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Short Communication

Increased bone morphogenetic protein 7 signalling in the kidneys of dogs affected with a congenital portosystemic shunt

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ABSTRACT

Dogs with a congenital portosystemic shunt (CPSS) often have enlarged and hyper-filtrating kidneys. Although expression of different growth factors has been well-described in the livers of dogs affected with a CPSS, their expression in the kidneys has yet to be determined. Bone morphogenetic protein 7 (BMP-7), hepatocyte growth factor (HGF) and transforming growth factor (TGF)-β have been implicated in renal development (BMP-7, HGF) or the onset of renal fibrosis (TGF-β). Moreover, BMP-7 and HGF have protective properties in renal fibrosis.

In this study, the expression and activity of BMP-7 were investigated in renal biopsies obtained from 13 dogs affected with a CPSS and compared to similar samples from age-matched healthy control dogs. Both quantitative reverse-transcriptase PCR and Western blotting showed up-regulated BMP-7 signalling in kidneys of CPPS-affected dogs. These research findings may help to explain the renal pathology/dysfunction in dogs affected with a CPSS.

Congenital vascular anomalies, resulting in development of a congenital portosystemic shunt (CPSS), can be detected in predisposed dog breeds (Hunt, 2004) and have been shown to be inherited in Cairn terriers and Irish wolfhounds (van Steenbeek et al., 2012). The clinical signs that usually develop at a young age are mainly due to liver dysfunction, including retarded growth, hepatic encephalopathy, and ammonium urate urolithiasis (Center and Magne, 1990). Interestingly, renal abnormalities also occur, such as enlargement of the kidneys and functional disorders such as polyuria and polydipsia, associated with glomerular hyper-filtration (Tisdall et al., 1996; Deppe et al., 1999).

There is little information available regarding the potential factors involved in development of the large, hyper-functional kidneys found in dogs affected with a CPSS. Bone morphogenetic protein 7 (BMP-7) and hepatocyte growth factor (HGF) are two growth factors involved in renal development and prevention of fibrosis (Meng et al., 2013). The aim of the present study was to investigate the renal signalling cascades regulated by BMP-7 and HGF, in hyper-functional kidneys of CPSS-affected dogs to identify whether these contrast with those in kidneys from healthy dogs.

Thirteen dogs affected with extra-hepatic CPSS and four dogs affected with intrahepatic CPSS, referred to the Faculty of Veterinary Medicine, Utrecht University, were recruited into the study (Table 1). The study was approved by the local ethical committee as required under Dutch legislation and informed owner consent was obtained in all cases.

Renal tissue samples (percutaneous, ultrasound guided, 16 G Tru-Cut needle) were obtained under anaesthesia, snap frozen in liquid nitrogen and stored at –70 °C until analysis. Control kidney tissue was obtained at post-mortem examination of clinically healthy dogs ($n = 13$), that had been euthanased as part of unrelated, ethically approved research projects (approval numbers: 2007.III.02.029, date of approval February 2007; 2007.III.06.080, date of approval June 2007; 2009.III.06.050, date of approval June 2009) and made available for other research projects in accordance with Utrecht University 3Rs policy.[1]

Kidney tissue samples were processed and analysed by quantitative PCR (qPCR) in a similar way to that described previously (Kummeling et al., 2012). The guidelines for real-time qPCR good research practice, adapted for the veterinary research community, were followed (Bustin and Penning, 2012) to calculate relative mRNA expression of BMP-7, HGF and its receptor c-MET, as well as TGF-β and its downstream target genes, namely, PAI-1, collagen-I and fibronectin. Primer sequences and reaction specifications are provided in Appendix: Supplementary Table S1. Water and non-reverse transcribed RNA were included as negative controls and remained negative by PCR. Five reference genes were included (glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein L8,

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[1] See: http://www.uu.nl/university/research/EN/researchatutrechtuniversity/animaltesting/Pages/Alternatievende.aspx.

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1090-0233/

Table 1
Summary of details of dogs affected with a congenital portosystemic shunt (CPSS) and control dogs, specified by breed, shunt-location (if present) and age at time of sampling.

Dog breed	Control/CPSS: intra or extra	Age (months)
Labrador retriever	Control	4
Labrador retriever	Control	5
Labrador retriever	Control	4
Labrador retriever	Control	6
Cross breed	Control	17
Cross breed	Control	17
Cross breed	Control	5
Cross breed	Control	5
Cross breed	Control	16
Cross breed	Control	16
Beagle	Control	17
Beagle	Control	18
Golden retriever	Control	36
Cairn terrier	CPSS-extra	6
Maltese	CPSS-extra	11
Labrador retriever	CPSS-intra	6
Irish wolfhound	CPSS-intra	5
Bernese mountain dog	CPSS-intra	10
Great Dane	CPSS-intra	5
Yorkshire terrier	CPSS-extra	16
Miniature poodle	CPSS-extra	42
Yorkshire terrier	CPSS-extra	12
West Highland white terrier	CPSS-extra	11
Yorkshire terrier	CPSS-extra	6
Jack Russell terrier	CPSS-extra	68
Jack Russell terrier	CPSS-extra	6

Intra, intrahepatic; extra, extrahepatic.

β2-microglobulin, β-glucuronidase, and ribosomal protein S19) in a GeNorm-analysis.[2]

To measure cell signalling proteins, biopsies were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium orthovanadate, 17 μg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF) (all from Sigma–Aldrich) as detailed in Spee et al. (2005). Protein concentrations were measured using a Lowry-based commercial assay (DC Protein Assay, BioRad). Electrophoresis (20 μg protein per lane) was performed using Criterion Tris–HCl polyacrylamide gels (BioRad). Protein was transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 1 h in a standard blocking solution containing 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with the primary antibody (rabbit anti human phospho-SMAD-1,5,8; Cell Signalling) diluted 1:700 in Tris-buffered saline, supplemented with 0.1% Tween-20 (TBST) and 5% BSA. Membranes were washed five times in TBST and subsequently incubated with the secondary antibody (horseradish peroxidase-labelled anti-rabbit IgG, Cell Signalling) diluted 1:20,000 in TBST and 5% non-fat dry skimmed milk for 1 h at room temperature. After washing five times in TBST and incubation with the ECL-advance Western Blotting Detection Kit (Amersham Biosciences), luminescence was measured with a ChemiDoc XRS Imager (BioRad). The cross-reactivity of the antibody for canine SMADs has been described previously (Larman et al., 2009). Details for the Western blotting analysis of β-actin for normalisation purposes are described elsewhere (Spee et al., 2005).

All statistical analyses were performed using commercial software (IBM SPSS version 16.0.1). Differences in expression levels were evaluated by two-sample Student's *t* test with $P < 0.05$ taken to represent statistical significance.

[2] See: http://www.biogazelle.com.

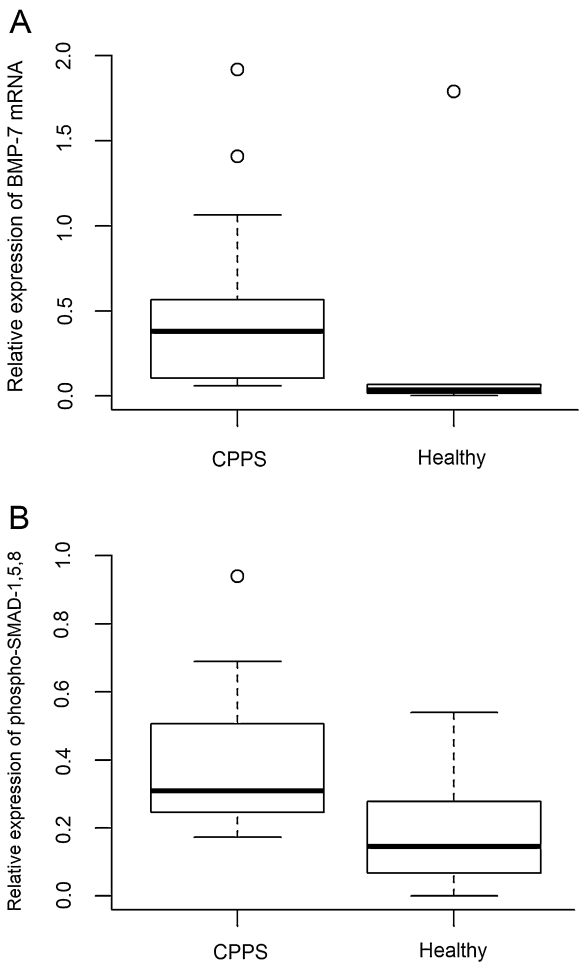

Fig. 1. (A) Bone morphogenetic protein 7 (BMP-7) mRNA expression in renal tissue samples of control dogs (healthy; $n = 13$) and dogs with a congenital portosystemic shunt (CPPS; $n = 13$). Normalisation was undertaken to the geometric mean of the five reference genes: glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein L8, β2-microglobulin, β-glucuronidase, and ribosomal protein S19. The data are represented by box and whisker plots. Outliers ($n = 2$, one in both groups) are depicted, but were not included in the statistical analysis. $P = 0.01$, comparing the two groups. (B) Expression of phospho-SMAD-1,5,8 was assessed in renal tissue samples of control dogs (healthy; $n = 13$) and dogs with a CPPS ($n = 13$) by Western blotting and image analysis. Data were normalised to β-actin expression and are represented by box and whisker plots. $P = 0.027$ comparing the two groups.

Renal BMP-7 mRNA expression was significantly greater in dogs with a CPSS compared with healthy dogs ($P = 0.01$; Fig. 1A). In contrast, there were no differences in mRNA expression of HGF, c-Met, TGF-β, and TGF-β-downstream target genes (PAI-1, collagen-I and fibronectin) comparing kidney biopsies of control and CPSS-affected dogs. Western blotting was used to assess expression of phospho-SMAD-1,5,8, which are activated by BMP-7 (see Appendix: Supplementary Fig. S1). There was evidence for increased BMP-7 activity, with a relative increase in phospho-SMAD-1,5,8 seen in tissues from CPSS-affected dogs ($P = 0.027$, Fig. 1B). These research findings are consistent with up-regulation of BMP-7 signalling in kidneys of dogs affected with a CPSS. However, BMP-7 mRNA expression and phospho-SMAD-1,5,8 levels did not significantly correlate with each other ($r^2 = 0.13$; $P = 0.07$), although we cannot exclude that this is due to technical differences in the assays or that this indicates the involvement of additional inhibitory or stimulatory factors.

Whether the increase in BMP-7 and SMAD-1,5,8 signalling is causally related to increased glomerular filtration or increased kidney

size remains to be established. As with many research studies investigating canine CPPS, the group size is somewhat limited and there are breed differences between the CPSS-affected and control groups (Kummeling et al., 2012), although the dogs affected with a CPSS and the control group were not significantly different in age ($P = 0.56$). It has been documented that renal function returns to normal upon surgical attenuation of the shunt (Deppe et al., 1999). Therefore, analysis of BMP-7 and its signalling pathways in the kidneys after surgical treatment might provide further evidence for its role in glomerular hyper-filtration. However, there are ethical concerns in undertaking additional invasive procedures, such as renal biopsy, from such clinical cases.

If it can be confirmed that there is a causative role for BMP-7 in the development of hyper-functional kidneys in dogs affected with a CPSS, this could lead to new treatment strategies to ameliorate deterioration of kidney function, as has been recently proposed for chronic kidney diseases (Meng et al., 2013).

Conflict of interest statement

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

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Appendix: Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2015.03.008.

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