Received: 11 January 2018 | Revised: 20 April 2018 | Accepted: 15 May 2018

DOI: 10.1111/jvim.15238

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

STANDARD ARTICLE

Hepatocyte expression and prognostic importance of senescence marker p21 in liver histopathology samples from dogs with chronic hepatitis

Andre J. Kortum | Emilie A. Cloup | Tim L. Williams | Fernendo Constantino-Casas | Penny J. Watson

Department of Veterinary Medicine, The Queen's Veterinary School Hospital, University of Cambridge, Madingley Road, Cambridge, United Kingdom

Correspondence
Andre J. Kortum, Department of Veterinary Medicine, The Queen's Veterinary School Hospital, University of Cambridge, Madingley Road, Cambridge CB3 OES, United Kingdom.
Email: ak974@cam.ac.uk

Funding information
Petplan Charitable Trust, Grant/Award Number: 217-263

Background: Chronic hepatitis (CH) occurs commonly in dogs but is associated with a variable and largely unpredictable prognosis. p21, a cell-cycle inhibitor and marker of cellular senescence, is upregulated in human liver disease and is a better prognostic marker than histological or clinical scoring systems.

Objective: To quantify hepatocyte p21 immunopositivity in histopathology samples from dogs with CH and determine its association with outcome.

Animals: Twenty-six client-owned dogs with histologically confirmed CH, and 15 dogs with normal liver histology.

Methods: Medical records and liver histopathology samples were retrospectively reviewed to identify cases of CH. Immunohistochemistry for p21 was performed on all samples and hepatocyte immunopositivity was visually quantified. Relationships between p21 and dog age and dog survival time were statistically evaluated.

Results: Hepatocyte p21 immunopositivity in dogs with CH was high (median percentage of positive hepatocytes: 90%, range: 20%-98%) and exceeded 70% in 23/26 cases with no association with age. In control dogs, p21 immunopositivity was low (≤15% positive hepatocytes in 12/15 cases) and was positively correlated with age (r_s = 0.63; P = .011). Dogs with p21 immunopositivity exceeding 91.8% (upper tercile) had significantly shorter survival compared to dogs with less than 88.9% immunopositivity (lowest tercile; 218 versus 874 days, P = .006). Increasing hepatocyte p21 immunopositivity was significantly negatively associated with survival time (HR 4.12; 95% CI 1.34-12.63; P = .013).

Conclusions and Clinical Importance: Marked p21 immunopositivity in dogs with CH might be indicative of widespread hepatocellular senescence. A significant association with survival time also suggests a potential value for p21 quantification in determining prognosis.

KEYWORDS

cell cycle arrest, DNA damage, immunohistochemistry, liver disease

1 | INTRODUCTION

Chronic hepatitis (CH) is a common liver condition in dogs which is characterised histologically by hepatocellular apoptosis or necrosis, a variable mononuclear or mixed inflammatory infiltrate, regeneration, and fibrosis.[1] Although a range of potential underlying aetiologies

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; CH, chronic hepatitis; CKD, cyclin-dependent kinase; GGT, gamma-glutamyltransferase; HR, hazard ratio; IQR, interquartile range; METAVIR, meta-analysis of histological data in viral hepatitis; SASP, senescence-associated secretory phenotype; WSAVA, World Small Animal Veterinary Association.

have been previously hypothesized, including infectious agents, toxins, and drugs, immune-mediated disease and breed-associated metabolic errors, only breed-related copper storage diseases have been extensively investigated and the cause unfortunately remains elusive in most cases.[2,3] Treatment of idiopathic CH is generally empirical and nonspecific, and life expectancy after diagnosis is highly variable, ranging from months to years.[4]

Hyperbilirubinaemia,[5] hypoalbuminaemia,[6] hypoglycaemia,[7] and the presence of ascites or cirrhosis[2,8] have been previously proposed as negative prognostic indicators in dogs with CH. However, none are reliable and typically only occur in advanced or end-stage disease. In addition, the severity of histological pathology can correlate poorly with the severity of clinical signs or survival, and dogs with severe clinical signs can have disproportionately minor changes on histology and vice versa.[9] There is therefore a need for an alternative prognostic marker in dogs with CH to better guide owners and veterinarians, particularly for those diagnosed at an earlier stage of disease before the development of cirrhosis and other clinical abnormalities indicative of end-stage CH.

Cellular senescence is a reversible state of cell cycle arrest in which cells are unresponsive to mitotic stimuli but remain metabolically active, and can be triggered by multiple mechanisms including irreversible DNA damage and telomere shortening.[10–13] These changes trigger a signal amplification cascade known as the DNA Damage Response leading to irreversible cell cycle arrest. p21 is a key mediator in this process and additionally contributes to the stability and maintenance of cellular senescence. p21 is a potent cell-cycle inhibitor, which is capable of inhibiting cyclin/cyclin-dependent kinase (CDK) complexes, thereby acting as a regulator of cell cycle progression.[10–13] Most notably, inactivation of CyclinA/CDK2 because of binding of the N-terminal domain of p21 results in hypophosphorylation of Retinoblastoma protein and sequestration of transcription factor E2F, thereby leading to cell cycle arrest at the G1/S phase checkpoint.[10–12] The actions of p21 are complex, and it has numerous additional roles in cell cycle regulation including CDK1 and CDK 4/6 inhibition and interaction with proliferative cell nuclear antigen.[10–12] Furthermore, p21 it also thought to play pivotal roles in inhibiting apoptosis, the regulation of transcription, and in DNA repair.[12,14,15] Although senescent cells have been previously reported to display mild morphological changes, such as increased nuclear size,[16] these features are not readily apparent using routine histological stains and their presence is inevitably likely to be overlooked.p21 is greatly upregulated in humans with alcoholic and nonalcoholic liver disease and is better correlated with survival time than histological and clinical scoring systems.[17,18] In addition to its role in determining prognosis in these cases, these findings add insight to the underlying pathophysiology of liver disease and might thereby facilitate the development of novel treatment strategies. To date, research in dogs has mainly focused on p21 expression in neoplasia[19–25]; however, no studies have previously investigated its relevance in dogs with liver disease. The aim of this study was therefore to investigate whether hepatocyte p21 expression is increased in CH in dogs and whether the degree of immunopositivity is associated with survival.

2 | METHODS

2.1 | Case selection

Medical records and histopathology reports were retrospectively reviewed for dogs diagnosed with CH at the Queen's Veterinary School Hospital, University of Cambridge between 2004 and 2017. Cases were selected based on fulfilment of the WSAVA histological criteria and compatible histories, physical examination findings, and clinicopathological abnormalities. Dogs with other concurrent disease processes likely to affect liver disease progression or survival time, or those treated with drugs, which could potentially result in hepatoxicity (such as carprofen, potentiated sulfonamides, phenobarbital, or ketoconazole) before liver biopsy acquisition were excluded.

Prior to inclusion, liver biopsy samples for all cases were subsequently blindly reviewed by two pathologists using routine stains (hematoxylin and eosin, rhodanine, Sirius red, and Periodic-acid-Schiff). Histological scoring and grading was performed based on the METAVIR scoring system used in human hepatology which has also been previously used to grade hepatic lesions in dogs.[26–28] The following variables were assessed: number of portal spaces, severity (scale 1–3) of hepatocyte injury (ie, apoptosis or necrosis); nature, distribution, and severity (scale 1–4) of parenchymal inflammation; and fibrosis stage (from 0 to 4). A cumulative final score was subsequently calculated and used to define the severity of CH (mild ≤5/11; moderate 6–8/11; marked >8/11). Copper accumulation was additionally scored (scale 0–3) using a previously established semi-quantitative scoring system.[9] Cases with histological changes suggestive of an infectious underlying etiology, such as those with pyogranulomatous or neutrophilic inflammatory infiltrates, were excluded. A control population was also included and comprised post mortem liver samples from dogs that were euthanased for reasons other than liver disease. No histological abnormalities within the liver were evident in any of these cases.

Clinical data (signalment, physical examination, and clinicopathological abnormalities) and treatment after diagnosis of CH was recorded for all cases. Survival data (all-cause mortality) was collected by contacting referring veterinary practices and, when available, the reason for death or euthanasia was recorded. Cases were also excluded if the cause of death or euthanasia was determined to be because of an unrelated disease process or if follow-up information was unavailable.

2.2 | Immunohistochemistry

Serial sections (3 μm) from formalin-fixed, paraffin wax-embedded liver biopsy samples were mounted on positively charged slides (Snowcoat, Surgipath Europe Ltd, Peterborough, UK) and dried overnight at 50°C in an incubator. Dewaxing, rehydration, and antigen retrieval was subsequently performed in a combined 3-in-1 procedure using the Dako PT link module (Dako, Carpinteria, California). Sections were then immersed in a preheated working solution (Dako Envision FLEX Target Retrieval solution) and, once the temperature had reached 97°C, were incubated for 20 minutes before being left to cool

to 65°C and immediately rinsed in buffer (Dako Envision Wash Buffer) at room temperature.

An automated immunohistochemistry system (Dako Autostainer) was used to process the prepared tissues. Endogenous peroxidase activity was inhibited using Dako Envision Flex, Peroxidase-Blocking solution for 30 minutes. The primary antibody used was polyclonal rabbit antihuman p21 (phospho T145) at 1 : 1000 dilution (Abcam, Cambridge, UK). After primary antibody incubation for 1 hour at room temperature, the immunosignal was detected using Dako Envision FLEX/HRP detection reagent for 30 minutes at room temperature. Labelling was demonstrated using 3,3′ diaminobenzidine for 10 minutes and slides were counterstained with Dako Envision Flex Hematoxylin for 5 minutes before rinsing, dehydrating, clearing, and covering. Sections of canine skin with positively labeled basal keratinocytes were used as a positive control (Inoue et al, 2006) and antibody diluent (Dako Envision Flex Antibody diluent) served as a negative control. Immunostaining was present in the positive control sections, but not detected in negative controls.

p21 immunopositivity for each case was visually quantified by two observers (EC and AK) by scoring p21-positive and p21-negative hepatocytes in eight high-power fields (400× magnification), and was expressed as an averaged percentage of total hepatocyte number. Observers were blinded to clinical information relating to the cases (ie, name, history, previous histology reports, treatment, and outcome) and to the scores of other observers. Hepatocytes were identified based on their typical polygonal shape with ample cytoplasm and central round nucleus. Hepatocytes with brown-stained nuclei were considered positive whereas hepatocytes with blue nuclei were considered negative; differences in stain intensity were not taken into consideration.

2.3 | Statistical analysis

Survival time was defined as the time between histological diagnosis of CH and death or euthanasia (all-cause mortality). Dogs that were alive or lost to follow up at the study end-point (March 2016) were censored from the survival analysis. Survival relationships were investigated using Kaplan-Meier and univariate and multivariable Cox regression analyses. Variables considered significant at the 20% level in the univariate Cox regression analysis were included into a backward, stepwise multivariable Cox regression model. Available clinicopathological data were divided into terciles and missing data for each variable was assigned to a missing data category for the purposes of the statistical analysis. p21 positivity was treated as both a continuous and categorical variable (divided into terciles) because of the possibility of a nonlinear relationship with survival time. Spearman's correlation coefficient was calculated to determine the relationship between hepatocyte p21 immunopositivity and age. Statistical analysis was performed using commercially available software (IBM SPSS Statistics version 25 for Windows, IBM Corp, Armonk, New York and GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla, California) and $P < .05$ was considered statistically significant. Unless otherwise stated, data are presented as median (range), or number (percentage).

3 | RESULTS

One hundred and thirty eight histopathology reports describing chronic inflammatory changes within the liver were initially reviewed, of which 73 were considered attributable to alternative aetiologies other than CH. Of the remaining 65 cases, histology samples were unavailable for review in 25 and a further 3 cases did not fulfil the WSAVA criteria for a diagnosis of CH on review. After review of clinical data, a further 3 cases were excluded because of a lack of follow-up information and 8 cases were excluded because of concurrent disease processes likely to affect liver disease progression or accounting for death or euthanasia. Twenty-six cases therefore ultimately satisfied the criteria for inclusion into the study.

The 26 included cases comprised 14 breeds: English Cocker Spaniel (5), Labrador Retriever (4), English Springer Spaniel (3), Cross-breed (3), Bedlington Terrier (2), American Cocker Spaniel (1), Rottweiler (1), Tibetan Terrier (1), Golden Retriever (1), Doberman Pinscher (1), Welsh Springer Spaniel (1), Staffordshire Bull Terrier (1), Jack Russell Terrier (1), and Standard Poodle (1). The median age for these dogs was 7.0 (0.75-12) years; 13/26 (50%) were neutered females, 10/26 (38%) were neutered males, 3/26 (12%) were entire females and 1/26 (4%) was an entire male. Routine clinicopathological data at the time of diagnosis is presented in Table 1. Fasting serum bile acids were measured in 16 cases and were elevated in 11. Bile acid stimulation testing was performed in 9 of these dogs and was abnormal in 5 cases. Treatment of cases varied according to clinician preference and included S-adenosyl methionine (19 cases), milk thistle (2 cases), vitamin E (2 cases), ursodeoxycholic acid (16 cases), spironolactone (8 cases), furosemide (3 cases), prednisolone (5 cases), amoxicillin-

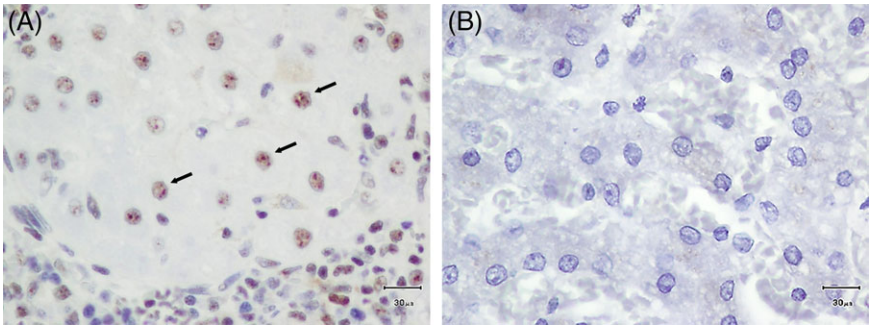

FIGURE 1 p21 immunostaining in a dog with CH (Image A) and normal liver (Image B) after immunohistochemistry. Hepatocytes with brown-stained nuclei were considered positive (arrows) and those with blue-stained nuclei were considered negative (400×)

TABLE 1 Serum ALT, ALP, and GGT activities, and total bilirubin and albumin concentrations for the study population of dogs with CH

	n	Median (range)	Number (%) of cases with abnormal values	Reference interval
ALT (IU/L)	23	401 (19–1344)	21 (91%)	14–67
ALP (IU/L)	23	682 (61–19213)	22 (96%)	26–107
GGT (IU/L)	23	15 (1–153)	18 (78%)	0–10
Total bilirubin (μmol/L)	23	9.8 (1.6–398.4)	8 (35%)	0–12
Albumin (g/L)	21	24 (12–33)	14 (67%)	25–41
Fasting Bile Acids (μmol/L)	16	46 (1–448)	11 (69%)	0–15
PostPrandial Bile Acids (μmol/L)	9	42 (0–357)	5 (56%)	0–22.5

Abbreviations: ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; GGT, gamma-glutamyltransferase.

clavulanate (6 cases), metronidazole (2 cases), marbofloxacin (1 case), ranitidine (4 cases), parenteral cobalamin supplementation (1 case), lactulose (1 case), and penicillamine (1 case).

Data regarding histological scoring for included cases is presented in Table 2. The median cumulative final score for the 26 cases was 9 (range 4–11), resulting in a final histological diagnosis of marked CH in 15 cases (58%), moderate CH in 8 cases (31%) and mild CH in 3 cases (12%). Lymphocytic or lymphocytic-plasmacytic periportal or lobular inflammatory infiltrates were seen in all cases, with smaller numbers of neutrophils and histiocytes reported in 20/26 (77%) and 8/26 (31%) cases, respectively. Four cases were suspected to be related to copper storage disease based on the degree and distribution of copper accumulation on rhodanine-stained tissue sections.

Fifteen control dogs were included and comprised 12 breeds: Labrador Retriever (3), German Shepherd dog (2), Golden Retriever (1), Bulldog (1), Cavalier King Charles Spaniel (1), Border Collie (1), Boxer (1), Chihuahua (1), Lakeland Terrier (1), Miniature Dachshund (1), Staffordshire terrier (1), and cross-breed (1). The median age for these dogs was 6.25 (0.75-16.7) years with no significant difference in age compared with dogs with CH (P = .203). 6/15 (40%) dogs were neutered males, 6/15 (40%) dogs were neutered females and 3/15 (20%) dogs were entire females. Reasons for euthanasia included: progressive neurological deterioration (5 cases), acute intervertebral disc extrusion (1 case), acute pulmonary oedema (1 case), aspiration pneumonia (1 case), cardiorespiratory arrest after recovery from anaesthesia (1 case), trauma (1 case), ulcerative gastritis (1 case), lymphoma of the distal ileum (1 case), and urothelial carcinoma (1 case). No histopathological changes suggestive of hepatic disease were present in any of the control population.

3.1 | Hepatocyte p21 immunopositivity

Regarding samples from dogs with CH, a median of 358 hepatocytes (65–501) were scored. The percentage of hepatocytes displaying immunopositivity for p21 was high in the majority of these cases (91 [20–98]%, Figures 1 and 2) with 24 of 26 cases (93%) demonstrating >70% p21-positive cells. Immunopositivity was similar for cases with and without copper-associated disease (91 [20–98]% versus 91 [89–94]% respectively, P = .67, Figure 3). No significant relationship was demonstrated between p21 immunopositivity and age in cases with CH (r_s = 0.025, n = 26; P = .90).

In the control population, a median of 268 hepatocytes (118–415) were scored. By comparison, a moderate positive correlation between age and hepatocyte p21 immunopositivity was identified in these dogs (r_s = 0.63, n = 15; P = .011). The percentage of hepatocytes displaying immunopositivity for p21 was significantly lower in control dogs compared with dogs with CH (P < .001) and was <15% in 12/15 (80%) cases (Figures 1 and 2). The remaining three control dogs had a higher degree of hepatocyte p21 immunopositivity, of 34, 64, and 86%, and 2 of these dogs were of older age (12.2, 7.5, and 16.7 years, respectively).

Variability between the two observers with regard to scoring of p21 immunopositivity was low. For dogs with CH, the median difference between scores was 5.64% (Interquartile Range [IQR] 2.43%-10.76%). For control cases, the median difference between scores was 1.05% (IQR 0.16%-2.89%).

3.2 | Survival analysis

Follow-up data and survival information was obtained for all dogs. The median survival time for the 26 included cases was 659 (12–2554) days. Five dogs were still alive at the time of data collection and were therefore censored from the survival analysis. Follow up time for these cases was 1684 (608–2554) days. The remaining 21 cases were all euthanased. Clinical signs relating to hepatic dysfunction (including jaundice, ascites, and neurological signs suggestive of hepatic encephalopathy) at the time of euthanasia or death were reported in the medical records of 8 dogs. In the remaining 18 cases, the reason for euthanasia or death was related to poor quality of life or was not recorded. Survival times were similar for cases with and

TABLE 2 Histological scores for included cases with CH[a]

	Number of portal spaces	Hepatocellular injury grade (1–3)	Inflammation grade (1–4)	Fibrosis stage (0–4)	Cumulative score[b] (0–11)	Copper score (0–3)
Median (range)	47.5 (1–50)	2 (1–3)	2 (1–4)	3 (0–4)	9 (4–11)	1 (0–3)

[a]The data set included 4 dogs with copper storage disease. Other than copper score (median 2; range 1–3), which was higher for cases with copper storage disease, all other scores were similar irrespective of copper status.
[b]The cumulate score is calculated based on the sum of scores for hepatocellular injury grade, inflammation grade and fibrosis stage.

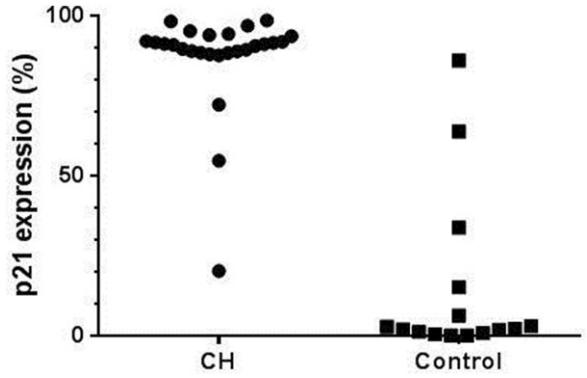

FIGURE 2 Scatter plot representing hepatocyte p21 immunopositivity in dogs with CH and healthy controls

without copper-associated disease (933 [84–1684] days versus 611 [12–2554] days, respectively; P = .17).

Cases were divided into terciles based on hepatocyte p21 immunopositivity with the upper, middle, and lower terciles comprising dogs with ≥92% (n = 8), 89%-92% (n = 9), and <89% (n = 9) p21 immunopositivity, respectively. Significantly shorter survival times were identified in dogs with p21 immunopositivity ≥92% compared with those with p21 immunopositivity <89% (P = .006). No significant difference in survival time was found between the upper and middle tercile (P = .69) or between the middle and lower tercile (P = .065, Figure 4). Median survival times for dogs in the upper, middle, and lower terciles were 218, 1055, and 874 days, respectively. Results were similar irrespective of whether cases with copper-associated disease were included or excluded. Univariate Cox regression analysis indicated that only hepatocellular p21 immunopositivity (P = .034), serum albumin concentration (P = .087) and overall histological score (P = .16) were significantly associated with survival time at the 20% level (Table 3). No significant associations were identified between survival time and serum ALT activity, serum ALP activity, serum GGT activity, serum bilirubin concentration, fasting serum bile acid concentration, postprandial serum bile acid concentration, or age (Supporting Information Table S1). Variables significant at the 20% level were included in the multivariable Cox regression model, and in the first iteration of the model, no variable was significantly associated with survival time (Table 4). However in the final iteration of the

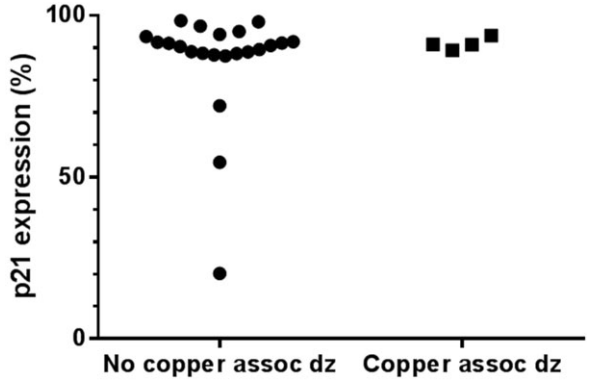

FIGURE 3 Scatterplot representing hepatocyte p21 immunopositivity in CH dogs with (left) and without (right) copper-associated disease

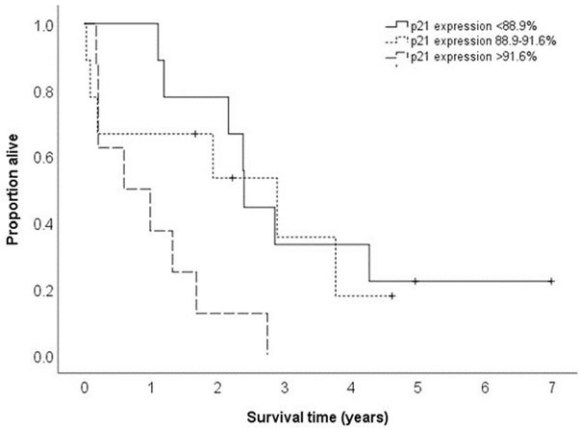

FIGURE 4 Kaplan-Meier Analysis of survival time in dogs with CH divided stratified by hepatocyte p21 immunopositivity. Dogs that were alive at the time of data collection were censored (n = 5)

automated, backward, stepwise model, only hepatocyte p21 immunopositivity remained significantly associated with survival time (P = .034). The results of the final iteration of the backwards, stepwise multivariable Cox regression analysis were equivalent of univariate analysis for p21 (Table 3) and are therefore not shown separately.

4 | DISCUSSION

The results of this study demonstrate that hepatocyte p21 is highly upregulated in dogs with CH. Compared with control dogs where p21 immunopositivity was less than 17% in 80% of dogs, 96% of dogs with CH had in excess of 70% p21 positive hepatocytes. Furthermore, the findings of this study also identified a negative association between hepatocyte p21 immunopositivity and survival of dogs with CH, suggesting a potential role for p21 in facilitating prognosis in these cases. In addition, whereas p21 immunopositivity was correlated with age in control dogs, there was no age correlation in dogs with CH. Similar findings have also been described in humans with liver disease and ascribed to accelerated cellular senescence in dogs with CH.[17,18]

TABLE 3 Results of univariate cox regression analysis showing variables significantly associated with survival time at 20% level[a]

Variable	n	Significance	HR (95% CI)
p21 (categorical model)	26	0.034[b]	
<88.9% (referent category)	9		1.00
<88.9% versus 88.9–91.8%	9	0.68	1.26 (0.42-3.78)
<88.9% versus >91.8%	8	0.013	4.12 (1.34-12.63)
Albumin (categorical model)	26	0.087[b]	
<21 g/L (referent category)	7		1.00
<21 g/L versus 21–25.2 g/L	7	0.039	0.29 (0.09-0.94)
<21 g/L versus >25.2 g/L	7	0.048	0.32 (0.10-0.99)
<21 g/L versus no data	5	0.050	0.20 (0.04-1.00)
Histological score (continuous variable)	26	0.16	1.19 (0.94-1.52)

Abbreviations: CI, confidence interval; HR, hazard ratio.
[a]Full results of the univariate Cox regression analysis are detailed in Supporting Information Table S1.
[b]Overall significance of variable when assessed categorically.

TABLE 4 Results of the initial iteration of the backwards, stepwise multivariable cox regression model comprising variables significantly associated with survival time in the univariate analysis

Variable	n	Significance	HR (95% CI)
p21 (categorical model)	26	0.41[a]	
<88.9% (referent category)	9		1.00
<88.9% versus 88.9–91.8%	9	0.75	1.21 (0.38-3.82)
<88.9% versus >91.8%	8	0.18	2.49 (0.65-9.51)
Albumin (categorical model)	26	0.46[a]	
<21 g/L (referent category)	7		1.00
<21 g/L versus 21–25.2 g/L	7	0.17	0.41 (0.12-1.45)
<21 g/L versus >25.2 g/L	7	0.47	0.59 (0.13-2.54)
<21 g/L versus no data	5	0.20	0.32 (0.06-1.84)
Histological score (continuous variable)	26	0.47	1.10 (0.85-1.42)

Abbreviations: HR, hazard ratio; CI, confidence interval.
[a]Overall significance of variable when assessed categorically.

As previously discussed, increased synthesis of p21 in response to DNA damage or telomere shortening induces a state of cellular senescence where cells exist is a state of replicative arrest, unable to undergo cell division or programed cell death. Importantly however, senescent hepatocytes are generally indistinguishable from normal cells on routine histology and their existence is therefore inevitably overlooked.[16–18] Considering the high percentage of hepatocytes displaying immunopositivity in this study, it is possible that these findings reflect a state of widespread hepatocyte senescence in the livers of dogs with CH. Although further studies are therefore required to further elucidate this relationship, the concept of cellular senescence warrants further consideration as replicative arrest affecting a large proportion of hepatocytes might significantly compromise the regenerative capacity of the liver in these cases. Furthermore, previous studies have suggested that p21 plays a key role in regulation of hepatocyte G1 phase progression and that transgenic mice with forced hepatic expression of p21 have markedly impaired regeneration after partial hepatectomy.[29,30]

In addition to the well-known effects on cell cycle regulation, accumulating evidence suggests that senescent cells also secrete an array of pro-inflammatory cytokines, chemokines and proteases, including TNF-α, IL-6, and metalloproteinases.[31–34] This senescence-associated secretory phenotype (SASP) is thought to result in various autocrine and paracrine effects which have the potential to significantly alter cellular function.[11] Furthermore, additional deleterious roles of the SASP in modulation of the extracellular matrix, causation of inflammatory diseases, and promotion of oncogenic transformation in neighboring cells have been suggested.[10,33–36] These findings consequently raise questions regarding the cause and effect relationship between cellular senescence and inflammatory diseases, such as CH. A positive correlation between the degree of hepatic fibrosis and p21 expression has been previously demonstrated in humans with alcohol-related liver disease,[17] however causality was not directly investigated and definitive conclusions in this regard can therefore unfortunately not be drawn.

Given that immunopositivity for p21 in dogs with CH exceeded 70% in the vast majority (92%) of cases and that direct cellular or genetic damage and the SASP might detrimentally affect cell function,[11] it is plausible that a state of widespread hepatocyte senescence could also have a major effect on overall liver function. Although a relatively poor correlation between histological severity and indicators of clinical status and hepatic function has been documented, markers of senescence were not evaluated.[2,9,37,38] Given the lack of readily identifiable morphological changes associated with hepatocyte senescence on routine histology, it could therefore be hypothesized that this disparity could be attributable to a high prevalence of senescent hepatocytes which, although functionally impaired, appear essentially identical to normal hepatocytes without the use of immunohistochemistry. Although further investigation of the relationship between p21 immunopositivity and liver function assays (eg, bile acid stimulation) would have been desirable, unfortunately, such results were only available for a minority of dogs due to the retrospective nature of this study. There is an association between p21 expression and serum albumin concentration and prothrombin time in humans[17]; however, further investigation is required to definitively elucidate the relationship between p21 immunopositivity, hepatocyte senescence, and liver dysfunction in dogs with CH.

Given that p21 synthesis is also induced by telomere shortening,[10,11,39] it is perhaps unsurprising that an increase in hepatocyte p21 immunopositivity occurred as an age-related phenomenon in negative control dogs in this study. Nonetheless, future studies focusing on telomere-related markers would be required to further investigate this relationship. These findings are however similar those in human medicine where an association between age and cellular senescence has been demonstrated.[10,11] Interestingly however, a subset of negative controls in this study had a considerably higher percentage of p21 positive hepatocytes than previously reported in human studies. Furthermore, hepatocyte p21 immunopositivity in dogs with CH in this study was considerably greater than that reported in previous studies investigating human liver diseases (86.7% compared with 35.6%).[17] Although these findings might be related to differences in natural lifespan and the rate of telomere shortening between species or differences in liver disease etiology, it is also possible that increased hepatocyte turnover occurs in dogs because of an increased tendency for dietary indiscretion in the canine population thereby predisposing to chronic, low-grade hepatotoxin exposure. Despite the association between age and hepatocyte p21 immunopositivity in the control population however, no such relationship was evident in dogs with CH, supporting the hypothesis that p21 expression, and potentially cellular senescence, occurs primarily because of disease-associated DNA damage in these cases. In this regard, the higher degree of p21 immunopositivity compared to humans with liver disease might reflect a tendency for dogs with CH to be presented at a later stage of clinical disease when more extensive injury to hepatocytes has already occurred. Alternatively, it is also possible that the inflammatory milieu and oxidative stress in dogs might result in more extensive DNA damage, or that the DNA Damage Response pathway is triggered after a lower degree of DNA damage has been sustained.

As also identified in recent studies of human liver disease,[17,18] the results of this study also suggest an association between hepatocyte p21 immunopositivity and survival. Hypoalbuminaemia,[6]

hypoglycaemia,[7] hyperbilirubinaemia,[5] and the presence of ascites and cirrhosis[2,8] have been previously suggested as negative prognostic indicators in dogs, however none are reliable and typically are only useful in the late stages of disease. In this study, dogs with hepatocyte p21 immunopositivity exceeding 91.6% had significantly shorter survival times than those with immunopositivity of <88.9%. In addition, although albumin concentration and histological score were associated with survival in the univariate Cox regression analysis, hepatocyte p21 immunopositivity was the only variable to be remain significantly associated with survival in the multivariate model. Furthermore, the association between p21 immunopositivity and survival time was also independent of the severity of CH based on overall histological score. These results therefore suggest that hepatocyte p21 quantification might be of potential use in determining the prognosis in dogs with CH; however, it should be considered that the differences in p21 immunopositivity between groups in the survival analysis are small and achieving this degree of accuracy might be practically difficult to achieve, particularly if the percentage of p21-positive hepatocytes is manually quantified. Nonetheless, considering the small population of dogs in this study, the strong relationship ($P = .006$) between high hepatocyte p21 immunopositivity and poor outcome was considered to be a significant finding and warrants further investigation. Interestingly, no significant relationship was identified between p21 immunopositivity and survival time in the univariate analysis when p21 was treated as a continuous variable. This could therefore imply that p21, perhaps because of the large reserve capacity of the liver, is not linearly associated with survival time but instead only affects prognosis once p21 immunopositivity exceeds a threshold value. In this regard, p21 expression might behave similarly to other biochemical markers, such as serum creatinine or bile acid concentrations. Although these results therefore suggest a potential prognostic role of p21 quantification CH, further investigations with larger populations of dogs are however required to substantiate these findings.

Several limitations of this study warrant consideration. Firstly, because of the retrospective nature of data collection, clinical and biochemical data were not available for all dogs. In particular, markers of hepatic dysfunction (such as serum bile acid and bilirubin concentrations) were not measured in all dogs and accurate assessment of the relationship between p21 immunopositivity and hepatic function was consequently not possible. Secondly, although p21 is a key mediator of cellular senescence and its consequences on cell function have been well described,[10,11,13,17] the actions of p21 are complex and diverse and expression of additional senescence and cell cycle markers (eg, P16, Mcm-2, cyclin A, and PH3) and direct indicators of cell function and replicative capacity (eg, Ki67) were not investigated in this study. Consequently, p21 immunopositivity cannot be viewed as synonymous with cellular senescence and further studies involving direct markers of cellular proliferation, DNA damage and telomere status are therefore required to clarify this relationship.

A further limitation is the potential for subjectivity in the recording of positive and negative cells, particularly as the intensity of nuclear staining is often variable. Despite this however, agreement between observers was generally good with minimal difference in calculated p21 immunopositivity for the majority of cases. Finally, because of the difficulty in defining liver-related cause of death from retrospective data, all-cause mortality was used as an end-point for the study. Although dogs with obvious alternative causes for euthanasia were excluded, clinical data pertaining to these situations was poorly recorded in many cases and it is therefore possible that some dogs might have been euthanased for reasons other than progression of CH, thereby resulting in falsely decreased survival times. Furthermore, motivations for euthanasia are notoriously variable between owners. Calculation of "time to disease progression" rather than "survival time" is increasingly used in studies in order to overcome these limitations but is difficult to apply to CH where progression is gradual and euthanasia is frequently considered before the development of easily measureable indicators of end-stage disease (such as ascites). Although, longitudinal analysis of hepatic histology would be ideal as a measure of liver disease progression, unfortunately repeat biopsy is frequently declined by clients and was therefore not available for the current study population.

In conclusion, this study demonstrates that the percentage of hepatocytes displaying immunopositivity for p21 is markedly increased in dogs with CH, suggesting that many hepatocytes might exist in a senescent state. Furthermore, quantification of hepatocyte p21 expression might have a role in determining the prognosis in dogs with CH. Further studies are however required to clarify whether p21 immunopositivity in canine hepatocytes is correlated with senescence and to determine the consequences of increased p21 expression on liver function and replicative potential.

ACKNOWLEDGMENTS

The Study was performed at the Queen's Veterinary School Hospital, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK. Financial support was provided by the Petplan Charitable Trust (Grant no 217–263). This data was presented as an oral research abstract at the ECVIM-CA congress in 2017.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

ORCID

Andre J. Kortum http://orcid.org/0000-0002-9760-733X

REFERENCES

1. Rothuizen J. *WSAVA standards for clinical and histological diagnosis of canine and feline liver disease*. Elsevier Health Sciences; 2006.

2. Poldervaart JH, Favier RP, Penning LC, et al. Primary hepatitis in dogs: a retrospective review (2002–2006). *J Vet Intern Med*. 2009;23:72–80.
3. Watson PJ. Chronic hepatitis in dogs: A review of current understanding of the etiology, progression, and treatment. *Vet J*. 2004;167:228–241.
4. Bexfield NH, Andres-Abdo C, Scase TJ, et al. Chronic hepatitis in the English springer spaniel: clinical presentation, histological description and outcome. *Vet Rec*. 2011;169:415
5. Gómez Selgas A, Bexfield N, Scase TJ, Constantino-Casas F, Watson PJ. Total serum bilirubin as a negative prognostic factor in idiopathic canine chronic hepatitis. *J Vet Diagn Invest*. 2014;26:246–251.
6. Raffan E, McCallum A, Scase TJ, Watson PJ. Ascites is a negative prognostic indicator in chronic hepatitis in dogs. *J Vet Intern Med*. 2009;23:63–66.
7. Strombeck DR, Miller LM, Harrold D. Effects of corticosteroid treatment on survival time in dogs with chronic hepatitis: 151 cases (1977–1985). *J Am Vet Med Assoc*. 1988;193:1109–1113.
8. Sevelius E. Diagnosis and prognosis of chronic hepatitis and cirrhosis in dogs. *J Small Anim Pract*. 1995;36:521–528.
9. Shih JL, Keating JH, Freeman LM, Webster CR. Chronic hepatitis in Labrador Retrievers: Clinical presentation and prognostic factors. *J Vet Intern Med*. 2007;21:33–39.
10. Muñoz-Espín D, Serrano M. Cellular senescence: From physiology to pathology. *Nat Rev Mol Cell Biol*. 2014;15:482–496.
11. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. *Cell*. 2007;130:223–233.
12. Cazzalini O, Scovassi AI, Savio M, et al. Multiple roles of the cell cycle inhibitor p21 CDKN1A in the DNA damage response. *Mutat Res*. 2010;704:12–20.
13. d'Adda di Fagagna F. Living on a break: Cellular senescence as a DNA-damage response. *Nat Rev Cancer*. 2008;8:512–522.
14. Dutto I, Tillhon M, Prosperi E. (2016) Assessing Cell Cycle Independent Function of the CDK Inhibitor p21CDKN1A in DNA Repair. In: Orzáez M, Sancho Medina M, Pérez-Payá E, eds. *Cyclin-Dependent Kinase (CDK) Inhibitors. Methods in Molecular Biology*. Vol 1336. New York: Humana Press, NY; 2016:123–139.
15. Campisi J, di Fagagna FD. Cellular senescence: When bad things happen to good cells. *Nat Rev Mol Cell Biol*. 2007;8:729–740.
16. Aravinthan A, Verma S, Coleman N, et al. Vacuolation in hepatocyte nuclei is a marker of senescence. *J Clin Pathol*. 2012;65:557–560.
17. Aravinthan A, Pietrosi G, Hoare M, et al. Hepatocyte expression of the senescence marker p21 is linked to fibrosis and an adverse liver-related outcome in alcohol-related liver disease. *PloS One*. 2013;8:e72904.
18. Aravinthan A, Scarpini C, Tachtatzis P, et al. Hepatocyte senescence predicts progression in non-alcohol-related fatty liver disease. *J Hepatol*. 2013;58:549–556.
19. Inoue M, Wada N. Immunohistochemical detection of p53 and p21 proteins in canine testicular tumours. *Vet Rec*. 2000;146:370–372.
20. Inoue M, Wu H, Une S. Immunohistochemical detection of p27 and p21 proteins in canine hair follicle and epidermal neoplasms. *J Vet Med Sci*. 2006;68:779–782.
21. Klopfleisch R, Gruber AD. Differential expression of cell cycle regulators p21, p27 and p53 in metastasizing canine mammary adenocarcinomas versus normal mammary glands. *Res Vet Sci*. 2009;87:91–96.
22. Wu H, Hayashi T, Inoue M. Immunohistochemical expression of p27 and p21 in canine cutaneous mast cell tumors and histiocytomas. *Vet Pathol*. 2004;41:296–299.
23. Kim HW, Ju JH, Shin JI, et al. Differential and correlated expressions of p16/p21/p27/p38 in mammary gland tumors of aged dogs. *J Vet Sci*. 2017;18:479–485.
24. Carrasco V, Canfrán S, Rodríguez-Franco F, et al. Canine gastric carcinoma: immunohistochemical expression of cell cycle proteins (p53, p21, and p16) and heat shock proteins (Hsp27 and Hsp70). *Vet Pathol*. 2011;48:322–329.
25. Chandler HL, Newkirk KM, Kusewitt DF, et al. Immunohistochemical analysis of ocular hemangiomas and hemangiosarcomas in dogs. *Vet Ophthalmol*. 2009;12:83–90.
26. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. *Hepatology* 1996;24:289–293.
27. Goodman ZD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *J Hepatol*. 2007;47:598–607.
28. Peyron C, Chevallier M, Lecoindre P, et al. Clinical, blood biochemical and hepatic histological data in 49 French Scottish Terriers dogs according to their plasma ALP activity, hepatic vacuolation and the presence or absence of hepatocellular carcinoma. *Revue Méd Vét*. 2014;165:245–251.
29. Wu H, Wade M, Krall L, et al. Targeted in vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development and regeneration. *Genes Dev*. 1996;10:245–260.
30. Albrecht JH, Poon RY, Ahonen CL, et al. Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver. *Oncogene*. 1998;16:2141.
31. Tchkonia T, Zhu Y, Van Deursen J, et al. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. *J Clin Invest*. 2013;123:966–972.
32. Martínez-Zamudio RI, Robinson L, Roux PF, Bischof O. SnapShot: Cellular Senescence in Pathophysiology. *Cell*. 2017;170:1044.
33. Coppé JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol Mech Dis*. 2010;5:99–118.
34. Watanabe S, Kawamoto S, Ohtani N, Hara E. Impact of senescence-associated secretory phenotype and its potential as a therapeutic target for senescence-associated diseases. *Cancer Sci*. 2017;108:563–569.
35. Xu M, Bradley EW, Weivoda MM, et al. Transplanted senescent cells induce an osteoarthritis-like condition in mice. *J Gerontol A Biol Sci Med Sci*. 2016;72:780–785.
36. McCulloch K, Litherland GJ, Rai TS. Cellular senescence in osteoarthritis pathology. *Aging Cell*. 2017;16:210–218.
37. Dirksen K, Burgener IA, Rothuizen J, et al. Sensitivity and Specificity of Plasma ALT, ALP, and Bile Acids for Hepatitis in Labrador Retrievers. *J Vet Intern Med*. 2017;31:1017–1027.
38. Oliver J, Roberts SH, Douglas AP, et al. Liver damage after paracetamol overdose. Comparison of liver-function tests, fasting serum bile acids, and liver histology. *Lancet*. 1975;306:579–581.
39. Herbig U, Jobling WA, Chen BP, et al. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21 CIP1, but not p16 INK4a. *Mol Cell*. 2004;14:501–513.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Kortum AJ, Cloup EA, Williams TL, Constantino-Casas F, Watson PJ. Hepatocyte expression and prognostic importance of senescence marker p21 in liver histopathology samples from dogs with chronic hepatitis. *J Vet Intern Med*. 2018;00:1–8. https://doi.org/10.1111/jvim.15238