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Transforming Growth Factor Beta 1 Activation, Storage, and Signaling Pathways in Idiopathic Pulmonary Fibrosis in Dogs

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Background: The pathogenesis of idiopathic pulmonary fibrosis (IPF) in dogs is poorly understood. In human, transforming growth factor β1 (TGF-β1) is considered central in the pathogenesis.

Objectives: To investigate TGF-β1 pathway in IPF.

Animals: Lung tissues from 12 affected and 11 control dogs. Serum from 16 affected West Highland white Terriers (WHWTs) and healthy dogs from predisposed (13 WHWTs, 12 Scottish Terriers and 13 Bichons Frise) and nonpredisposed breeds (10 Whippets, 10 Belgian shepherds, 8 Labradors).

Methods: In this prospective study, immunohistochemistry was used to evaluate expression and localization of TGF-β1 protein and proteins involved in TGF-β1 signaling (TGF-β receptor type I and phospho-Smad2/3). Pulmonary expression of TGF-β1 and molecules involved in its storage (latent TGF-β binding proteins [LTBP] 1, 2, and 4), activation (αvβ6 and αvβ8 integrins, thrombospondin-1) and signal inhibition (Smad 7) was analyzed by quantitative reverse transcriptase PCR. Circulating TGF-β1 concentration was measured by ELISA.

Results: In IPF, high level of TGF-β1 protein was found in areas of fibrosis, epithelial cells had strong expression of TGF-β receptor type 1 and phospho-Smad2/3, gene expression was decreased for LTBP 4 ($P = .009$) and β8 integrin ($P < .001$) and increased for thrombospondin-1 ($P = .016$); no difference was seen for Smad7, LTBP1 and 2. Serum TGF-β1 concentration was higher in predisposed compared with nonpredisposed breeds ($P < .0001$).

Conclusions and Clinical Importance: This study identified an enhanced TGF-β1 signaling activity in IPF. TGF-β1 storage and activation proteins with altered expression represent potential therapeutic targets. Higher circulating TGF-β1 concentration in predisposed breeds might partly explain their susceptibility for IPF.

Key words: Integrin; Latent binding protein; Smads; Thrombospondin-1.

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Cases were recruited at the University of Liège and at the Finland Veterinary Teaching Hospital. The laboratory work was performed at the University of Liège (gene expression analysis), at the University of Brussel (serum TGFß-1 measurement, immunohistochemistry for TGFß-1 and TGFβR1) and at the Helsinki University Central Hospital (immunohistochemistry for P-Smad2/3).

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Abbreviations:

IPF	idiopathic pulmonary fibrosis
ITGB6	integrin chain β6
ITGB8	integrin chain β8
LTBP	latent transforming growth factor binding protein
P-Smad2/3	phosphorylated Smad2/3
qRT-PCR	quantitative reverse transcriptase PCR
RPS18	ribosomal protein S18
TBP	TATA box binding protein
TGFβR1	transforming growth factor β receptor type 1
TGF-β	transforming growth factor β
THBS1	thrombospondin-1
WHWT	West Highland white Terrier

Idiopathic pulmonary fibrosis (IPF) in dogs has been recently completely characterized clinically[1–3] and histologically.[4] This histological description has confirmed the fibrotic nature of the disease with an underlying mature fibrosis in all dogs and multifocal areas of accentuated subpleural and peribronchiolar fibrosis with occasional honeycombing and alveolar epithelial changes found in most dogs (type II pneumocytes atypia and hyperplasia). Inflammatory changes are limited to mild-to-moderate interstitial lymphoplasmacytic infiltration. Another striking figure of IPF is the major breed predisposition for the West Highland white Terrier (WHWT). Rare cases have been described in other Terrier breeds including the Staffordshire Terrier[5] and the Scottish Terrier[6] and in other small breeds such as the Bichon Frise (C. Clercx , E. Krafft, personal observation).

Breed predisposition suggests a genetic basis for the disease; however, the pathogenesis of IPF is poorly

understood. Endothelin-1[6] and procollagen aminopeptide type III[7] have been studied as biomarkers of IPF and are probably involved in the pathogenesis. Recently, a pulmonary gene expression profile analysis has showed increased pulmonary expression of genes encoding cytokines such as CCL2, CCL7, IL8, CXCL14, and the fibroblast activation protein.[8]

Transforming growth factor β1 (TGF-β1), a member of the TGF-β superfamily, plays important regulatory roles in cell growth, morphogenesis, differentiation, and apoptosis. It is a potent fibrogenic factor which increases extracellular matrix accumulation by enhancing collagen synthesis and suppressing protease production.[9] In human patients with IPF, increased TGF-β1 concentrations are found both in bronchoalveolar lavage fluid[10] and plasma.[11] Moreover, TGF-β1 mRNA and protein are overexpressed in lung tissue of human patients with IPF.[12–14] For all these reasons, dysregulated or aberrant TGF-β1 signaling TGF-β1 is now considered to be one of the primary causative agents of pulmonary fibrosis in man.[15]

Transforming growth factor β1 is produced as an inactive form, the small latent complex, which is the active mature TGF-β1 peptide bound to its propeptide. This small latent complex binds to latent TGF binding proteins (LTBPs) to form a large latent complex before being released from the cell. After excretion, most latent complexes are targeted to the extracellular matrix through LTBPs. This storage is a means of regulating TGF-β1 signaling.[16] In vivo, latent complexes are activated by integrins αvβ6[17,18] and αvβ8[19] and by interaction with thrombospondin-1 (THBS1).[20–22] The active form of TGF-β1 binds to its specific type II receptors, which is followed by the recruitment of type I receptors (TGFβR1). Various intracellular signaling pathways downstream to the TGF-β1 receptors have been described, including the Smad proteins.[23] Activation of TGFβR1 results in Smad2/3 phosphorylation leading to a complex with Smad4. This complex translocates to the nucleus where it activates target genes by binding to specific promoter element. Smad7 inhibits TGF-β1 signaling by binding to TGFβR1 and interfering with Smad2 and Smad3 phosphorylation.

The aim of this study was to evaluate TGF-β1 biochemical pathways in healthy dogs and dogs with IPF. Specifically, the concentration of circulating TGF-β1 was evaluated in addition to lung expression and localization of this molecule as well as proteins involved in its storage (LTBPs) and activation (αvβ6 and αvβ8 integrins and THBS1). We also analyzed proteins involved in TGF-β1 signaling including TGFβR1, phosphorylated Smad2/3 (P-Smad2/3) and Smad7.

Materials and Methods

All samples were obtained from privately owned dogs. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee of Experimental Animals of the University of Helsinki, Finland (permit number: ESLH-2008-05403) and of the University of Liège, Belgium (permit number 1435).

Animals

Lung tissue samples were obtained from 12 WHWTs with IPF (mean age ± SE of the mean: 12.8 ± 0.4 years) and 11 control dogs of various breeds (3 Beagles, 3 mixed-breed, one dog from each of the following breeds: Yorkshire Terrier, Jack Russell Terrier, Border collie, Newfoundland, Leonberger) (5.2 ± 1.4 years).

Serum was collected from 16 WHWTs with IPF (11.7 ± 0.3 years) and from 66 healthy control dogs from breeds with different predispositions to IPF. These included highly predisposed WHWTs (13 dogs, 9.6 ± 0.8 years), two breeds reported to be mildly or moderately predisposed, the Scottish Terrier (12 dogs, 5.6 ± 0.8 years), and the Bichon Frise (13 dogs, 5.4 ± 1.0 years) and 3 breeds considered to be nonpredisposed: the Whippet (10 dogs, 8.3 ± 0.9 years), the Belgian shepherd (10 dogs, 5.6 ± 0.1 years), and the Labrador (8 dogs, 4.9 ± 1.2 years).

Clinical diagnosis of IPF was based on compatible clinical signs and exclusion of other causes of chronic respiratory disease by thoracic radiography, bronchoscopy, bronchoalveolar lavage fluid analysis, echocardiography, and fecal analysis (Baermann and flotation methods), and on the results of HRCT.[1,3] Euthanasia was indicated because of progressive respiratory failure in 12 dogs; diagnosis of IPF was confirmed postmortem by microscopical examination of lung tissues.[4] The health status of the control dogs was assessed based on history (absence of clinical signs consistent with respiratory or cardiac disease, or any other disease with systemic consequences), physical examination, hematology, and serum biochemistry. In the healthy WHWTs, blood gas analysis, bronchoscopy, and thoracic HRCT were also performed.[1] The 11 control (non-WHWTs) dogs used for lung analyses were euthanized for nonpulmonary related reasons and histopathological examination confirmed normal lung architecture.

Lung and Blood Samples

Full-thickness lung tissue samples were obtained within 30 minutes after euthanasia. Samples for RNA extraction were either placed in a 1.5 mL cryotube,[a] snap frozen in liquid nitrogen and stored at −80°C (n = 4) or transferred into in a cryotube[a] containing RNA later,[b] refrigerated at 4°C for up to 24 hours, and then frozen at −80°C until further processing (n = 19). Samples for histopathology and immunohistochemistry were placed in 10% neutral buffered formalin. Since the lesion distribution is heterogeneous in IPF, samples for RNA extraction and for histopathological analysis were collected from adjacent areas to ensure microarray and quantitative reverse transcriptase PCR (qRT-PCR) analysis was performed on lesional tissue. Blood samples collected in plain tubes were centrifuged 30 minutes after collection at 4°C for 15 minutes at 3,000 × *g* and the serum was stored at −20°C until analysis.

Immunohistochemistry

Paraffin wax-embedded sections of control (n = 5) and IPF lung (n = 7) were dewaxed in toluene and rehydrated in graded alcohol. For P-Smad2/3 only, antigens were retrieved by heating the sections in 0.01 M citrate buffer (pH 6.0) in a microwave oven set to full power until the solution came to boil and for 10 minutes after this point. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase.

TGF-β1 and TGFβR1

Tissue sections were incubated for 1 hour in blocking solution (10% normal goat serum in PBS) and then exposed to rabbit anti-human TGF-β1[c] or anti-human TGFβR1-ALK5[c] (as described previously[24–27]), each diluted at 1 : 400 in the blocking solution at 4°C overnight. Secondary antibody (biotinylated goat anti-rabbit IgG[d]) was applied to the sections for 1 hour at room temperature at a concentration of 1 : 300 in the blocking solution. Immunoreactivity was detected by the use of a peroxidase-labeled avidin-biotin complex kit[d] followed by incubation with 3,3′-diaminobenzidine tetrahydrochloride.[d] Sections were then counterstained with toluidine blue. For negative controls, the primary antibody was replaced by rabbit IgG[d] (1 : 5,000) to determine the specificity of the labeling. Positive control tissue consisted of sections of canine myocardium.

P-Smad2/3

Tissue sections were exposed to the primary antibody: rabbit antibody against human, mouse and rat P-Smad2/3,[e] as described previously,[28] at 4°C overnight. The bound antibodies were visualized by use of the Novolink Polymer Detection System (Novocastra[f]) and 3,3′diaminobenzidine.[f] The sections were counterstained with Mayer's hematoxylin. For negative control, the sections were treated with isotype-specific antibody for rabbit.[g]

Quantitative RT-PCR

Total RNA was isolated from lung tissue samples (12 dogs with IPF, 11 control dogs) using the Micro to Mini Total RNA extraction kit[h] and its quality was checked, as described previously.[8] TATA box binding protein (TBP) and ribosomal protein S18 (RPS18) were used as nonregulated reference genes for normalization of gene expression.[29] The primer and probe sequences for TGF-β1, RPS18, and TBP were the same as previously described.[29,30] Primers and probes were designed using the GenBank sequences for integrin chain β6 (ITGB6; XM_852055), integrin chain β8 (ITGB8; XM_532487), THBS1 (XM_544610), SMAD7 (XM_845400), LTBP1 (XM_546547), LTBP3 (XM_540857.3), and LTBP4 (XM_533664.4), as described previously[30] (Table 1). Quantitative RT-PCR was carried out in a two-tubes, two enzymes format using a combination of a reverse transcriptase[i] and Hot-Start Taq Master Mix[j] as described previously.[30] A negative control of nuclease free water and a positive control sample with a known Ct value were included in each run; with only one transcript quantified on each plate. Duplicate reactions were run for each cDNA sample and a mean Ct value was calculated for each sample.

Measurement of Serum Concentration of TGF-β1

Transforming growth factor β1 concentration was measured in serum using a canine ELISA kit,[k] as per the manufacturer's instructions. The range of detection of this assay is 31.2–2,000 pg/mL. As indicated in the manufacturer's instructions for canine serum samples, a 40-fold dilution was performed and the concentration measured at the end of the procedure was multiplied by the dilution factor to obtain the real concentration.

Statistical Analysis

Statistical analysis was performed using commercially available software.[l] Relative quantification of gene expression was performed using the ΔC_t method. Mean relative qRT-PCR expressions were compared using the Mann–Whitney *U*-test. Serum

Table 1. Primers and probes sequences used for quantitative reverse transcriptase PCR (Eurogentech Ltd., Romsey, Hampshire, UK).

Primer Set	Product Size (pb)	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Fluoro-phore 5′	Probe Sequence (5′–3′)	3′ Quencher
ITGB6	99	TCTTCTCATTGGGGTTGTCC	GCCTTTGACCGTTCTGCTT	FAM	TGCTGTGCATTTGGAAGCTGTTGG	BHQ1
ITGB8	96	AAGGGCCAAGTGTGTAGTGG	GGGGCAGTGTTCACAGAAG	FAM	CGGCCAATGCTCCCAGGATC	BHQ1
THBS1	129	CATTCAGGAGTGCGACAAGA	GGGCTAGGAGAATTGCAGAG	FAM	CCACTGGTCCCCGTGGTCGT	BHQ1
LTBP1	136	TATCTGTGGAGCCGGACACT	GGAGCAGAGTTGTTGGACCT	FAM	CGAGGGCTACAAGTTCAGTGAGCAACA	BHQ1
LTBP3	80	GGCTACACCCAAGACAACAAC	GCCCCAAACAATATGCACTC	FAM	CTACGGCATCCCCAGCCCACC	BHQ1
LTBP4	121	GAGCTGCCCTGTGTGAGAA	GGGAATGTGCCAGGAGAAG	FAM	TCAGGGCTGGTGGGGCAGAC	BHQ1
SMAD7	104	TCTCCCCCTCCTCCTTACTC	AAATTCGTTCCCCCTGTTTC	FAM	CCAGATGCTGTGCCTTCCTCCG	BHQ1

FAM, 6-carboxy-fluorescine; BHQ1, black hole quencher 1.

TGF-β1 concentration in WHWTs with IPF was compared with healthy WHWTs with a *t*-test. Multivariate analysis was performed in healthy dogs to check influence of age and breed on serum TGF-β1 concentration. If a factor was found significant, it was further studied using posthoc contrasts in the GLM procedure with correction for multiple testing (Tukey Kramer). Statistical significance was defined as $P < 0.05$. No quantitative analysis was performed for immunohistochemical results.

Results

TGF-β1 Gene Expression, TGF-β1 and its Receptor TGFβR1 Immunoreactivity

Expression of TGF-β1mRNA was not significantly different between samples of lung from dogs with IPF and from control dogs ($P = .086$) (Fig 1A). In sections of lungs from all control dogs (Fig 2A), strong and diffuse TGF-β1 labeling was seen in the layer of fibrous connective tissue surrounding bronchi and bronchioles and a weaker granular expression was seen in bronchial/bronchiolar and vascular smooth muscle (comparable intensity in all dogs). Endothelial cells and bronchial epithelial cells showed no or a very weak expression of TGF-β1. In the interstitium between alveoli, the labeling was less consistent with some areas showing no labeling and others having weak expression (Fig 2E). In contrast, bronchial and bronchiolar epithelial cells had distinct apical expression of TGFβR1 in all dogs but there was only weak granular labeling of the smooth muscle and no expression within the layer of fibrous connective tissue surrounding bronchi or bronchioles (Fig 2C). There was no TGFβR1 labeling of alveolar tissue in any dog.

In sections of lung from all WHWTs with IPF, the same pattern of immunoreactivity (same localization and comparable intensity) was observed for both antibodies in bronchial areas (Fig 2B,D). In areas of pulmonary fibrosis in all dogs, there was uniformly strong and diffuse expression of TGF-β1 in the fibrous matrix (Fig 2F). In contrast, TGFβR1 was not visible within the fibrotic tissue, but there was very strong labeling of individual alveolar epithelial cells and particularly hyperplastic pneumocytes (Fig 2G,H). In those sections of lung from dogs with IPF in which there was a concurrent inflammatory response, there was no expression of TGF-β1 by macrophages or neutrophils,

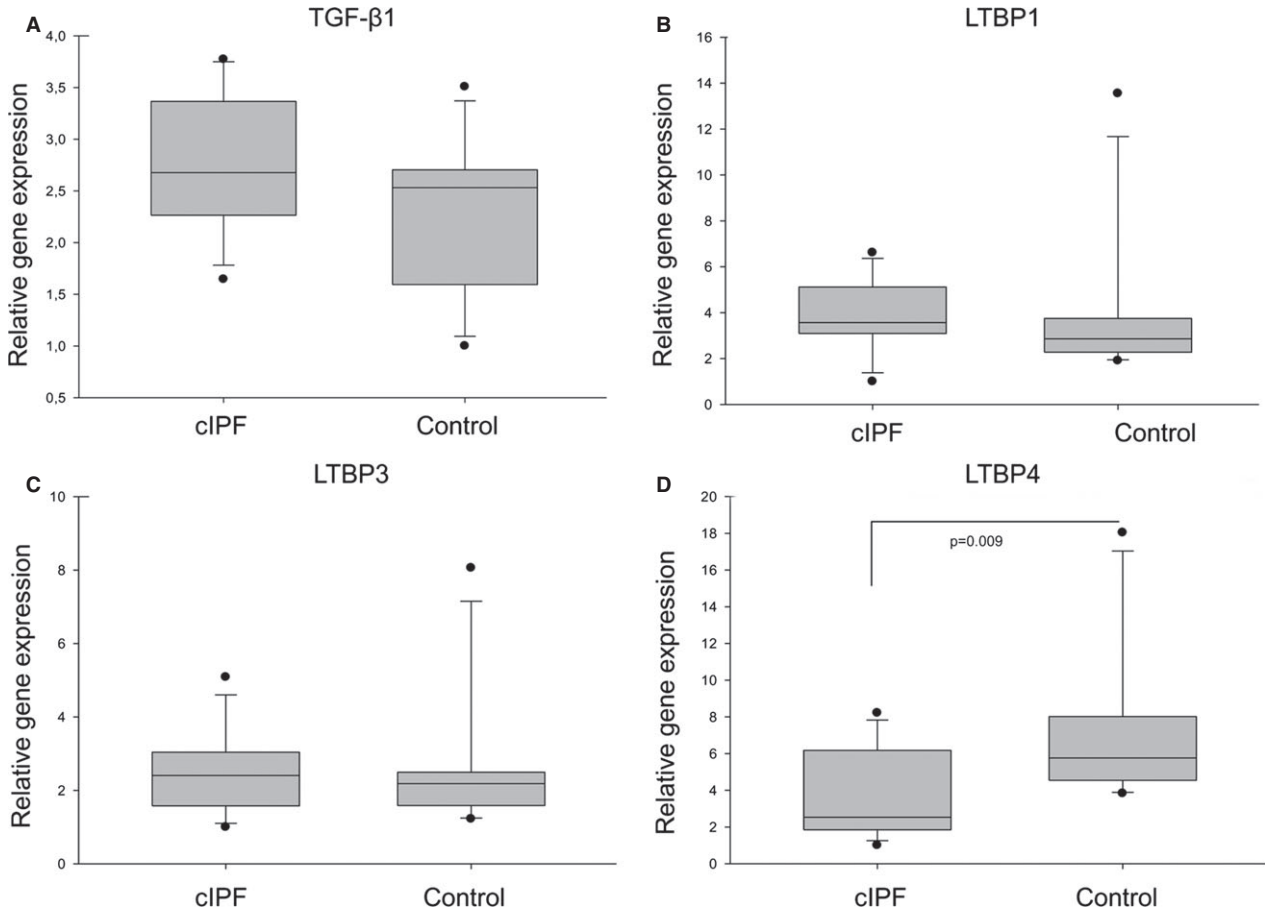

Fig 1. Pulmonary gene expression levels of **(A)** transforming growth factor β1 (TGF-β1), **(B)** latent transforming growth factor binding protein 1 (LTBP1), **(C)** LTBP3 and **(D)** LTBP4 measured by real-time quantitative reverse transcriptase-PCR in West Highland white Terriers with idiopathic pulmonary fibrosis (IPF, n = 12) versus control dogs (n = 11). Results are presented as box plots: the lower, middle, and upper lines of each box represent the 1st, 2nd, and 3rd quartiles, respectively. The whiskers delineate the 10th and 90th percentiles. Dots show the maximum and minimum values.

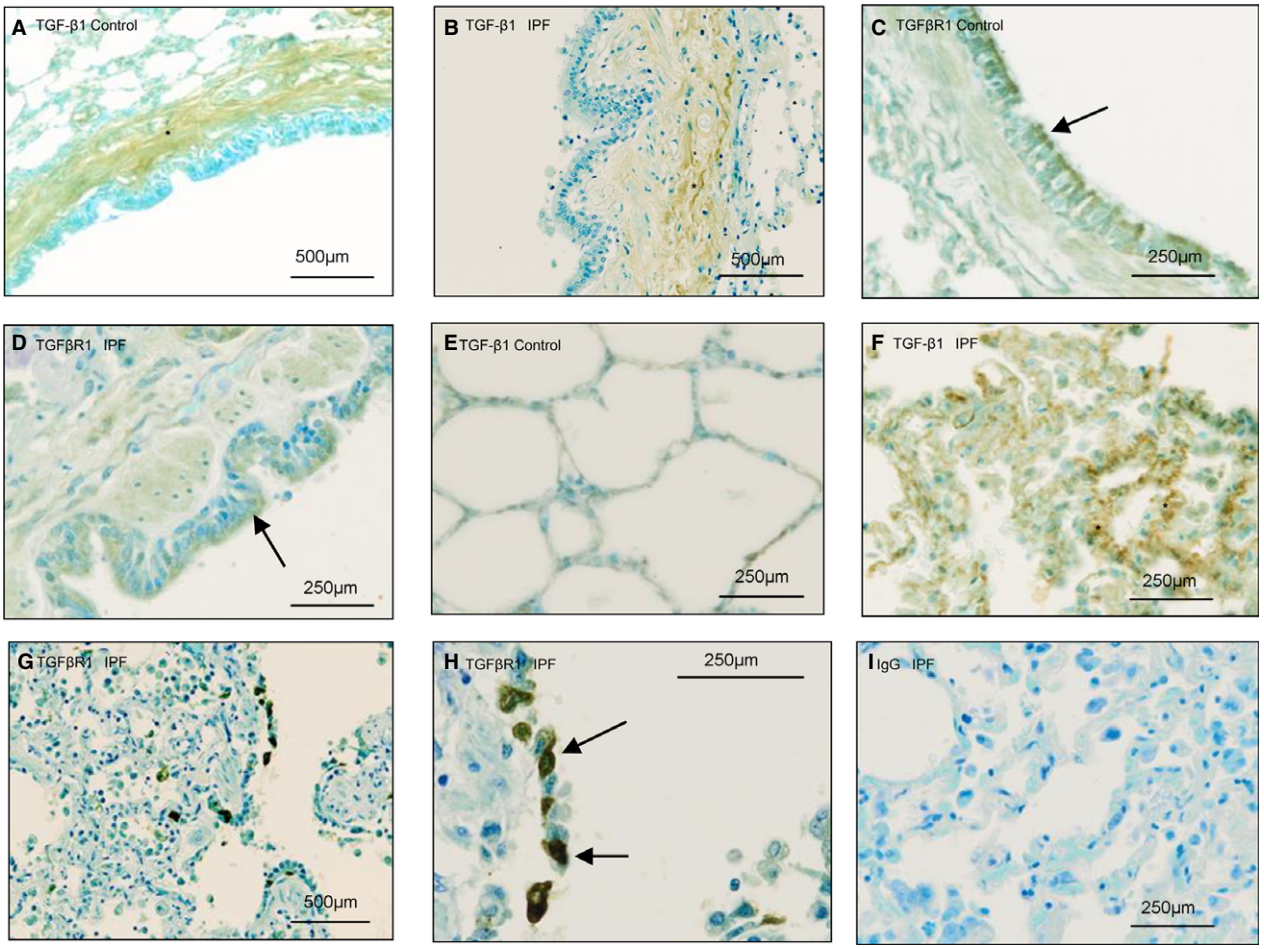

Fig 2. Transforming growth factor β1 (TGF-β1) immunoreactivity in healthy control (**A**: bronchial area, **E**: alveolar area) and in West Highland white Terrier with idiopathic pulmonary fibrosis (IPF) (**B**: bronchial area, **F**: alveolar area). TGF-β receptor type 1 (TGFβR1) immunoreactivity in healthy control lung (**C**: bronchial area) and in affected WHWT (**D**: bronchial area, **G**: alveolar area, and **H** closer view). Control staining with rabbit IgG in an affected WHWT in **I**. *Diffuse and intense staining. Arrows: distinct apical expression in bronchial and bronchiolar epithelial cells in C and D, strong labeling of individual alveolar epithelial cells in H. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

but some alveolar macrophages appeared to express TGFβR1.

For all staining, control sections were negative; a representative image in an affected lung is shown in Figure 2I.

TGF-β1 Signal Transducer P-Smad2/3 Immunoreactivity

In healthy control lungs, P-Smad2/3 nuclear labeling was observed in some alveolar and bronchial epithelial cells (Fig 3A). In the lungs of dogs with IPF, intense positive nuclear labeling was observed in the pathological alveolar epithelium (Fig 3B).

Gene Expression of TGF-β1 Storage and Activation Proteins

Relative LTBP1 and LTBP3 gene expression was not significantly different between the lungs of dogs with IPF and control dogs, but LTBP4 gene expression was decreased in IPF (Fig 1B–D). Integrin αvβ6 and αvβ8 are heterodimeric molecules containing 2 distinct chains: the αv chain is common to many integrins, whereas the β6 chain is specific to αvβ6 and the β8 chain to αvβ8. The expression of the 2 β subunits (ITGB6 and ITGB8) was measured. ITGB6 gene expression was not significantly different between the two groups ($P = .054$). Expression of ITGB8 was significantly lower ($P < .001$) and THBS1 expression was significantly higher ($P = .016$) in IPF relative to controls (Fig 4).

Gene Expression of the Inhibitory Smad 7

The expression level of Smad7 in pulmonary tissues of dogs with IPF was not significantly different from that of control dogs ($P = .834$) (Fig 4D).

Serum TGF-β1 Concentration

No significant difference in serum TGF-β1 concentration was observed between WHWTs with IPF

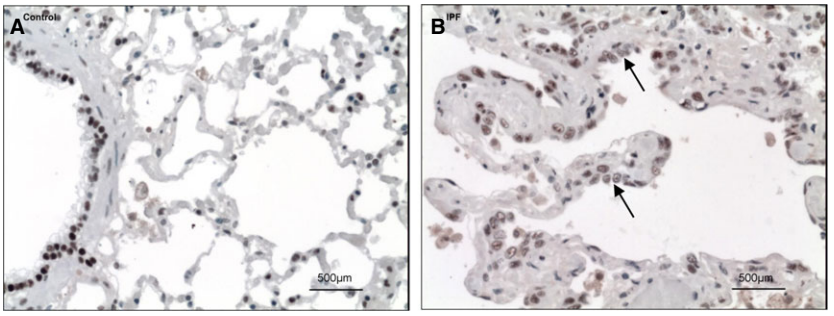

Fig 3. Phosphorylated Smad2/3 immunoreactivity in healthy control lung **(A)** showing positive (brown) bronchial epithelial nuclear staining and in West Highland white Terrier with idiopathic pulmonary fibrosis (**B**, IPF) showing an intense positive nuclear labeling in the pathological alveolar epithelium (arrows). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

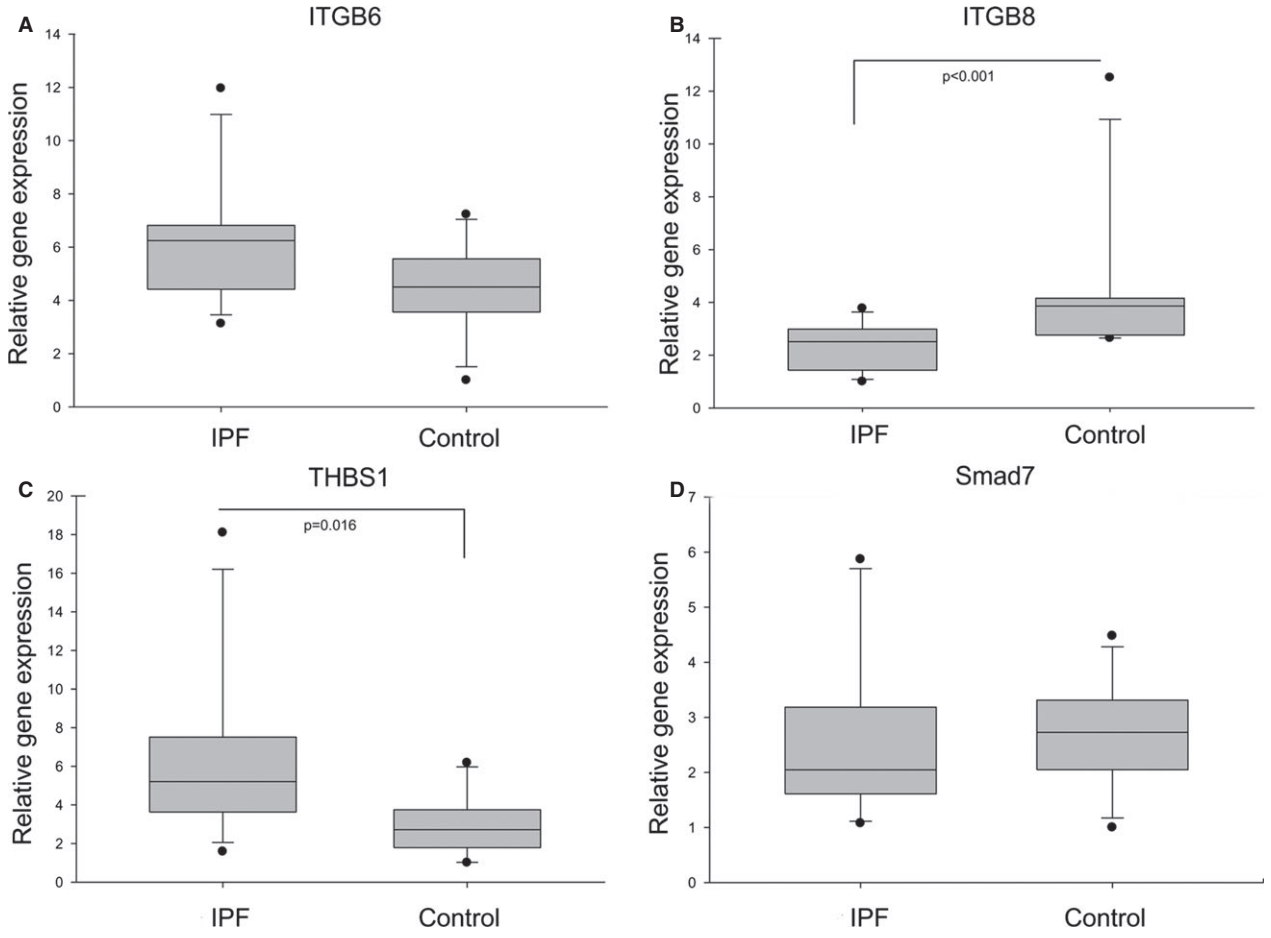

Fig 4. Pulmonary gene expression levels of **(A)** integrin chain β6 (ITGB6), **(B)** integrin chain β8 (ITGB8), **(C)** thrombospondin-1 (THBS1), and **(D)** Smad 7 measured by real-time quantitative reverse transcriptase-PCR in West Highland white Terriers with idiopathic pulmonary fibrosis (IPF, n = 12) versus control dogs (n = 11) and represented as box plot.

(mean ± SE of the mean: 59.2 ± 3.9 ng/mL) and healthy WHWTs (66.0 ± 4.1, $P = .202$) (Fig 5). The multivariate analysis performed on all healthy dogs shown no age influence ($P = .2363$), but a highly significant breed effect ($P < .0001$). Serum TGF-β1 concentration was significantly higher in WHWTs compared with all breeds except for the Scottish Terrier. Serum concentration in the Scottish Terrier was significantly higher than those in the Whippet, Belgian shepherd, and Labrador. Serum concentration was also higher in the Bichon Frise compared with the Whippet, Belgian shepherd, and Labrador (Fig 6).

Discussion

This study demonstrated that an active TGF-β1 signaling exists in affected lungs, especially at the level of

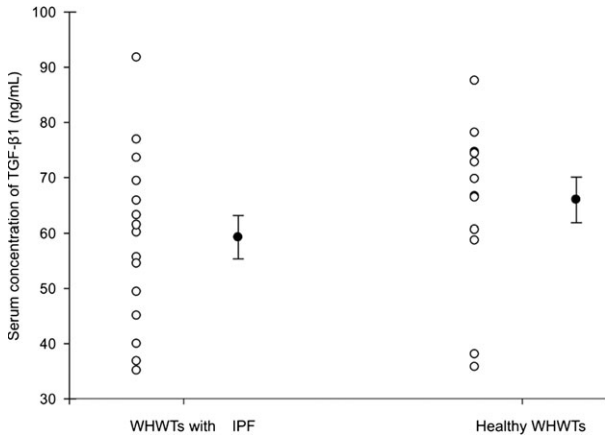

Fig 5. Serum transforming growth factor β1 (TGF-β1) concentration measured by ELISA in West Highland white Terriers (WHWTs) with idiopathic pulmonary fibrosis (IPF, n = 16) and in healthy WHWTs (n = 13). Results are presented as dot-plots. The vertical bars indicate the mean ± SE of the mean.

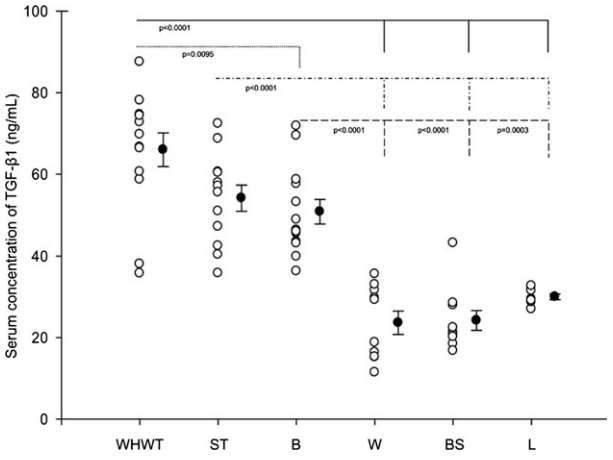

Fig 6. Serum transforming growth factor β1 (TGF-β1) concentration measured by ELISA in healthy dogs (n = 66) from breeds with different predispositions to idiopathic pulmonary fibrosis (IPF), including the highly predisposed West Highland white Terrier (WHWT, n = 13), 2 breeds reported to be mildly or moderately predisposed: the Scottish Terrier (ST, n = 12), and the Bichon Frise (B, n = 13), and 3 breeds considered to be nonpredisposed: the Whippet (W, n = 10), the Belgian shepherd (BS, n = 10), and the Labrador (L, n = 8). Results are presented as dot-plots. The vertical bars indicate the mean ± SE of the mean.

the pathological epithelium, as revealed by an intense staining for the intracellular messenger P-Smad2/3 and for TGFβR1 in epithelial cells. While there was no clear overexpression of TGF-β1 gene, increased TGF-β1 protein content was found in affected lungs with intense interstitial labeling. This study suggested that activation and storage pathways are also modified in IPF with decreased expression of LTBP4 and ITGB8 and increased expression of THBS1. TGF-β1 circulated in higher concentration in predisposed breeds, which might partly explain their susceptibility. On the basis of TGF-β1 well-known profibrotic properties and the findings of this study, we can speculate that TGF-β1 is probably involved in IPF pathogenesis in dogs and modulation of its storage, activation or signaling represents potential therapeutic targets.

Potential mechanisms for TGF-β1 signal regulation include changes in TGF-β1 gene transcription, stability, and translation of TGF-β1 mRNA, post-translational modifications, storage, and activation of TGF-β1 latent complexes, inhibition of TGF-β1 intracellular signaling and inactivation of TGF-β1. Some of these mechanisms were examined in dogs with IPF. In human IPF, TGF-β1 mRNA and protein have been reported to be elevated.[12,13] In this study, no significant overexpression for TGF-β1 gene expression was found in the lung tissue of affected WHWTs compared with controls, while clear extracellular-TGF-β1 protein labeling was detected in fibrotic areas. These findings suggested that regulation of TGF-β1 gene transcription is not an important regulatory mechanism for the TGF-β1 pathway in IPF. Indeed, this pathway is known to be mainly modulated at posttranscriptional stages.[16,31]

Immunohistochemistry was used to identify cells producing TGF-β1, areas of TGF-β1 storage and TGF-β1 target cells. In normal canine lungs, there was extracellular accumulation of TGF-β1 in connective tissue surrounding bronchi and bronchioles. This indicates that, in dogs, TGF-β1 is present in the normal lung and mainly stored in the extracellular matrix. In the lungs of dogs with IPF, there was intense TGF-β1 labeling of the fibrous matrix. This is a common finding in human IPF,[13,32,33] consistent with high amount of TGF-β1 in fibrotic areas and increased TGF-β1 extracellular storage. Even in areas with inflammation, no TGF-β1 expression was seen in alveolar macrophages, suggesting that, unlike in human, in dog, macrophages are not a source of TGF-β1.[31–33] However, in general, no strong intracellular labeling was seen in normal lung or in IPF tissues, which precluded identification of cells producing TGF-β1. The antibody used in this study is raised against an epitope of the C-terminus tail of TGF-β1. As this epitope is common to TGF-β1 precursor and mature forms, this antibody should recognize both precursor and mature TGF-β1. But, as already shown for antibodies directed again the aminoterminal region of TGF-β1, one antibody may preferentially cross-react with the intracellular form of TGF-β1, while another reacts with the extracellular TGF-β1 storage form.[34] So the absence of intracellular labeling found here could either be because of the technical use or to an absence of TGFB1 expression.

Labeling for TGFβR1 was seen in normal lung and in the lung of dogs with IPF in bronchial and bronchiolar epithelial cells as well as a weak expression in the smooth muscles. In normal lungs, pneumocytes did not express TGFβR1. In contrast, there was strong labeling of individual alveolar epithelial cells and particularly hyperplastic pneumocytes in IPF tissues. These hyperplastic pneumocytes appear to be an important target for TGF-β1 in IPF. In the lungs of

dogs with IPF, some alveolar macrophages also expressed TGFβR1. Therefore, lung macrophages may be another TGF-β1 target cell in IPF. Surprisingly, there was mainly intracellular labeling for TGFβR1, while membrane labeling might have been expected. However, TGF-β1 receptors are constitutively internalized via endocytic pathways[35] and this might explain the observed intracellular labeling.

The finding of an increased extracellular storage of TGF-β1 in fibrotic areas and of a trend toward increased gene expression of TGF-β1 prompted evaluation of whether increased TGF-β1 signaling was also present in affected lungs. P-Smad2/3 was used as an indicator of TGF-β1 signaling in IPF lung tissues. P-Smad2/3 nuclear expression was observed in both healthy control lungs and lungs of dogs with IPF, as shown previously in human lungs.[36] In healthy control lungs, some positive alveolar epithelial cells and bronchial epithelial cells were seen, consistent with a basal TGF-β1 signaling activity. In the lungs of dogs with IPF, intense positive nuclear labeling was observed in the diseased alveolar epithelium, suggesting enhanced TGF-β1 signaling at the level of activated epithelial cells.

Lung expression of LTBPs was evaluated. Of the 4 isoforms, all but LTB2 can associate with the small latent TGF-β1.[37] Besides acting as matrix components, LTBPs have important functions in the regulation of TGF-β1 activity. They facilitate latent TGF-β1 secretion, mediate distribution of latent TGF-β1 in the extracellular matrix for storage and regulate latent TGF-β1 activation. This study showed that LTBP-1, -3, and -4 are all expressed in normal canine lungs. In the lungs of dogs with IPF, LTBP1, and LTBP3 gene expression was unchanged compared with controls, while LTBP4 expression was lower in diseased lungs than in control lungs. It has been suggested that each LTBP isoform has specific properties,[38,39] but little is known about their specific functions.

Thereafter, the expression of 3 proteins involved in TGF-β1 latent complex activation (integrin αvβ6, integrin αvβ8 and THBS1) was evaluated. In normal human lungs, integrins αvβ6, and αvβ8 are present at low levels.[36,40] The results of this study suggest that in dogs, both integrins and THBS1 are expressed in normal lungs. Activation via integrin αvβ6 is speculated to be mainly implicated in pathological responses such as in injury and inflammation.[18,40] In human IPF, overexpression of αvβ6 is found within pneumocytes lining alveolar ducts and alveoli[41] and in epithelial cells of fibrotic areas.[36] Integrin αvβ8 is normally highly expressed in human airway epithelium,[19] but its expression is sparse in the lung of patients with IPF.[36] In the lungs of dogs with IPF, ITGB6 gene expression was not significantly different compared with normal lungs, but ITGB8 gene expression was decreased. In this study, increased expression of THBS1 was documented as well, suggesting that interaction with THBS1 might also be an important mechanism for TGF-β1 activation in IPF as it is suspected in human IPF.[42,43]

Expression of Smad 7 is stimulated by TGF-β1, suggesting a negative feedback mechanism.[23] A defective negative retro-control could enhance TGF-β1 signaling. No difference in Smad 7 gene expression was seen between IPF and control dogs, suggesting that this inhibitory pathway is neither stimulated nor defective at the transcriptional level in IPF.

Transforming growth factor β1 serum concentration was evaluated with the hypothesis that a high circulating concentration would be a marker of high TGF-β1 lung activity in dogs with IPF and that it could be used as a biomarker. Surprisingly, no difference was seen between affected and healthy WHWTs, which precludes its use as a biomarker. However, the data demonstrated that TGF-β1 circulates at increased concentrations also in healthy dogs from breeds predisposed to IPF. In human, blood TGF-β1 concentration is heritable[44] and genetic variants have been associated with increased susceptibility for IPF or to disease progression in IPF.[45–47] This study suggested that serum TGF-β1 concentrations might be genetically determined in the dog as well and that there might be a cause-effect relationship between high circulating TGF-β1 and development of IPF. However, because not all dogs from predisposed breeds develop the disease, a high serum TGF-β1 concentration is not sufficient by itself but might trigger an inappropriate lung response to injury leading to pulmonary fibrosis, as suggested in mouse.[48]

The main limitation of this study was that the control group was poorly age- and breed-matched. Concerning the effect of age, no significant effect was found on serum TGF-β1 concentration; however, we could not exclude that age might have an impact on the TGF-β1 pathways locally and might have impacted the qRT-PCR and immunohistochemistry results. Unfortunately, pulmonary tissue from healthy WHWTs was not available and although control dogs mainly included small-breed dogs from which 2 terriers, we could not excluded that breed, independently from the status affected or free of IPF, has an impact on the TGF-β1 pathway locally. However, part of these results has been corroborated in a very recent immunohistochemical study[49] comparing healthy and affected WHWTs. In this study, the authors also found an increased P-Smad2/3 immunoreactivity in WHWTs with IPF at the level of the pathological epithelium in comparison with 3 healthy WHWTs; confirming the existence of an enhanced TGF-β1 signaling activity in affected WHWTs. This study also analyzed the expression of LTBP1. Increased peribronchial and perivascular LTBP-1 immunoreactivity was seen in WHWTs with IPF compared with controls. Alveolar LTPB-1 immunolabeling in diseased WHWTs was seen mainly in the altered alveolar epithelium. Lack of concordance between these results and our findings about LBTP1 may refer to the different technical approaches used. Using qRT-PCR on whole lung samples, we cannot exclude a localized overexpression of LTBP1 by a minor cell type population, which will show up using immunohistochemistry.

Another hypothesis is that regulation of LTBP1 gene transcription is not an important regulatory mechanism determining the amount of protein present in the tissue.

In conclusion, TGF-β1 and its activating, storage, and signaling pathways are modified in dogs with IPF with increased TGF-β1 labeling and increased signaling activity in the pathological epithelium. TGF-β1 activating pathways is altered with a shift toward increased activation via THBS1; highlighting a potential therapeutic target with modulation of TGF-β1 activity via inhibition of THBS1. High circulating TGF-β1 concentration is found in healthy dogs from predisposed breeds which might at least partly explain their high susceptibility to IPF.

Footnotes

[a] CM.LAB, Vordinborg, Denmark
[b] Ambio Inc., Huntington, UK
[c] Santa Cruz Biotechnology Inc, Santa Cruz, CA
[d] Vector Laboratories Inc, Burlingame, CA
[e] Ser465/467; Millipore, Billerica, MA
[f] Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK
[g] Invitrogen, Carsbad, CA
[h] Invitrogen, Merekbeke, Belgium
[i] ImProm-II Reverse Transcription System; Promega Corporation, Southampton, UK
[j] Qiagen Ltd, Crawley, UK
[k] Mouse/Rat/Porcine/Canine TGF-β1 Quantikine ELISA kit; R&D systems, Minneapolis, MN
[l] SigmaStat, Systat Software, Inc, San Jose, CA and Statistical Analysis Systems, SAS Institute, Cary, NC

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