu>100, n=7), chronic hepatitis with liver Cu less than 100 mg/kg wt.w. (CH-Cu<100, n=13) and control group (C, n=20). Different letters indicate statistically significant differences between groups (P<0.001).
Trace elements in canine chronic hepatitis

Seven of the 20 dogs (35%) affected by CH in our study showed a hepatic Cu accumulation above the normal range (<100 mg/kg) (Thornburg 2000, Webb et al. 2002, Hoffmann et al. 2006, Johnston et al. 2013). Not much information is available, but our data is in accordance with a recent study in The Netherlands that demonstrated that 36% of cases of primary hepatitis had increased Cu accumulation on rubeanic stained sections of liver tissue (Poldervaart et al. 2009). Again, some breeds are clearly related to this pathology. The only well-known genetic disorder is that affecting Bedlington terrier (Van De Sluis et al. 2002). In other breeds, primary Cu metabolism disorders are more complex and have not been fully determined. For Labrador retrievers, an inherited genetic defect has also been proposed (Hoffmann et al. 2006, Johnston et al. 2013). In young Dalmatians, CACH shares some similarities with the Bedlington disease and may represent a primary metabolic defect in hepatic Cu metabolism (Webb et al. 2002). In other breeds, CACH appears to be overrepresented, including Doberman pinscher (Mandigers et al. 2004), West Highland white terrier (Thornburg et al. 1986) and Skye terrier (Haywood et al. 1988), but in none of these does it appear to be a true “storage disease” like it is in Bedlington terriers. In contrast to Bedlington terriers, Cu does not build up throughout life and the amount of Cu stored is often not correlated with the degree of underlying disease and/or is not present very early in the disease process (Thornburg 2000). In these breeds, Cu accumulation appears to be secondary to cholestatic liver injury (Rothuizen & Twedt 2009). Cu accumulation should then be understood as cause and effect of CH. In general, susceptibility to accumulation has a genetic basis and the expression of disease is due to environmental factors, such as diet (Johnston et al. 2013, Fieten et al. 2014). Johnston et al. (2013) described an increase of hepatic Cu in dogs over time, reflecting increased ingestion and more bioavailable forms of Cu in the diet.

The impact of primary hepatic disease on the metabolism of other trace elements has been documented in dogs (Schultheiss et al. 2002, Smedley et al. 2009, Pressler et al. 2010). Fe is possibly the element that has received most attention and both Cu and Fe-induced hepatic damage is thought to be the result of free radical-generated oxidant injury (Center 1999). A high proportion of dogs in this study had increased liver iron concentrations (normal range 88 to 438 mg/kg w.wt. or 350 to 1750 mg/

Table 2. Toxic metal concentrations (µg/kg wet weight) in the liver of dogs with chronic hepatitis with liver Cu greater than 100 mg/kg w.t.w. (CH-Cu>100, n=7), chronic hepatitis with liver Cu less than 100 mg/kg w.t.w. (CH-Cu<100, n=13) and control (C, n=20) dogs

<table>
<thead>
<tr>
<th></th>
<th>CH-Cu&gt;100</th>
<th>CH-Cu&lt;100</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Cd</td>
<td>9·8</td>
<td>7·5 to 16·4</td>
<td>8·9</td>
</tr>
<tr>
<td>Hg</td>
<td>122</td>
<td>38 to 195</td>
<td>54</td>
</tr>
<tr>
<td>Pb</td>
<td>42·9</td>
<td>6·8 to 214·9</td>
<td>70·4</td>
</tr>
<tr>
<td>As</td>
<td>90</td>
<td>28 to 245</td>
<td>49</td>
</tr>
</tbody>
</table>

As arsenic, Cd cadmium, Hg mercury, Pb lead.

FIG 3. Relationship between liver Cu concentrations (mg/kg wt.w.) and liver Co, Mn and Zn concentrations (mg/kg wt.w.). Discontinuous line (---) all animals (•: chronic hepatitis with liver Cu>100 mg/kg (CH-Cu>100, n=7); ○: chronic hepatitis with liver Cu less than 100 mg/kg (CH-Cu<100, n=13); □: control (C, n=20). Continuous line (——) animals with chronic hepatitis with liver Cu greater than 100 mg/kg (CH-Cu>100, n=7)

heterogenous group, clearly different in number and breed-mixtures in the different countries and possibly with different access to veterinary services compared to pure-breed dogs. Although there are differences by breeds, females are generally more affected than males (Watson 2004, Bexfield et al. 2012) and median age at diagnosis is around 6 to 8 years (with a range from 5 months to 18 years) (Poldervaart et al. 2009, Bexfield et al. 2012, Kilpatrick et al. 2014, Rifkin & Miller 2014).

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The impact of primary hepatic disease on the metabolism of other trace elements has been documented in dogs (Schultheiss et al. 2002, Smedley et al. 2009, Pressler et al. 2010). Fe is possibly the element that has received most attention and both Cu and Fe-induced hepatic damage is thought to be the result of free radical-generated oxidant injury (Center 1999). A high proportion of dogs in this study had increased liver iron concentrations (normal range 88 to 438 mg/kg w.wt. or 350 to 1750 mg/
Hepatic Fe concentrations were not statistically higher in ‘CH-Cu greater than 100’ compared to ‘CH-Cu less than 100’ or ‘C’ dogs, although the CH-Cu greater than 100 group showed the highest proportion of dogs with high hepatic Fe concentrations. In one study using dogs of various breeds, it was found that dogs can have a wide range of hepatic Fe concentrations. Increased hepatic Fe concentrations have been documented in naturally-occurring and experimental canine CH (Schultheiss et al. 2002). The pathogenic mechanism of Fe on CH, and particularly in dogs with Cu accumulation, is not completely understood and no consistent associations have been found between both metals (Center 1999). Fe and Cu concentrations do not appear to increase in direct proportion to each other (Schultheiss et al. 2002). In contrast to Cu, Fe is not excreted into bile, so Fe concentration would not be expected to influence abnormalities in the biliary system (Center 1999). In the liver of CH-affected dogs, Fe histologically appears adjacent to areas of chronic inflammation within the Kupffer cells which could support the hypothesis of haemolytic disease (Center 1999). In any case, hepatic Fe accumulation in our study appears to be a consequence of the liver disease, but probably not directly the cause of liver lesions (Schultheiss et al. 2002).

In contrast to Fe accumulation that could be related to inflammatory and haemolytic processes, the increase and interaction of Cu with other essential metals (Zn, Mn and Co) appears to be secondary to a cholestatic phenomenon. Hepatic Zn concentrations were statistically higher in CH-Cu greater than 100 compared to CH-Cu less than 100 or C dogs, with normal liver Zn concentrations in the three groups (<200 mg/kg w.wt. or 800 mg/kg d.w., Schultheiss et al. 2002). The role of Zn on CH is even less well understood. Zn is a relatively low-toxic element and Zn concentrations above physiological levels are uncommon in dogs affected by liver disease (Schultheiss et al. 2002), and have not been correlated with Fe and Cu levels or histologic lesions in CH-affected dogs. Moreover, whereas some dogs with CH and Cu accumulation were deficient in hepatic Zn (Webb et al. 2002) in others the Zn concentrations were higher than in controls (Schultheiss et al. 2002). Inconsistent results between studies could be explained considering the relative concentrations of both metals in the body. It is well known that Cu and Zn share chemical properties and have important metabolic relationships in the healthy animal (López-Alonso et al. 2002) because of their capacity to induce and bind to MT. In fact, these common homeostatic mechanisms are the basis of Zn therapy in CH. Zn induces the production of MT in the intestinal mucosa, binding Cu in the intestinal cell and therefore preventing its absorption (Hoffmann et al. 2009, Fieten et al. 2013). Zn treatment can generate a negative Cu balance and therefore indirectly remove free Cu from the liver (Hoffmann et al. 2006, 2009, Fieten et al. 2013, 2014). Zn can induce MT in hepatocytes thereby binding free Cu and protecting cells against oxidative stress induced by free Cu. The increase of Mn concentrations in the liver of CH-Cu greater than 100 dogs in our study could also be explained by a cholestatic phenomenon, although Gow et al. (2015) demonstrated that resolution of congenital portosystemic shunts in dogs resolves the encephalopathy but not the hypermanganesemia. The liver plays a pivotal role in Mn metabolism because the majority of the gastrointestinally-absorbed element is removed by the liver and excreted into bile, allowing for only approximately 2% of absorbed Mn to reach the systemic circulation (Aschner & Aschner 2005). Very limited information is available on the role of Mn in canine liver disease, even though recent studies have demonstrated that whole-blood Mn concentrations were higher in dogs with primary hepatitis (Kilpatrick et al. 2014) and hepatic encephalopathy (Gow et al. 2015). Studies in humans have shown increased blood Mn concentrations and deposition in the central nervous system (CNS) in cases of hepatic insufficiency (Zheng et al. 2011, Tuschl et al. 2013). The deposition of Mn in the CNS is considered to play a role in hepatic encephalopathy and a direct relationship has been demonstrated between blood Mn concentration, Mn deposition in the brain and severity of encephalopathic score in humans (Bowman et al. 2011).

Co is a relatively low-toxic element, poorly retained in tissues and scarcely studied in dogs. Although the main route of excretion of Co is via the kidney (Simonsen et al. 2012), a small but significant amount is excreted by the bile. Cholestasis could explain the higher concentrations of Co in the CH-Cu greater than 100 dogs in our study. Pressler et al. (2010) suggested that COMMD+/- beagle-Bedlington terriers, although asymptomatic, may have an uncharacterised defect that results in liver Co accumulation. However, since hepatic Co reference values have not yet been established for dogs, we are unable to assess the relevance of the increase of Co concentrations in the CH dogs.

Finally, to point out that we are conscious that the present study has several limitations. This is an initial descriptive study, with a limited number and heterogenous group of animals; there are not reference intervals in dogs for all the metals analysed in this study and the reference ranges for Cu are established on dry tissue basis and our results were in wt.w. However, our results indicate that some trace elements other than Cu (mainly Co, Mn and Zn) are increased in the liver of dogs affected by CH. Taking into account that multi-element analysis by ICP-MS is nowadays relatively cheap, easily-available and requires minimal sample size, the determination of main trace elements in the liver as well as their interactions could help to better understand the disease and enhance diagnosis of liver disease in dogs.

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Conflict of interest

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.
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