Markers of hepatic regeneration associated with surgical attenuation of congenital portosystemic shunts in dogs

Michael S. Tivers a,b,*, Victoria J. Lipscomb a, Kenneth C. Smith c, Caroline P.D. Wheeler-Jones d, Arthur K. House e

a Department of Veterinary Clinical Sciences, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, AL9 7TA, United Kingdom
b Cave Veterinary Specialists, George’s Farm, West Buckland, Nr. Wellington, TA21 9LE, United Kingdom
c Department of Pathology and Infectious Diseases, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, AL9 7TA, United Kingdom
d Department of Comparative Biomedical Sciences, Royal Veterinary College, Royal College Street, London, NW1 0TU, United Kingdom
e Veterinary Referral Hospital, 18/151-159 Princess Hwy, Hallam, Vic, Australia

ARTICLE INFO

Article history:
Accepted 14 February 2014

Keywords:
Liver
Dog
Portosystemic shunt
Regeneration
Biomarker

ABSTRACT

Dogs with congenital portosystemic shunts (CPSS) have liver hypoplasia and hepatic insufficiency. Surgical CPSS attenuation results in liver growth associated with clinical improvement. The mechanism of this hepatic response is unknown, although liver regeneration is suspected. This study investigated whether markers of liver regeneration were associated with CPSS attenuation. Dogs treated with CPSS attenuation were prospectively recruited. Residual liver tissue was collected for gene expression analysis (seven genes) from 24 CPSS dogs that tolerated complete attenuation, 25 dogs that tolerated partial attenuation and seven control dogs. Relative gene expression was measured using quantitative polymerase chain reaction (qPCR). Blood samples were collected before, 24 h and 48 h post-surgery from 36 CPSS dogs and from 10 control dogs. Serum hepatocyte growth factor (HGF) concentration was measured using a canine specific enzyme-linked immunosorbent assay (ELISA). HGF mRNA expression was significantly decreased in CPSS compared with control dogs (P = 0.046). There were significant increases in HGF (P = 0.050) and methionine adenosyltransferase 2A (MAT2A; P = 0.002) mRNA expression following partial CPSS attenuation. Dogs with complete attenuation had significantly greater MAT2A (P = 0.024) mRNA expression compared with dogs with partial attenuation. Serum HGF concentration significantly increased 24 h following CPSS attenuation (P < 0.001). Hepatic mRNA expression of two markers of hepatocyte proliferation (HGF and MAT2A) was associated with the response to surgery in dogs with CPSS, and serum HGF significantly increased following surgery, suggesting hepatocyte proliferation. These findings support the concept that hepatic regeneration is important in the hepatic response to CPSS surgery.

© 2014 Elsevier Ltd. All rights reserved.

Introduction

Dogs with congenital portosystemic shunts (CPSS) have liver hypoplasia associated with hepatic insufficiency. Successful CPSS attenuation results in resolution of clinical signs and improvement in hepatic function as assessed with dynamic bile acids or ammonia tolerance testing (Hunt and Hughes, 1999; Hunt et al., 2004). In the short term following CPSS attenuation, liver volume as measured by computed tomography (CT) or magnetic resonance imaging (MRI) increases (Stieger et al., 2007; Kummeling et al., 2010). These findings suggest that this rapid return of the liver to a normal size is achieved by hepatic regeneration, although evidence for this is circumstantial.

Liver regeneration is complex and involves a large number of factors, although hepatocyte growth factor (HGF) plays a key role. In experimental studies, HGF expression in liver and its serum concentration increase following partial hepatectomy (PH) in association with liver regeneration (Lindroos et al., 1991; Zarnegar et al., 1991). Serum HGF also increases following PH in humans, and it is suggested that this is associated with regeneration (Efimova et al., 2005).

No published studies have specifically investigated the mechanisms governing the hepatic response to CPSS attenuation in dogs. One study demonstrated that the main components of the HGF signalling pathway were reduced but intact in dogs with CPSS (Spee et al., 2005). If it can be demonstrated that liver regeneration occurs following CPSS attenuation, this could have important
implications for the development of therapy for this condition in dogs as well as in other species.

Table 1

Table showing details of reference gene and gene of interest primer pairs for quantitative polymerase chain reaction (qPCR).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>PCR amplicon length (bp)</th>
<th>GenBank accession number</th>
<th>Primer sequence reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMBS</td>
<td>Forward: TCACCATCGGAAGCCACTCT</td>
<td>112</td>
<td>XMS45491</td>
<td>Peters et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCTCCACACGCTCCTCTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL13A</td>
<td>Forward: GGGGGAGTGTTATGCTCTCT</td>
<td>87</td>
<td>AJ388525</td>
<td>Peters et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGGAGGCGGCAATGAAATATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL32</td>
<td>Forward: TGCTTACGCAGGACCACACAGAAGAA</td>
<td>100</td>
<td>XM_848016</td>
<td>Peters et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCACATACAGCCAGGATCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS18</td>
<td>Forward: TGTCATGGTATGATGAGGAA</td>
<td>116</td>
<td>XM_532106</td>
<td>Peters et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCTTATACGCGCGATGATCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGF</td>
<td>Forward: AAAGGAGATGAGAAACGCAAACAG</td>
<td>92</td>
<td>NM_001002964</td>
<td>Kummeling et al.2012</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGGCTTACGGCGGATGACAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGFac</td>
<td>Forward: ACAAGACCTTGGTGGCCTGACAGTG</td>
<td>128</td>
<td>AY581482</td>
<td>Kummeling et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Reverse: AAACCTGAGGGCCGATGCAGCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cMET</td>
<td>Forward: CCAGGCCTGGCTGGTGCTCTC</td>
<td>112</td>
<td>NM_001002963</td>
<td>Kummeling et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGCCTGGTGCTCTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAT2A</td>
<td>Forward: TTAAAACTGGCCATCCGTGTA</td>
<td>121</td>
<td>XM_532980</td>
<td>Kummeling et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTTTTTGGGCGGGGGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFα</td>
<td>Forward: CCGCCCTGCTGGTGGTCTCTC</td>
<td>136</td>
<td>AY58143</td>
<td>Spee et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGGGCCTGGCTCTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>Forward: CAGAAGCTCCTGGCGGAGCTGGA</td>
<td>113</td>
<td>L34965</td>
<td>Spee et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGGAGACCTTTGCCTGTGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβR2</td>
<td>Forward: GACCTGGCTGCTGGAGCTGCTT</td>
<td>116</td>
<td>XM_534237</td>
<td>Kummeling et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCACCTGCGGAGGAGCTGCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Quantitative polymerase chain reaction (qPCR) was used to measure the relative hepatic expression of seven genes related to hepatic regeneration, including HGF, HGF activator (HGFac), HGF receptor (cMET), methionine adenosyltransferase 2 A (MAT2A), transforming growth factor alpha (TGFα), TGF beta 1 (TGFβ1) and TGFβ receptor 2 (TGFβR2). Previously published canine gene specific primers for the genes of interest (Spee et al., 2005; Kummeling et al., 2012) and four liver specific reference genes (Peters et al., 2007) hydroxymethyl-bilane synthase (HMBS), ribosomal protein L13a (RPL13A), ribosomal protein L32 (RPL32) and ribosomal protein S18 (RPS18) were used (Table 1).

For quantification each liver sample had two cDNA samples analysed in duplicate. Reactions were carried out in 25 μL using a Bio-Rad CFX96 Real-Time PCR Detection System thermocycler (Bio-Rad Laboratories). Each reaction consisted of 1 μL cDNA as the template with Immobuffer (1× concentration), Hi-Spec Additive (1× concentration), dNTP (final concentration 1 mM), magnesium chloride (final concentration 2.5 mM for genes of interest, 4.5 mM for reference genes), 1 unit Immolase DNA polymerase (all Bioline) and EvaGreen dye (Biotium) (0.06 μM diluted 1:4 with nuclease-free water). Samples were incubated at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 10 s. An appropriate primer-dimer melting temperature for 1 s was programmed before fluorescence readings were taken at the end of each cycle. A melting curve analysis from 65 °C to 95 °C with a plate read every 0.5 °C was performed at the end of 40 cycles. Bio-Rad CFX Manager Software (Bio-Rad Laboratories) was used for the initial qPCR analysis.

Analysis of raw data was performed using GenEx professional version 4.4.2 software (Multlid Analyse). Relative gene expression was quantified as previously described (Vandesompele et al., 2002). Quantification cycle (Cq) values were corrected using the calculated efficiencies for each primer set. Normalisation of each sample Cq for the genes of interest was performed relative to the geometric normalisation of the four reference genes. The relative expression of the mRNA of each genes of interest in each cDNA sample was then calculated using the normalised Cq of each sample relative to the average Cq of all of the samples.

Serum HGF concentration

Blood samples were taken from CPSS dogs and exploratory laparotomy controls preoperatively for diagnostic purposes and after surgery for post-operative monitoring and, where available, residual blood was collected for the study. Residual blood samples were also taken immediately before euthanasia in Beagle control dogs. The serum was separated and stored at −80°C. A Canine ELISA Kit (Biorbyt) was used to measure the serum concentration of HGF. Samples were analysed in duplicate using an ELx808 absorbance microplate reader (BioTek Instruments). Sample concentration was calculated from the standard curve using Gen5 V1.07.5 software (BioTek Instruments).

Statistical analysis

Analysis was performed using PASW Statistics 18.0.0 statistical software package (Education SPSS). Continuous data were visually assessed for normality. Median and range were reported for skewed data, which was compared with the Mann–Whitney U test. Repeated measures were compared with the Friedman's
two-way analysis of variance by ranks with pair wise comparison. The qPCR data were transformed to normal distribution (square root, log or inverse log). The data were then compared with an independent \( t \) test or paired sample \( t \) test. For all tests, significance was set at the 5% level (\( P \leq 0.05 \)).

Results

Gene expression

Liver samples from 49 dogs with CPSS were included. The median age was 275 days (range, 97–4374). Thirty-eight (77.6%) dogs had an extrahepatic CPSS and 11 (22.4%) had an intrahepatic CPSS. Twenty-four dogs (49%) had complete attenuation and 25 dogs (51%) had partial attenuation. Partial attenuation dogs had repeat surgery a median of 110 days (range, 69–358) post-operatively. Liver samples from seven Beagle control dogs were included. The median age was 628 days (range, 515–1544), which was statistically significantly greater than CPSS dogs (\( P = 0.036 \)). The livers of all control dogs were histopathologically unremarkable.

For each gene the following comparisons were made: CPSS vs. control; partial attenuation vs. complete attenuation; before and after partial attenuation (paired samples). Statistically significant results are shown in Fig. 1.

Relative HGF mRNA expression was statistically significantly decreased in CPSS dogs compared with control dogs (\( P = 0.046 \)). Relative HGF mRNA expression statistically significantly increased following partial attenuation (\( P = 0.050 \)). Relative MAT2A mRNA expression was statistically significantly greater in complete attenuation dogs compared with partial attenuation dogs (\( P = 0.024 \)). Relative MAT2A mRNA expression statistically significantly increased following partial attenuation (\( P = 0.002 \)). Relative TGF\( \alpha \) mRNA expression was statistically significantly greater in CPSS dogs compared with control dogs (\( P = 0.005 \)). Relative TGF\( \beta \)R2 mRNA expression was statistically significantly greater in complete attenuation dogs compared with partial attenuation dogs (\( P = 0.016 \)). There were no statistically significant associations evident for the relative expression of HGFac, cMET or TGF\( \beta \) mRNA.

Serum HGF concentration

Serum samples taken before surgery and 24 h and 48 h post-surgery from 36 dogs with CPSS were included. The median age was 239 days (range, 104–4374). Twenty-eight (77.6%) dogs had extrahepatic CPSS and eight (22.2%) had intrahepatic CPSS.

Serum samples from five healthy Beagles and five dogs undergoing abdominal surgery were included as preoperative control samples. The dogs were undergoing abdominal surgery for the investigation or treatment of insulinoma, adrenal carcinoma, splenic carcinoma, phaeochromocytoma and inflammatory bowel disease. In addition to the preoperative sample, the five dogs undergoing abdominal surgery also had a paired 24 h post-surgery sample taken. The median age of control dogs was 1763.5 days (range, 526–4204), which was statistically significantly greater than CPSS dogs (\( P < 0.001 \)). There was no statistically significant difference in preoperative HGF concentrations between CPSS dogs and controls. In CPSS dogs there was a statistically significant difference in the concentration of HGF at the different time points (\( P < 0.001 \)) (Fig. 2). Pair wise comparison of this data set confirmed that HGF at 24 h was statistically significantly greater than pre-surgery (\( P < 0.001 \)). For the five control dogs with pre- and post-operative samples, there was no statistically significant difference in the concentration of HGF at the different time points.

Discussion

Liver regeneration is a complex process involving a wide variety of factors and multiple, interconnected pathways (Fausto et al., 2006; Michalopoulos, 2007). A previous study assessed the mRNA expression of factors involved in the HGF signalling pathway and found that they were intact but down-regulated in CPSS dogs compared with normal dogs (Spee et al., 2005). Thus, impaired liver development in CPSS dogs is associated with down-regulation of growth factors and receptors associated with hepatic regeneration. In the current study it was hypothesised that an up-regulation of these factors would occur following partial CPSS attenuation.

Dogs that tolerate complete attenuation have better hepatic function and intrahepatic portal vasculature than those that only tolerate partial attenuation (Lee et al., 2006). Therefore, it was anticipated that dogs tolerating complete attenuation would show up-regulated hepatic expression of genes associated with regeneration compared with dogs that could only tolerate partial attenuation.

The mRNA expression of five genes associated with hepatocyte proliferation (HGF, HGFac, cMET, MAT2A and TGF\( \alpha \)) and two implicated in the termination of regeneration (TGF\( \beta \) and TGF\( \beta \)R2) were assessed (Michalopoulos, 2007; Lu and Mato, 2008). HGF mRNA expression was significantly decreased in CPSS dogs compared with controls and significantly increased following partial CPSS attenuation. MAT2A mRNA expression was also significantly greater in complete attenuation dogs compared with partial attenuation dogs and significantly increased following partial CPSS attenuation. HGF is a key hepatocyte mitogen that is vital for normal hepatic regeneration (Matsumoto and Nakamura, 1992; Schmidt et al., 1995; Uehara et al., 1995). Increased MAT2A gene expression has been shown to be associated with hepatocyte proliferation during liver regeneration and in vitro (Huang et al., 1998; Garcia-Trevijano et al., 2000; Latasa et al., 2001; Rodriguez et al., 2007). Therefore, both of these factors play an important role in hepatocyte replication and, hence, liver regeneration. The significant association between these factors and liver development in dogs with CPSS suggests that hepatocyte replication plays an important role in the hepatic response to surgery.

A further finding was that TGF\( \beta \)R2 mRNA expression was significantly increased in complete attenuation dogs compared with partial attenuation dogs. TGF\( \beta \)1 is an inhibitor of hepatocyte proliferation and is implicated as being involved in the termination of liver regeneration (Carr et al., 1986; Braun et al., 1988; Houck and Michalopoulos, 1989). The increased expression of TGF\( \beta \)R2 mRNA in complete attenuation dogs would suggest increased binding capacity for TGF\( \beta \)1, consistent with these dogs having greater liver development and hence increased inhibition.

Previous studies have demonstrated altered mRNA expression of HGF, HGFac, cMET, TGF\( \alpha \) and TGF\( \beta \)R2 in CPSS dogs and that the mRNA expression of HGFac and MAT2A are associated with a good response to surgery (Spee et al., 2005; Rummeling et al., 2012). These studies support our findings with HGF and MAT2A being important factors in dogs with CPSS. However, the current study did not find any significant associations for HGFac and cMET mRNA expression, which contrasts with the previous study that found that HGFac mRNA expression was significantly increased and cMET mRNA expression was significantly decreased in CPSS dogs compared with controls (Spee et al., 2005). In addition, we also found that TGF\( \alpha \) mRNA was significantly increased in CPSS dogs. TGF\( \alpha \) is a hepatocyte mitogen and increased expression in CPSS dogs seems counter-intuitive (Mead and Fausto, 1989; Evans et al., 1992). Indeed, this finding is in contrast to the previous study where it was significantly decreased in CPSS dogs (Spee et al., 2005). The reason for these discrepancies is unclear, but
Hepatocyte growth factor (HGF) expression in control versus CPSS dogs

Methionine adenosyltransferase 2 A (MAT2A) expression in CPSS dogs tolerating partial attenuation versus complete attenuation

Transforming growth factor alpha (TGFα) expression in control versus CPSS dogs

Transferring growth factor beta receptor 2 (TGFβR2) expression in CPSS dog tolerating partial attenuation versus complete attenuation

Fig. 1. Relative growth factor and growth factor receptor mRNA expression in liver biopsies from dogs with congenital portosystemic shunts (CPSS) and control dogs. The graphs only show statistically significant findings for the seven genes assessed. Statistical significance is highlighted with the appropriate P value.
Due to the nature of this study there were limitations on tissue available for analysis. In experimental studies, changes in growth factors are assessed in the first 3 days post-surgery and changes are normally maximal during this time. In the current study repeat samples were taken between 69 and 358 days after initial surgery. Thus, any acute changes in gene expression may have normalised by this time. It would be interesting to compare hepatic biopsies at first surgery with samples taken in the immediate post-operative period. In addition, it is important to note that whilst mRNA expression was measured, this does not necessarily indicate altered protein expression.

Although hepatic tissue immediately post-surgery was not available, this study was able to measure serum HGF concentration immediately before and after CPSS attenuation, thus allowing the assessment of more acute changes. Serum HGF concentration was significantly increased 24 h following CPSS attenuation compared with pre-surgery. In experimental studies of hepatic regeneration following PH or carbon tetrachloride injury, there are immediate increases in HGF concentration in peripheral blood (Kinoshita et al., 1991; Lindroos et al., 1991; Pediaditakis et al., 2001). Administration of an anti-HGF antibody in rats following liver injury inhibited hepatocyte proliferation, suggesting a strong link between HGF and hepatocyte proliferation (Burr et al., 1998).

There is a significant post-operative increase in serum HGF, peaking at approximately 24 h following PH or liver transplantation for treatment of hepatic neoplasia, liver cirrhosis and fulminant liver failure (Matsunami et al., 1992; Nishizaki et al., 1995; Kimura et al., 1996; Higaki et al., 1999; Hu et al., 1999; Chijiiwa et al., 2002). Decreased HGF following hepatectomy is associated with poor liver regeneration, suggesting an increase in HGF is indicative of regeneration (Nishizaki et al., 1995). The precise reason for this increase in HGF is unclear. Although HGF is produced in the non-parenchymal cells of the liver, it is also present in other organs including lung, pancreas, kidney and spleen (Zarnegar et al., 1990; Matsumoto and Nakamura, 1992; Schirmacher et al., 1992; Maher, 1993). The early rise in HGF after PH seems to originate from extrahepatic sources as it precedes the HGF mRNA increases in the liver (Kinoshita et al., 1991). The liver is responsible for the clearance of HGF from the blood (Liu et al., 1992, 1993). It is possible that HGF increases due to decreased clearance as a result of the reduction in hepatic volume and hepatic dysfunction caused by pre-existing liver damage rather than as a result of hepatic regeneration (Tomiya et al., 1992; Tani et al., 1994; Higaki et al., 1999). HGF can also increase following laparotomy for non-hepatic surgery as a result of surgical trauma and tissue inflammation (Tomiya et al., 1992; Kimura et al., 1999).

The increase in HGF in humans undergoing hepatectomy is greater than for other abdominal and thoracic surgeries, suggesting that HGF is produced by the liver as a result of regeneration over and above that induced by inflammation (Kimura et al., 1999). It is difficult to directly translate these results to CPSS dogs as humans had increased serum HGF before surgery and had hepatic tissue removed. CPSS dogs should not suffer any hepatic injury as a result of the surgery. In addition, rather than having decreased hepatic mass with constant portal blood flow, they have an increased portal blood flow with a constant hepatic mass (or possibly an increase in functional hepatic mass with the increased hepatic perfusion). Thus, the increase in HGF in the CPSS dogs would not be due to decreased hepatic clearance, and it can be concluded that this is due to increased production of HGF.

A control group of breed- and age-matched dogs undergoing non-hepatic abdominal surgery would provide further information on the specificity of increases in HGF. Unfortunately such a control group was not available. A small number of dogs undergoing abdominal surgery with pre- and post-operative samples did not show a significant increase following surgery, although this could be a type II statistical error. This would suggest that the increase in HGF in CPSS dogs is not a non-specific response to surgery. In healthy humans undergoing PH for liver donation, there is a significant post-operative increase in serum HGF peaking at 24 h (Matsunami et al., 1992; Efimova et al., 2005). Conversely, another similar study did not detect post-operative changes in HGF concentration (Eguchi et al., 2003). Another report described post-operative increases in serum HGF in three brothers with CPSS who underwent attenuation (Ikeda et al., 1999). Although the evidence is not consistent, these findings would support the fact that increases in HGF following CPSS attenuation are indicative of hepatocyte replication rather than a non-specific response.

In the current study residual blood was collected when samples were taken for routine diagnostic and monitoring purposes. This meant that a limited amount of blood was available and that it could only be collected at certain time points. Blood samples taken earlier than 24 h post-operatively may have demonstrated even greater increases in HGF consistent with the hypothesis. We did not investigate the effect of medical management on serum HGF concentrations or relative mRNA expression. Whilst all dogs would have been on some form of medical management prior to sampling, this is aimed at treating the clinical signs of hepatic encephalopathy due to hepatic insufficiency and would not be expected to affect serum HGF concentrations or relative mRNA expression.

The findings of the current study have important implications for our understanding of CPSS in dogs and for the development of novel treatment strategies for dogs with CPSS. A recent study demonstrated that the administration of recombinant HGF to dogs with CPSS resulted in an increase in liver volume (Kruitwagen et al., 2011). Our findings also indicate that surgical CPSS attenuation could represent a preclinical research model of hepatic regeneration.
Conclusions

Hepatic mRNA expression of two markers of hepatocyte proliferation (HGF and MAT2A) was significantly associated with the degree of liver development and increased in response to partial CPSS attenuation. Serum HGF was also significantly increased 24 h post-surgery, suggesting active hepatocyte replication. This suggests that factors associated with liver regeneration are important in the hepatic response to surgery, and that liver regeneration occurs following CPSS attenuation and is responsible for the improvement in liver function.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors are very grateful to the Kennel Club Charitable Trust for funding this study. The authors would like to acknowledge Professor Dirk Weening and Dr. Bettina Schmidt for their help and advice. The authors would also like to acknowledge the veterinary surgeons, veterinary nurses and undergraduate students who were responsible for the care of the animals whilst treated at the Royal Veterinary College. We are very grateful to Dr. Fiona McClure and colleagues at GlaxoSmithKline Research and Development, Ware, UK for their advice.

References


