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Original article

Lipogranulomas and pigment granulomas in livers of dogs with portosystemic shunt

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Abstract

Lipogranulomas are lesions found in histopathological liver examination in humans and in various animal species, including dogs, especially those with portosystemic shunts. They consist of macrophages and other inflammatory cells, and sometimes they contain iron salts (pigment granuloma). This study aimed at determining the number of granulomas and cellular composition of lipogranulomas in dogs with the congenital extrahepatic portosystemic shunt, and to identify factors associated with their development. 44 archival liver samples from dogs with portosystemic shunt were stained using HE, Perl's method and – in randomly-selected cases – immunohistochemically against CD56, CD20 and CD3 (DAKO). A reduction in the size of the liver was observed in all dogs during laparotomy, and the diameter of the vessel circumventing the liver was also measured (in 24 dogs). Lipogranulomas were found in 52.3% of samples; iron salts were present in 47.8% of them; 72% of cells in lipogranulomas were macrophages. In lipogranulomas both types of lymphocytes – T and B – were seen. The presence of lipogranulomas in liver samples in dogs was connected with fatty degeneration of hepatocytes and was correlated with the age of animals and with the diameter of the abnormal vessel circumventing the liver. Their formation appears to be triggered by severe ischemia and shortage of nutrient supply.

Key words: lipogranuloma, pigment granuloma, liver, portosystemic shunt, dog

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Introduction

Lipogranulomas are lesions found in histopathological examination of the liver in various animal species and in people. They consist of groups of macrophages with lipid vacuoles, as well as lipofuscin, hemosiderin and ceroid in their cytoplasm (van Winkle et al. 2006, Stalker and Hayes 2007, Isobe et al. 2008b). They are formed with the possible participation of lymphocytes, plasma cells and stellate cells (van Winkle et al. 2006, Stalker and Hayes 2007). These structures are formed in the liver parenchyma as well as in the connective tissue of the portal area, around the hepatic triad.

It has been shown that lipogranulomas are found in human patients with fatty liver caused by alcohol consumption, diabetes or obesity and in chronic hepatitis caused by the hepatitis C virus (Petersen and Christoffersen 1979, Delladetsima et al. 1987, Zhu et al. 2010). Using an electron microscope it has been shown that lipid droplets are extracellular, surrounded by macrophages and lymphocytes with hepatocytes debris among them (Petersen and Christoffersen 1979). Some regard lipogranulomas in steatofibrosis of the human liver as a marker of previous steatosis of hepatocytes (Zhu et al. 2010).

When it comes to animals, lipogranulomas have been found in experimental rodents (rats), mainly in correlation with fatty liver of various aetiology. Koutsos et al. (2001) reported that lipogranulomas were also found in livers of cockatiels when they were given a high-protein diet, as a metabolic adaptation of the birds to this diet.

Lipogranulomas were found in 44% of dogs with hepatic fibrosis and, as in humans, it might be a potential indicator of iron and lipid accumulation in the liver (Isobe et al. 2008b). However, no correlation was found between their presence and the content of collagen I/III (Isobe et al. 2008b). Lipogranulomas were also found in the liver in over 55% of dogs with portosystemic shunt, especially in those above 1 year of age (Isobe et al. 2008a). These lipogranulomas consisted of macrophages, which were demonstrated by immunohistochemical staining for macrophage scavenger receptor class A (MSR-A) and their cytosol was found to contain ceroid and hemosiderin.

A hypothesis was put forward in older pathomorphological studies of the dogs' liver that the formation of lipogranulomas might play a role in the pathogenesis of nodular hyperplasia (Bergman 1985). Moreover, Van Winkle et al. (2006) proposed that granulomas in dogs should be classified depending on the content of macrophage cytoplasm, into "lipogranulomas" – where ceroid is found in macrophage cytoplasm along with lipids and "pigment granulomas" – where macrophages contain lipids, ceroid and iron compounds. However, pathogenesis and the role of lipogranulomas in the liver of dogs have not so far been clarified.

Portosystemic shunt (PSS) is an abnormal vascular connection between the portal system and the caudal vena cava or azygos vein. PSS is usually congenital (CPSS) and may be either extrahepatic, when it circumvents the liver, or intrahepatic, when it penetrates liver parenchyma. Congenital portosystemic shunts constitute pathological vessels that developed during embryonic life; in general, they are single (both extra- and intrahepatic shunts) and their presence is not associated with portal hypertension. Congenital shunts are more often reported in pedigree dogs than in crossbreeds (Johnson SE 2000). Intra-hepatic shunts are usually diagnosed in large breeds (Doberman Pinscher, Golden Retriever, Labrador Retriever, Irish Setter, Samoyed, Irish Wolfhound), whereas extra-hepatic shunts are reported in small breed dogs (such as Yorkshire Terrier, Miniature Schnauzer, Cairn Terrier, Maltese, Miniature Poodle, Dachshund (Richter 2003). Acquired PSS is usually extrahepatic and consists of multiple vessels. It occurs as a result of end stage liver disease (cirrhosis) and portal hypertension.

Our study aimed at determining the number of granulomas and cellular composition of lipogranulomas in dogs with congenital extrahepatic portosystemic shunt and to identify factors associated with their development.

Materials and Methods

Archival liver samples collected from dogs with extrahepatic CPSS were used as the study material.

A tentative diagnosis of extrahepatic CPSS was made on the basis of suggestive clinical signs (gastrointestinal signs, neurological signs and urate urolithiasis); elevated pre- and postprandial levels of bile acids, ammonemia; and Doppler abdominal ultrasound. A definitive diagnosis was established and liver sections were collected during laparotomy. The diameter of the vessel circumventing the liver was also measured during laparotomy.

Liver sections were fixed in 10% buffered formalin and embedded in paraffin (Paraplast). The sections were 4-μm thick and they were stained with hematoxylin and eosin (HE) and, according to Perl's method, staining for iron (Bancroft and Cook 1994).

Specimens were viewed in a light microscope (Olympus BX 43, Poland) and lipogranulomas were counted in 10 fields of vision at a lens magnification of 40x. Subsequently, the cell morphology was assessed in each patient in three randomly-selected lipogranulomas and cells of each type were counted: macrophages, lymphocytes, plasma cells and neutrophils.

Material collected from 10 randomly-selected dogs was stained immunohistochemically with the following antibodies: CD56 for macrophages (monoclonal mouse antibody CD56 Clone 123C3; IS62830-2; Dako, Glostrup, Denmark; Ready to Use; incubation at room temperature, 30 min), CD20 for B lymphocytes (monoclonal mouse antibody CD20 Clone L26; IS60430-2; Dako, Glostrup, Denmark; Ready to Use; incubation at room temperature, 30 min) and CD3 for T lymphocytes (polyclonal rabbit antibody CD3 IS50330-2; Dako, Glostrup, Denmark; Ready to Use; incubation at room temperature, 30 min), in order to identify cells in lipogranulomas.

Sections of lymphatic tissue from human cecum and tonsils were used as a control.

Photographic documentation was made with an Olympus BX 43 optical microscope coupled with an SC30 camera.

Statistical analysis

Numerical variables were presented as the median, interquartile range (IQR) and range, and compared between groups using a Mann-Whitney U test. Categorical variables were given as the count and percentage, and compared between groups using a Pearson chi-square test or a Cochrane-Armitage chi-square for trends (Agresti 2007). A Spearman rank-order correlation coefficient (r_s) was used to assess the relationship between the age and the number of lipogranulomas. A significance level (α) was set at 0.05. Statistical analysis was performed in Statistica 12 (StatSoft Inc., USA).

Results

Forty-four dogs, half males and half females, were diagnosed with PSS and enrolled in the study. Among them there were 23 Yorkshire terriers, 4 miniature schnauzers, 2 Cairn terriers, Maltese dogs, Pekingese dogs and mongrels, and a single representative of Jack Russel terrier, Chihuahua, Brussels Griffon, Pinscher, Papillion, American Staffordshire terrier, Pug, English Cocker Spaniel and Greyhound. Their age ranged from 3 months to 7 years, with the median of 18 months and IQR from 12 to 48 months, and no difference between sexes was observed (p=0.734; data available for 41 dogs). All dogs had either post-prandial bile acid concentrations elevated above 25 μmol/L (measured in 10 dogs; reference: ≤20 μmol/L pre-prandial, ≤25 μmol/L post-prandial, Bunch SE 2008) or hyperammonemia above 70 μmol/L (measured in 10 other dogs), or the vessel circumventing the liver was visible in ultrasonography (performed in all 44 dogs). A reduction in the size of the liver was also observed in all 44 dogs during laparotomy. The diameter of the abnormal vessel was measured in 24 dogs and ranged from 2 mm to 12 mm, with a median of 5 mm and IQR from 4 to 6 mm.

Lipogranulomas, both in the parenchyma and at the portal area, were found in the liver sections of 23 dogs (52.3%; Fig 1A, 1B) – 11 Yorkshire terriers, 2 miniature schnauzers and Pekingese dogs, and a single Cairn terrier, Maltese dog, Pinscher, American Staffordshire terrier, Pug, English Cocker Spaniel, Greyhound and mongrel. The presence of iron salts was revealed in 11 dogs (4 female, 7 male; 47.8% of all dogs with lipogranulomas; in 40% of females and 63.6% males with granulomas in the liver samples) – these were pigment granulomas (Fig. 1C). Macrovesicular (Fig. 1A) or microvesicular (Fig. 1B and 1C) fatty degeneration (focal or diffuse) was found in all animals with lipogranulomas; moreover necrosis was seen in 16 animals (in 11 of them – disseminated, in 5 – necrosis of single hepatocytes) and steroid-induced hepatopathy – in 2 cases. Intensified liver parenchyma fibrosis was observed in four dogs (2 males and 2 females).

Sex distribution was similar among dogs with and without lipogranulomas – females accounted for 52.2% and 47.6% of dogs in these two groups, respectively (p=0.723). No difference in the number of lipogranulomas was found between males and females (p=0.665).

Formation of lipogranulomas proved to be linked with age. They were found in none of 6 dogs up to the age of 6 months, 7 of 11 dogs (64%) between 7 and 12 months, 7 of 12 dogs (58%) between 13 and 24 months, and 9 of 12 dogs (75%) older than 24 months (p=0.014). Also, the number of lipogranulomas tended to rise along with age (r_s = 0.56, p=0.005) (Fig. 2).

The total number of inflammatory cells (macrophages, lymphocytes, plasma cells and neutrophils) in the lipogranuloma ranged from 9 to 49, with a median of 17 and IQR from 15 to 30. Macrophages and lymphocytes accounted for the vast majority of inflammatory cells, while the share of neutrophils and plasma cells was marginal (Fig. 3). The total number of inflammatory cells in the lipogranuloma tended to increase with age (r_s = 0.77, p<0.001), which resulted from the increasing number of macrophages (r_s = 0.81, p<0.001) but not lymphocytes (r_s = 0.15, p=0.503) (Fig. 4).

Formation of lipogranulomas was also linked to the diameter of the abnormal vessel – the vessel was significantly wider in dogs with lipogranulomas (n=13; median of 6 mm, IQR of 5 to 8 mm and range from 4 to 12 mm) than in dogs without lipogranulomas (n=11; median of 4 mm, IQR from 3 to 5 mm, and range from 2 to 6 mm; p=0.005).

Expression of CD56 was demonstrated in an immunohistochemical examination in all lipogranulomas in

the dog livers (Fig. 5A). The expression was also focused in various places of the parenchyma and around the hepatic triads. Expression of CD20 (B lymphocytes) was found in lipogranulomas in 9 out of 10 dogs (Fig. 5B); in two of them it was only slightly marked and found only in individual cells. Moreover, expression of CD20 was visible in the liver parenchyma in all 10 dogs and around the hepatic triads in 9 out of 10 dogs. Expression of CD3 (T lymphocytes) was observed in all 10 dogs; however, it was only mildly marked in half of them (Fig. 5C). Expression around the hepatic triads was visible in 9 out of 10 dogs (weaker in 4 animals, extremely intensive in 1 dog) and in the single cells in the liver parenchyma in all animals. In pigment granuloma interpretation of immunohistochemical staining was hampered by the background composed of iron salts.

Discussion

Portosystemic shunts are abnormal vascular connections between the portal vein system and the systemic vein circulation that allow for a flow of blood directly into the systemic circulation, bypassing the liver (Johnson SE 2000, Maxie 2007). In the liver it manifests macroscopically by a decrease in this organ's size and it was a visible change in all our examined dogs. Histopathological changes, both in an intra- and extrahepatic portosystemic shunt, include proliferation of blood vessels and regressive changes in hepatocytes (atrophy, fatty degeneration, necrosis). However, the intensity of changes – both macro- and microscopic – depends on the deviation from the normal vascular system (Cullen et al. 2006) and on the drugs given to the patients (signs

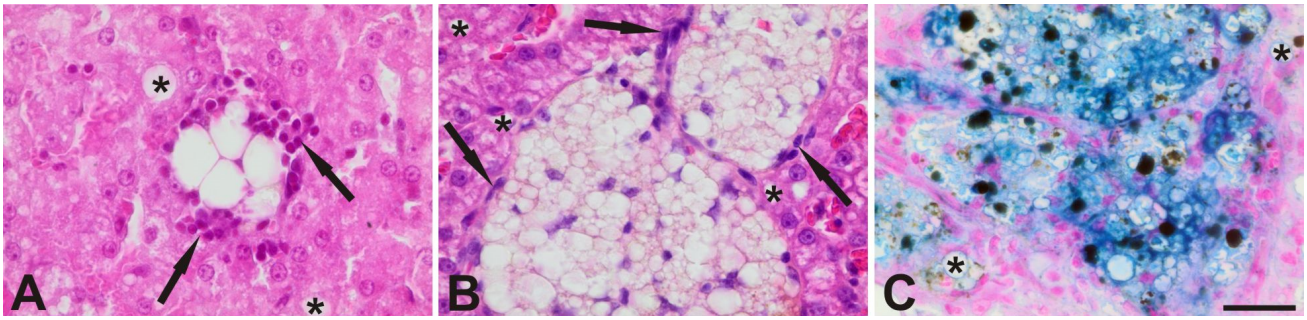

Fig. 1. Lipogranulomas: A - small one with a high infiltration of lymphocytes (arrows), B - large one with only few lymphocyte infiltrations and pigment granuloma (C) in the liver of dogs with portosystemic shunt and macrovesicular (A) and microvesicular (B, C) fatty degeneration (asterisks) of hepatocytes. A, B: morphology of granulomas in routine HE staining; C: iron salts in pig ment granuloma (stained blue). Staining with HE (A, B) and according to Perl's method for iron salts (C). Magnification as shown in Fig. 1C, Bar = 20 μm.

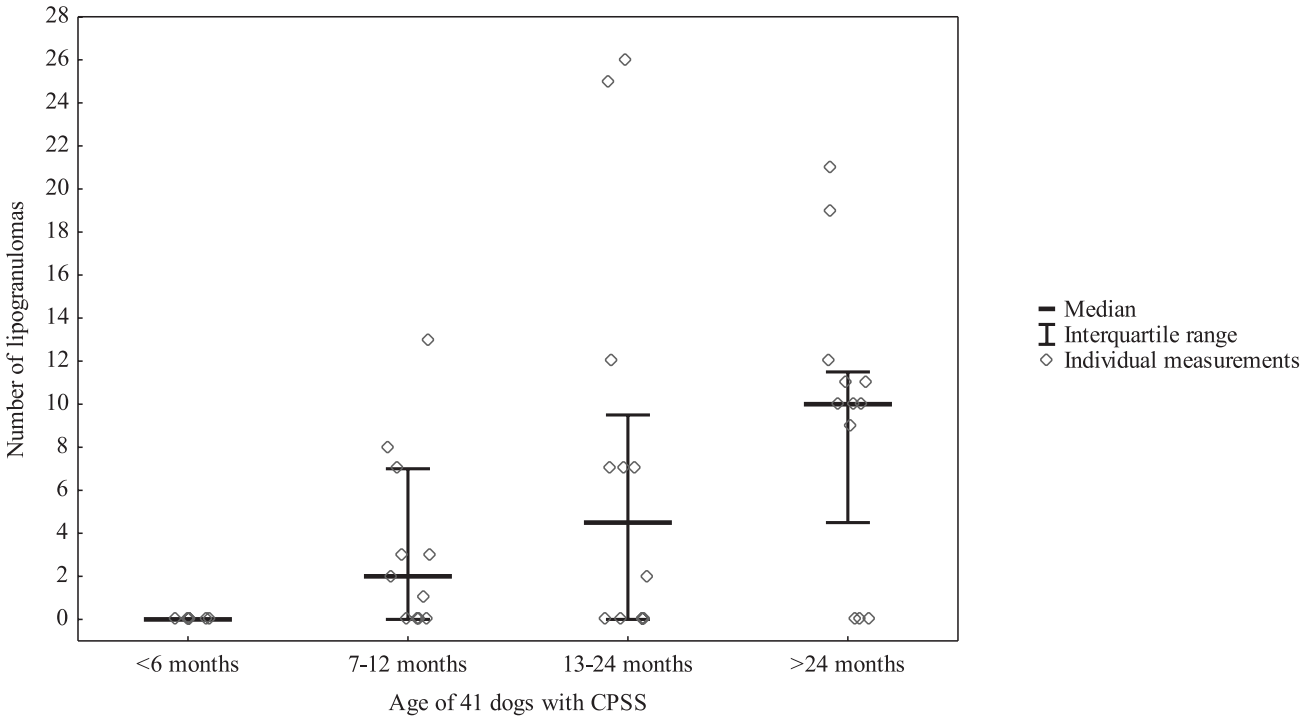

Fig. 2. Number of lipogranulomas found in the liver of 41 dogs with congenital portosystemic shunt (CPSS) in four consecutive age categories.

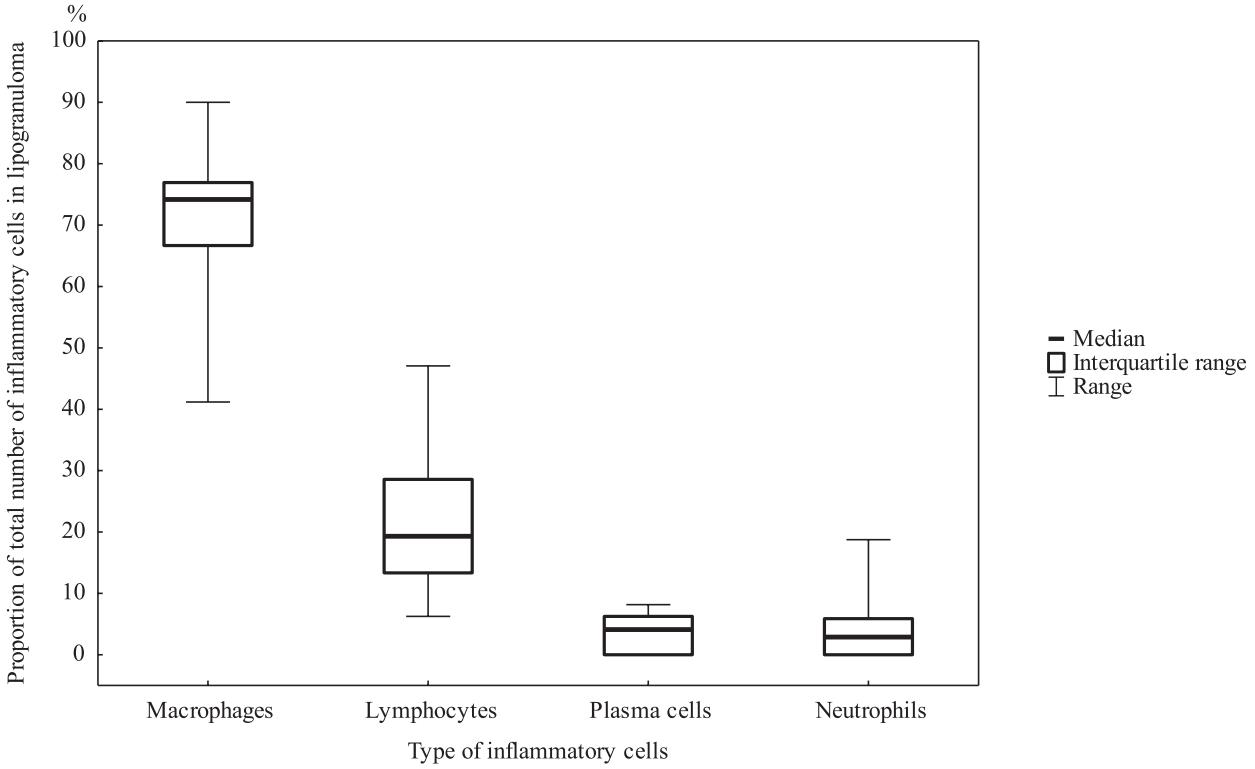

Fig. 3. Share of different types of inflammatory cells in the lipogranuloma in the livers of 23 dogs with congenital portosystemic shunt (CPSS).

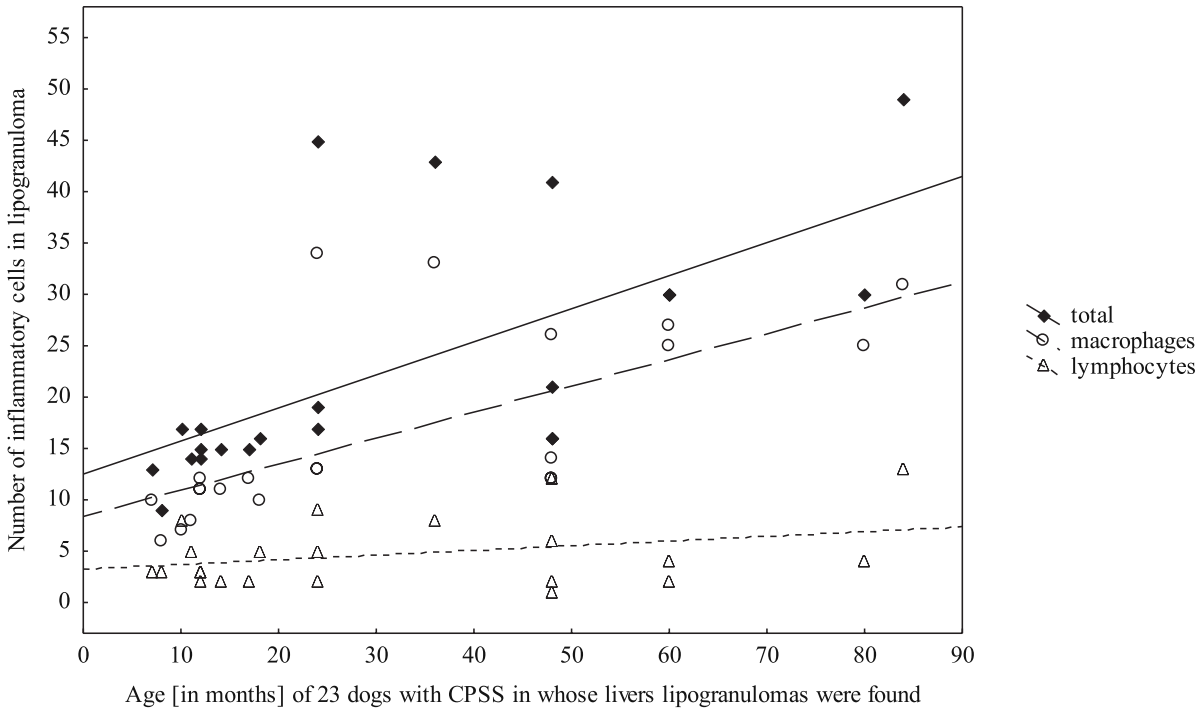

Fig. 4. Correlation between number of inflammatory cells found in the livers lipogranulomas of 23 dogs with congenital portosystemic shunt (CPSS) and their age. Solid diamonds (◆) signify the total number of inflammatory cells, open circles (○) – the number of macrophages, and open triangles (Δ) – the number of lymphocytes. Lines show the general trend.

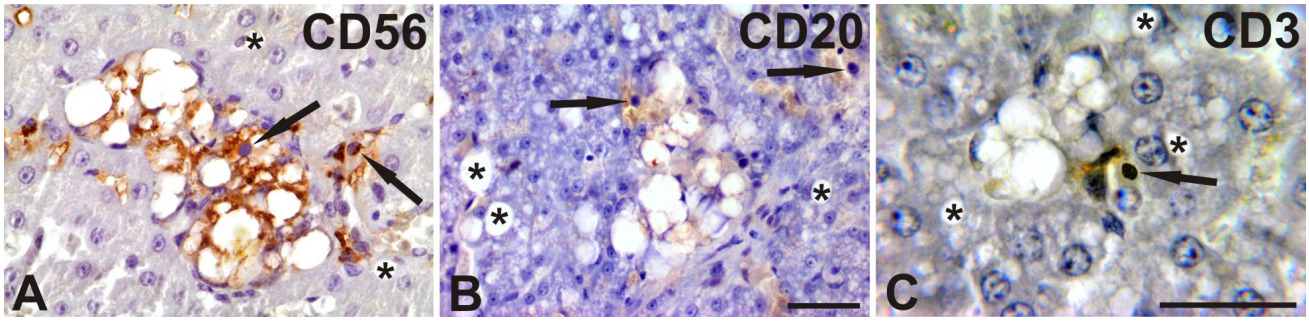

Fig. 5. Immunohistochemical staining of lipogranulomas and fatty degeneration of hepatocytes (asterisks) in the livers of dogs with a portosystemic shunt: A - expression of CD56 (brown colour, arrows) indicative of the presence of macrophages; B: expression of CD20 (brown colour, arrows) indicative of the presence of B lymphocytes; C: expression of CD3 (light brown colour, arrow) indicative of the presence of T lymphocytes. Magnification in Fig. 5A and 5B as shown in Fig. 5B, Bar = 20 μm; magnification in Fig. 5C – Bar = 40 μm.

of steroid-induced hepatopathy were observed in some animals under study). As with people and rodents, the presence of lipogranulomas in the liver of dogs under study was associated with macrovesicular and microvesicular fatty degeneration (focal or diffuse) of hepatocytes (Petersen and Christoffersen 1979, Zhu et al. 2010). In our study it was not associated with hepatic fibrosis, which was observed only in 4 out of 23 animals under study in which lipogranulomas were found. Such results correlate with conclusions drawn by Isobe et al. (2008b).

The liver sections in which lipogranulomas were found was collected from dogs of various breeds (both sexes) in which a portosystemic shunt had been confirmed. Yorkshire terriers and dogs similar to this breed dominated among the patients, which can be attributed not only to frequent occurrence of the portosystemic shunt in Yorkshire terriers (Johnson SE 2000, Richter 2003, Maxie 2007), but also to the high popularity of this breed as an assistance companion dog in Poland. In our study granulomas were found in liver sections taken from dogs older than 6 months, whereas Isobe et al. (2008a) defined this threshold age as "more than 1 year". Granulomas were found in 52.3% of animals with the portosystemic shunt in our study. Similar incidence of such changes was observed by Isobe et al. (2008a), however this percentage was much lower (15%) in people with hepatitis C, hepatic steatosis and fatty liver disease (Zhu et al. 2010).

The number of lipogranulomas was found to increase with the increasing age of the animals. The total number of inflammatory cells in the lipogranuloma and the size of lipogranuloma also tended to increase with age, which resulted predominantly from the increasing number of macrophages. However, the number of granulomas did not correlate with the sex of the animals under study.

It has been shown in histopathological diagnostics in people that portal granulomas occur in 2.4% of cases and in a half of samples only single granulomas are visible in material taken by biopsy (Delladetsima et al. 1987). Unlike in people, one liver section taken from a dog in our study contained several granulomas, both in the liver parenchyma and in the portal area. Conversely, single granulomas were visible in few dogs and they were associated with young age (12 months or less).

Lipogranulomas and pigment granulomas are largely made up of macrophages. This was concluded from the cell morphology in routine (HE) staining and confirmed in immunohistochemical staining. It is consistent with the observations of other authors (Isobe et al. 2008a, Nagy et al. 2013).

Cullen and Stalker (2016) describe degenerative changes – including fatty degeneration in dog liver – using the term "fatty cyst", defined as "groups of the fat-laden cells" with a tendency to rupture and to merge. In their interpretation, it is an epithelial structure, which transforms into granulomas. A similar pattern in the current study (seen in preparations stained with HE and in an analysis with CD56 antibodies) indicates that these are clusters of macrophages. This makes one consider the rate of changes in the liver in cases of vast fatty degeneration of hepatocytes and the mobilisation rate of Browicz-Kupffer cells (hepatic macrophages) as well as the moment at which the epithelial structure described by Cullen and Stalker (2016) turns into a granuloma made up of macrophages. In our opinion, the rate of changes occurring in the liver could be determined only in an experiment.

It was found that cells that make up lipogranulomas include macrophages, but also cells with the morphology of lymphocytes. Immunohistochemical staining revealed that these were both T and B lymphocytes. The presence of both populations of lymphocytes in the liver of dogs is justified, when we consider the role of the liver in the organism's defence against pathogens, flowing with blood from other organs such as the alimentary tract. The presence of lymphocytes in granulomas is

confirmed by the fact that no dysfunctions of the immune system were found in these animals, either in the clinical examination or in the laboratory blood check-up.

Routine and histochemical staining in the area containing granulomas reveals iron salts, although not in equal amount in each dog. When describing illustrations which show granulomas, Cullen and Stalker (2016) use the terms "lipogranuloma" and "pigment granuloma" interchangeably, although in the text of their paper they define the latter as "containing iron with ceroid". They conclude that macrophages, especially those containing iron, accumulate in the liver along with age of the animals and hepatocyte turnover. The cytoplasm of macrophages which made up granulomas in the animals under study was found to contain iron, but the relationship between the animal age and the presence and level of iron salts was not examined. However, it was found that pigment granulomas predominated in male dogs and those without iron salts in female dogs. Moreover, it was clearly visible in our study, as was concluded by Cullen and Stalker (2016), that the total number of inflammatory cells, mainly macrophages, tended to increase along with dogs' age.

Conclusions

Roughly a half of dogs with the congenital extrahepatic portosystemic shunt have lipogranulomas or pigment granulomas in their livers. Their occurrence is related to increasing age (so to longer duration of the disease) and to the diameter of the abnormal vessel circumventing the liver. Therefore, their formation appears to be triggered by severe ischemia and shortage of nutrient supply.

Lipogranulomas contain macrophages and both populations of lymphocytes – T and B. Moreover, immunohistochemical methods proved effective in verifying histopathological examination based on routine staining.

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