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Analysis of gene expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis: A transcriptomic analysis

Morgane Vanherberghen^{a,*}, Fabrice Bureau^b, Iain Robertson Peters^c,
Michael Joseph Day^c, Cécile Clercx^a, Dominique Peeters^a

^a Companion Animal Clinical Sciences, Faculty of Veterinary Medicine, University of Liège, Belgium

^b Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, University of Liège, Belgium

^c School of Clinical Veterinary Science, University of Bristol, UK

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ABSTRACT

Sino-nasal aspergillosis (SNA) and lymphoplasmacytic rhinitis (LPR) are two common causes of nasal discharge in dog. SNA is typically due to an invasion of *Aspergillus fumigatus* in the surface of nasal mucosa. The etiology of LPR is poorly understood and a possible implication of fungi is suspected.

The purpose of the present study was to explore the immunopathogenesis of these diseases by comparing gene expression in the nasal mucosa from dogs affected by SNA or LPR with healthy dogs, using a canine-specific microarray and quantitative real-time reverse transcriptase polymerase chain reaction for confirmation of the findings of the microarray study. Total RNA was isolated from biopsies of nasal mucosa and gene expression was analyzed via hybridization to the Affymetrix GeneChip[®] Canine Genome 2.0 Array. Selected Affimetrix probes sets identifiers were downloaded into the Database for Annotation, Visualization and Integrated Discovery. Genes of interest were chosen after their fold change and their possible implication in immunopathogenesis of SNA or LPR. The results presented here were in concordance with previous studies on SNA and LPR and highlighted new molecules potentially involved in the pathogenesis of SNA. The over-expression of interleukin (IL)-16, natural killer cell group 7 and chemokine ligand 10 might be related to a potential protective Th1 immunity counterbalanced by other molecules such as DNA-binding protein Ikaros.

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1. Introduction

Sino-nasal aspergillosis (SNA) and idiopathic lymphoplasmacytic rhinitis (LPR) are among the most common causes of nasal discharge in dogs (Windsor et al., 2004).

The clinical signs associated with both diseases are typical of chronic rhinitis, although specific signs such as ulceration or depigmentation of the nostril are only found in the case of SNA (Sharp et al., 1991).

SNA is most often caused by *Aspergillus fumigatus* (Billen et al., 2009). Although the etiology of LPR is currently unknown, a possible role of commensal fungi or bacteria in the pathogenesis of this disease has been suggested (Windsor et al., 2006).

The mucosal immune response in SNA and LPR has been studied through the quantification of messenger RNA (mRNA) encoding cytokines and chemokines in nasal mucosal biopsies (Peeters et al., 2006, 2007). The cytokine profiles are clearly distinct in both diseases, with a

* Corresponding author at: Division of Small Animal Internal Medicine, Faculty of Veterinary Medicine, University of Liège, Bat B44, Boulevard de Colonster, Sart-Tilman, B-4000 Liège, Belgium. Tel.: +32 4 366 42 43; fax: +32 4 366 42 41.

E-mail addresses: mvanherberghen@ulg.ac.be (M. Vanherberghen), fabrice.bureau@ulg.ac.be (F. Bureau), i.r.peters@bristol.ac.uk (I.R. Peters), m.j.day@bristol.ac.uk (M.J. Day), cclercx@ulg.ac.be (C. Clercx), dpeeters@ulg.ac.be (D. Peeters).

dominant Th1 immune response in the nasal mucosa of dogs with SNA and a partial Th2 immune response in that of dogs with LPR (Peeters et al., 2007).

Despite a strong local Th1 immune response, dogs with SNA are unable to clear the *Aspergillus* infection. A local immune defect has been suspected in affected dogs (Peeters et al., 2007); however, up-regulation of the IL-10 gene suggests a potential local immune suppressive response which might counter-balance the active immunity in order to limit secondary immunopathology. A hypersensitivity to bacterial or fungal organisms is suspected to trigger the inflammation in canine LPR (Windsor et al., 2006).

The aim of the present study was to further explore the immunopathogenesis of these diseases by comparing gene expression in the nasal mucosa from dogs affected by SNA or LPR, using a canine-specific microarray and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for confirmation of the findings of the microarray study.

2. Materials and methods

2.1. Animals

Seven dogs with SNA were included in the study. Diagnosis was made based on physical examination, observation of typical intranasal/intranasal fungal plaques associated with severe turbinate destruction on rhinoscopy, and culture of *A. fumigatus* from suspected lesions in all dogs. The ages of these dogs ranged from 3 to 11 years (median 8 years) with two neutered females and five males (two neutered).

Seven dogs with LPR were included in the study. Diagnosis was made based on clinical signs (unilateral or bilateral nasal discharge of at least 6 months duration, without nasal pain, nostril depigmentation or ulceration, and without any signs of systemic disease), rhinoscopic findings (unilateral or bilateral mild to moderate turbinate lysis with mucosal congestion and oedema, mucoid to mucopurulent secretions; and failure to demonstrate a foreign body, neoplasia or fungal plaques in the nasal cavities by rhinoscopy), histopathological findings, and on a negative fungal culture of nasal biopsy and a negative serum antibody titer for *A. fumigatus*. A CT-scan of the head was performed in 3 dogs that excluded aspergillosis in the frontal sinuses. No antifungal therapy had been administered to the patients. The age ranged from 1 to 13 years (median 9 years) with four females (three neutered) and three entire males.

Seven client-owned dogs, humanely destroyed for various independent reasons, were included as control animals (CTRL). None of these dogs had clinical history or signs of upper respiratory tract, immune-mediated or immune suppressive disease. The age ranged from 3 to 11 years (median 6 years) with two females (one neutered) and five males (one neutered).

2.2. Tissue samples

In diseased dogs (SNA and LPR), the nasal cavities and frontal sinus were explored with a rigid endoscope (Cytoscope K Storz SL 30°, Karl-Storz-Endoscopy Belgium SA, Strombeek-Bever, Belgium) and/or a flexible bronchoscope

(Fujinon BRO-YP2, Onys, SA, Brussels, Belgium) by the same investigator (CC). Endoscopic mucosal biopsies were collected from visibly inflamed mucosa. In the control dogs, endoscopic mucosal biopsies were taken, within 10 min of euthanasia by overdose of pentobarbital, from the posterior third of the right nasal cavity. Biopsies were placed in a 2 ml cryotube (CM.LAB, Vordingborg, Denmark), immersed in RNAlater (Ambion, Inc., Huntington, UK) at 4 °C for 24 h and stored at –80 °C until use.

2.3. RNA isolation

Total RNA was isolated from the biopsies using the Micro to Midi Total RNA Extraction Kit (Invitrogen, Merelbeke, Belgium). The tissue was added to a 2-ml Safe-Lock microcentrifuge tube (Eppendorf, Cambridge, UK) containing 350 µl of lysis buffer (from the extraction kit) and a 5 mm stainless steel bead (Qiagen Ltd., Crawley, UK). Samples were disrupted using a TissueLyser (Qiagen Ltd., Crawley, UK) by shaking the tubes at 30 cycles per second for 2 min, and the resulting lysate was used in the RNA extraction as per the manufacturer's protocol. Further DNase digestion of the RNA solution was carried out using RQ1 RNase-Free DNase (Promega Corporation, Southampton, UK) as per the manufacturer's instructions. The RNA was stored at –80 °C until use.

2.4. Microarray procedure

The quantity and quality of RNA was assessed by spectrophotometry (NanoDrop 1000, Thermo Scientific) and automated gel electrophoresis (Experion, Bio-Rad Laboratories). RNA samples presenting a 260:280 nm ratio ranging from 1.8 to 2.2, a 230:260 nm ratio ranging from 1.8 to 2 and a RNA quality indicator (RQI) above 8.4 were selected for microarray analysis.

Aliquots of RNA (4 ng) from all dogs of each group (SNA, LPR and CTRL) were pooled in equal proportion and gene expression was analyzed via hybridization to the Affymetrix GeneChip® Canine Genome 2.0 Array (Affymetrix, UK Ltd., High Wycombe, UK) using the one cycle amplification protocol according to the manufacturer's instructions.¹ The GeneChip Canine Genome 2.0 Array contains over 42,800 *Canis familiaris* probe sets to monitor gene expression for over 18,000 *C. familiaris* transcripts and over 20,000 non-redundant predicted genes. Sequence information for this array contains public data sources obtained from *C. familiaris* UniGene (April 2005), GenBank (April 2005) and the Boxer Dog Genome Project (May 2005). The remainder of the RNA from each dog was kept separate for use in the qRT-PCR.

2.5. Microarray data analysis

Raw data were obtained with the high-density Affymetrix GeneChip Scanner 3000 [Array express: Reviewer_E-MEXP-3166; password: Jobmi1kf]. The Affymetrix call detection algorithm was used to assess which probe sets

¹ http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf [consulted 15 July 2009].

Table 1
Sequences of primers and probes used in the qRT-PCR reactions.

Primer set	Entrez gene ID	GenBank accession number	Product size (pb)	Forward primer (5'–3')	Reverse primer (5'–3')	Probe sequence (5'–3')
RPL13a	479612	XM_533620	87	GCCGGAAGGTTGTAGTCGT	GGAGGAAGGCCAGGTAAC	TGTGAAGGCATCAACATTGCA
RPS18	403685	NM_001048082	118	TGCTCATGTGGTATTGAGGAA	TCTTACTGGCGTGGCTG	CACTGAGGATGAGGTGGATGG
TBP	611193	NC_006594	96	CTATTTCTTGGTGTGCATGAGG	CCTCGGCATTACAGCTTTT	TCGCTAAGAAGCGGTACGGG
IL-5	403790	NM_001006950	158	GACTGGTGGCAGAGACCTTGA	CGTGGGCAGTTGGTTCTC	CGAACTTGGCTGATAGGCGGG
IL-16	488762	XM_855695	99	CCAGTCCAAGGGGATTACAG	TGAGAATGAGCGGTTGAG	ACCGGTGCTGACGCCAAAGC
IL-17RC	610118	XM_847527	82	TGCTCGCTGATGGTGATGATG	TTGGTTCCAGTACAAGAG	TGGATGCTCTGAGGAGCAGC
CCL3	448787	NM_001005251	90	GCCCGGTATTATCTCGAAAC	CTTCAGATCGGCCACATTC	AGAAAAGGCCGCCAGGCTG
CCL4	448786	NM_001005250	112	CGTCTTTCTCTCTTGTGCT	GAATCTTCGCGAGGGTAA	CCGGCGCTCTCAGACCAAT
CCL19	448793	NM_001005256	83	GAATATCGTGGGAGCCTTTC	CGACCTTCAGTGTGGTGA	GATGGCTGTAGACTGCCTGG
CL21	448796	NM_001005258	101	GATCGAGCGCTGTGGACAA	TTGGAGCCCTTTCCCTTC	AGGGGGCTCCCAAGTCTGGC
CCL28	448795	NM_001005257	105	CTGTGCACTGAGGTTTCA	ATGACAGCAGCCAAAGTCA	CGCATCCAGAGAGCTGATGGG
CXCL10	478432	XM_535609	79	TGAGATGATTCTGCAAGTCC	GACATCTTTCTCCCATTT	TCCACATGTTGAGATCATTACA
GZMA	487207	XM_544335	139	GAGTTTCCTATCCGTGCTTT	GTCCCGGTTTCACATCAC	TCTCCGGCTCCCAAGAGG
IFN- γ	403801	NM_001003174	113	TCAACCCCTTCTCGCACT	GCTGCCTACTTGGTCCCTA	CCCCACCCGAACCTCTTCT
IKZF1	483231	XM_540350	91	TGCACTTCTTAACGGCAAC	CAGTGTGGCTTCTCTGTG	TCCGCCCAATGTGCTCATG
NKG7	476398	XM_533601	132	GGCCAGCTTTACATTTCAC	ACCAGGAAGATCACCGAC	ATCCGTGCGACGACAGCCT
STAT1	488449	XM_545571	97	GAACACGACCCGATTACAAA	TGCTTTCCACCACAAAG	TGTGGGACCCGACCTTCAACC
STAT4	478845	XM_536007	140	GGGGCTTAACCTCAGATCAAC	CAAGCCATGTCCAAAATAA	CCTGGGCCAAGTTCTGAAGA

Probe transformation: Fluorophore 5': 6-carboxy-fluorescein (FAM). 3'Quencher: Black Hole Quencher 1 (BHQ1).

were reliably detected above background on each array. Probe sets were discarded for further analysis if they were not detected on at least one array or if their detection was classified as marginal. Within the remaining probe sets, for each disease sample, those with at least a two-fold increase or decrease in expression, relative to the control sample, were considered for further analysis. The Affymetrix probe set identifiers (e.g. CfaAffx.11027.1.S1_at) were separated based on their up- or down-regulation compared to control dogs, and then they were uploaded onto the Database for Annotation, Visualization and Integrated Discovery (DAVID)² to identify the corresponding gene names. The genes were then classified in biological annotations (e.g. immune response, T-cell receptor signaling pathways) based on functional categories sorting (e.g. gene ontology terms [GO term], Kyoto Encyclopedia of Genes and Genomes pathways [KEGG pathways]) (Huang et al., 2009). In addition, the DAVID tool highlighted statistically the biological annotations which were over-represented (enriched) within the submitted list and calculated a fold-enrichment. The fold-enrichment is defined as the ratio of the proportion of genes with a significantly modified expression with a given biological annotation to the proportion of genes with that annotation within the population background (e.g. genes analyzed in the whole microarray); the higher the value, the greater the annotation is enriched. For this microarray data analysis the Benjamini–Hochberg multiple testing correction was chosen and the level of significance was set at $p < 0.05$.

Further analysis focused on genes involved in inflammatory or immune processes, which may play a role in the immunopathogenesis of SNA or LPR. Gene sequences with a significant difference in expression were obtained using the canine genome annotations or the NCBI NetAffx Blast search direct link on the Affymetrix website.³

2.6. Primer and hydrolysis probe design

Primers and probes for interleukin (IL)-16, IL-17 receptor C (RC), chemokine (C–C motif) ligand 3 (CCL3), CCL4, CCL19, CCL21, CCL28, chemokine (C–X–C motif) ligand 10 (CXCL10), granzyme A (GZMA), DNA-binding protein Ikaros (IKZF1), natural killer (NK) cell group 7 sequence (NKG7), Signal Transducers and Activators of Transcription (STAT) 1 and STAT4 were designed via Primer 3⁴ (Rozen and Skaletsky, 2000) and M-fold⁵ using the canine specific GenBank sequences as described previously (Peters et al., 2003) (Table 1). As the sequence data for IL-16, IL-17RC, CCL3, CCL4, CCL19, CCL21, CCL28, CXCL10, GZMA, IKZF, NKG7, STAT1 and STAT4 was derived by gene prediction, additional primer pairs were designed to amplify a longer portion, including the qPCR target, in order to confirm the mRNA sequence of products amplified from complementary DNA (cDNA) (Table 2). The IL-5 and IFN- γ assays were the same as used previously (Peeters et al., 2005). Primers and probes were synthesized by Eurogentec S.A. (Seraing, Belgium) and were reconstituted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) before use.

2.7. Quantitative real-time RT-PCR

Synthesis of cDNA was carried out using the ImProm-II Reverse Transcription System (Promega Corporation) using 500 ng of total RNA and 0.5 μ M of random hexamers in a final volume of 20 μ l, according to the manufacturer's instructions. The resulting cDNA was diluted 1:5 in 80 μ l of TE buffer and stored at -20°C prior to use.

Quantitative real-time PCR (qPCR) was performed using HotStarTaq Master Mix (Qiagen Ltd.) using 0.2 μ M of each

² <http://david.abcc.ncifcrf.gov/home.jsp> [consulted September 2009].

³ <http://www.affymetrix.com> [consulted October 2009].

⁴ http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi [consulted November 2009].

⁵ <http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form> [consulted November 2009].

Table 2

Sequences of primers used for the sequencing of the mRNA products.

Primer set	Entrez gene ID	GenBank accession number	Product length	Forward primer (5'–3')	Reverse primer (5'–3')
IL16	488762	XM_855695	589	TGAACCTCTCCACGGACTCT	CATGACTCTGCCCTTCGTTT
IL17RC	610118	XM_847527	356	CTGCCCTGGCTCAATGT	AGTGGCCTCAGCAAGCA
CCL3	448787	NM_001005251	287	GTCTCTCTGCACCATGTC	GAGGTCACTGGGTCTCCAA
CCL4	448786	NM_001005250	269	CTGTGTGACCGCTCTTCTCT	CAGTTCAGTTCAGATCATCCA
CCL19	448793	NM_001005256	231	GCCAATGATGCTGAAGACTG	CTAACTGCTGTGGCCCTTGT
CCL21	448796	NM_001005258	392	TGTCTCCATCACTGGCTCTG	TTGGGGTCTGTGGCTGTT
CCL28	448795	NM_001005257	363	AGGACTCACTCTCGCTCTCGT	TTTATGGCTGTGGATTCTGG
CXCL10	478432	XM_535609	184	TGACTCTGAATGGTACTCAAGGAA	CAGACATCTTTCTCCCACTC
GRZA	487207	XM_544335	689	GTTCGCGCATCTCTTTCT	CGAGGGTCTCCGATTTC
IKZF	483231	XM_540350	1019	ATGTGCGGAGGATTACGAG	TGGTTGGTCAGGTAGATGAGG
NKG7	476398	XM_533601	611	CCTATCGGTCTTGGCTCTAC	AACAAATGAAGGGGTCTAGG
STAT1	488449	XM_545571	982	AATGATGAAGTGGTGGAGTGG	ATCCAAAGCCAGAAAGGAAAA
STAT4	478845	XM_536007	666	TCAAGATTGTCTTTGTGG	GGTTCAGGCATTATATCCTT

primer, 0.1 μ M of probe or SYBR Green 1 (1:100,000) (Sigma–Aldrich Ltd.) and 5 μ l of diluted cDNA in a final volume of 25 μ l. Sample incubations were performed in an iCycler IQ5 (Bio–Rad Laboratories Ltd.). The thermal cycling conditions were the following: 95 °C for 15 min followed by 45 cycles of 95 °C for 10 s and 60 °C for 15 s. The thermocycling protocol was extended when SYBR Green I was used (for the longer amplicons) by heating samples from 75 °C to 95 °C in 0.5 °C increments with a dwell time at each temperature of 10 s in order to create a melt curve.

The absence of genomic DNA (gDNA) contamination of the RNA samples was confirmed prior to the RT reactions. None of the samples showed evidence of amplifiable DNA with the RPL13a qPCR assay which is capable of amplifying gDNA (Peters et al., 2007). Negative controls consisting of nuclease-free water and a no template reverse transcription (RT) were included in all plates. A positive control sample with a known *Ct* value was included in all sample runs to control for run-to-run *Ct* variation. Each qPCR reaction was run in duplicate. Samples were grouped together to minimize the number of sample runs and the difference in *Ct* values between replicates was less than 0.5.

2.8. Product sequencing, reaction efficiency and copy number calculation

The qRT-PCR product obtained with the primer set designed for sequencing were separated by 2% (w/v) agarose gel electrophoresis and appropriate sized product were excised, with a clean scalpel blade, and purified using the NucleoSpin® Extract II (Machery–Nagel) as per the manufacturer's instructions. Sequencing was performed using Big dye terminator, DMSO, buffer 5 $\times$ PE, 2.5 μ l of cDNA and 2 μ l of primer 2.5 μ M in final volume of 10 μ l. Sample incubation was performed in the PTC-200 DNA engine (Bio–Rad Laboratories) at 96 °C for 4 min then 35 cycles of 96 °C for 6 s, 50 °C for 5 s and 60 °C for 4 min. Products were purified and sequenced on ABI PRISM 3730 DNA analyzer (Applied Biosystems). The sequence data were compared with the predicted GenBank sequences using SEQSCAPE V2.1 (Applied Biosystems).

Ten-fold serial dilution curves (1:10 dilution) of the sequenced product were produced using standard qPCR

protocol, in duplicate, for each reaction. A graph of *Ct* versus log₁₀ relative copy number of the sample from the dilution series was produced, and the reaction efficiency calculated, using the IQ5 Optical System Software V.2 (Bio–Rad Laboratories Ltd.).

Relative expression data for each of the mRNA targets were calculated using the qBase applet for Microsoft Excel⁶ (Hellemans et al., 2007). This applet calculates a relative copy number for each sample, normalized against the measured housekeeper mRNAs and the assay efficiency. The sample with the fewest mRNA copies is given a relative copy number of 1 with all other samples given values relative to this. Samples which gave a negative result for an assay were given a relative copy number of 0. These relative copy number results were used for the statistical analysis and data presentation. The housekeeper genes TATA box binding protein (TBP), ribosomal protein L13 (RPL13a) and ribosomal protein S18 (RPS18) were used as they have previously been established as stably expressed genes in canine nasal mucosa (Peeters et al., 2007).

A comparison was made between the fold change detected by the microarray and that determined by qRT-PCR. For qRT-PCR, the fold change was calculated for each gene using the ratio between the mean relative copy numbers in the diseased versus the control groups.

2.9. Statistical analysis

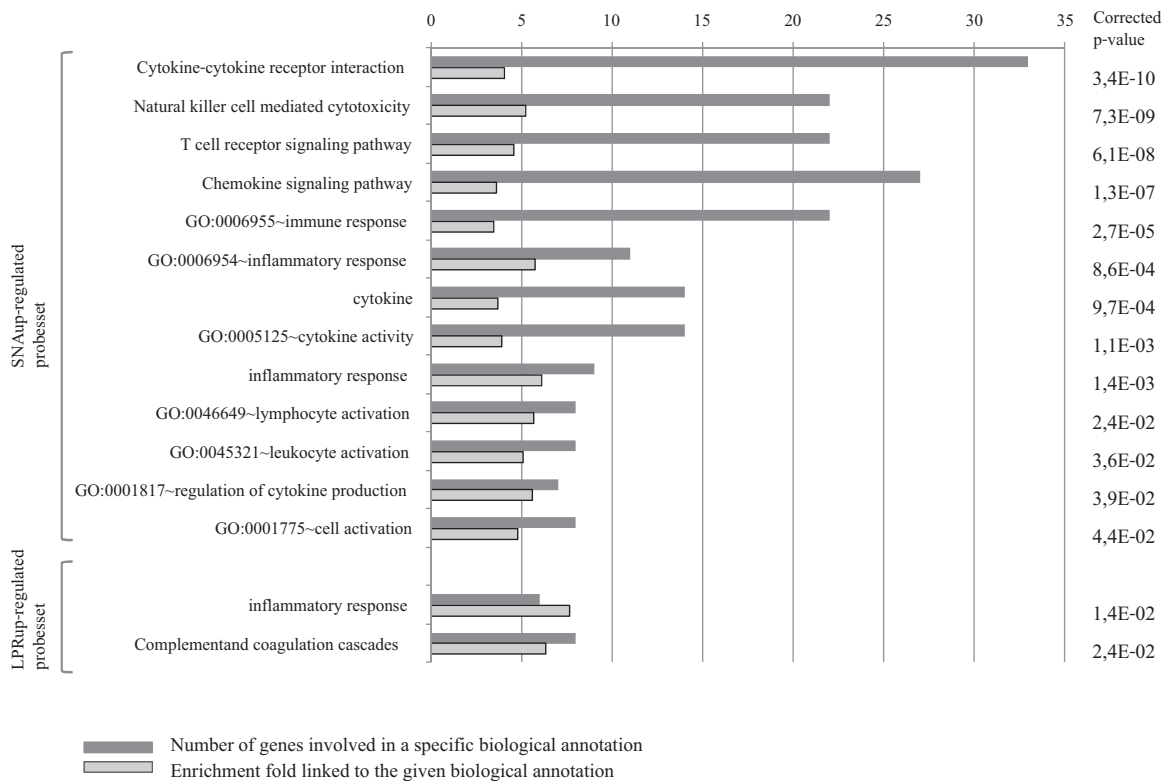
The non-parametric (Kruskal–Wallis) analysis of variance was used to compare the expression of each gene evaluated by qRT-PCR among the three groups of dogs. Significant results were further characterized using the Mann–Whitney test. The level of significance was set to $p < 0.05$ for all analyses.

3. Results

3.1. Microarray analysis

A list of 1562 and 1131 probes with a minimum of a two-fold difference in expression were uploaded in the

⁶ <http://medgen.ugent.be/qbase/>.



SNA, sino-nasal aspergillosis.

LPR, idiopathic lymphoplasmacytic rhinitis.

Fig. 1. Microarray analysis: selected biological annotations related to immune and inflammatory processes. SNA, sino-nasal aspergillosis. LPR, idiopathic lymphoplasmacytic rhinitis.

DAVID search engine for the SNA and the LPR groups, respectively. From these probes, the DAVID tool identified 1246 and 885 genes (Gene ID) in the SNA and LPR groups, respectively. For each disease, the identified genes were divided on the basis of whether they were up- or down-regulated and the biological annotations for each of the identified genes were mapped by the DAVID tool. For each biological annotation, a statistical corrected *p*-value, the number of genes involved and the enrichment fold were calculated. DAVID mapped the probes into 49 up-regulated and 7 down-regulated biological annotations for SNA, and 12 and 23 up- and down-regulated biological annotations for LPR. The biological annotations related to immune and inflammatory process were selected; only up-regulated annotations were found: 13 for SNA and 2 for LPR (Fig. 1).

Sixteen genes were selected for qRT-PCR analysis based on a combination of their potential role in immunological processes e.g. antifungal immunity, and/or the immunopathogenesis of SNA and LPR, as well as their fold change in gene expression (Fig. 2).

3.2. qRT-PCR validation

The assays for IL-16, IL-17RC, CCL3, CCL4, CCL19, CCL21, CCL28, CXCL10, GZMA, IKZF1, NKG7, STAT1 and STAT4 had

reaction efficiencies in excess of 95% and were able to detect a minimum of one gene copy.

The fold change in gene expression determined by microarray and qRT-PCR were generally in agreement; however, the fold change determined by qRT-PCR was generally greater than that from the microarray (Fig. 2). By qRT-PCR, dogs with SNA had a statistically significant increase in expression of mRNA encoding GZMA, NKG7, CCL4, IFN- γ , IKZF1, CCL3, IL-16, CCL19, STAT4 and CXCL10 and a decrease in expression of CCL21 and CCL28 mRNA, relative to control dogs (Fig. 3). These results confirmed the pattern of expression change seen with the microarray; however, there was no significant difference in expression of mRNA encoding IL-5, STAT1 or IL-17RC, which is in contrast with the microarray results. In dogs with LPR, there was a statistically significant increase in expression of mRNA encoding CCL4 and IL-5 mRNA relative to control dogs, confirming the microarray result; however, no significant difference in expression was found by qRT-PCR for GZMA, NKG7, CXCL10, IFN- γ or IL-17RC (Fig. 3).

When the gene expression determined by qRT-PCR was analyzed separately for each dog, one dog with LPR had a higher expression of GZMA, NKG7, CCL4, IFN- γ and CXCL10 relative to the other dogs in this group. In addition, only

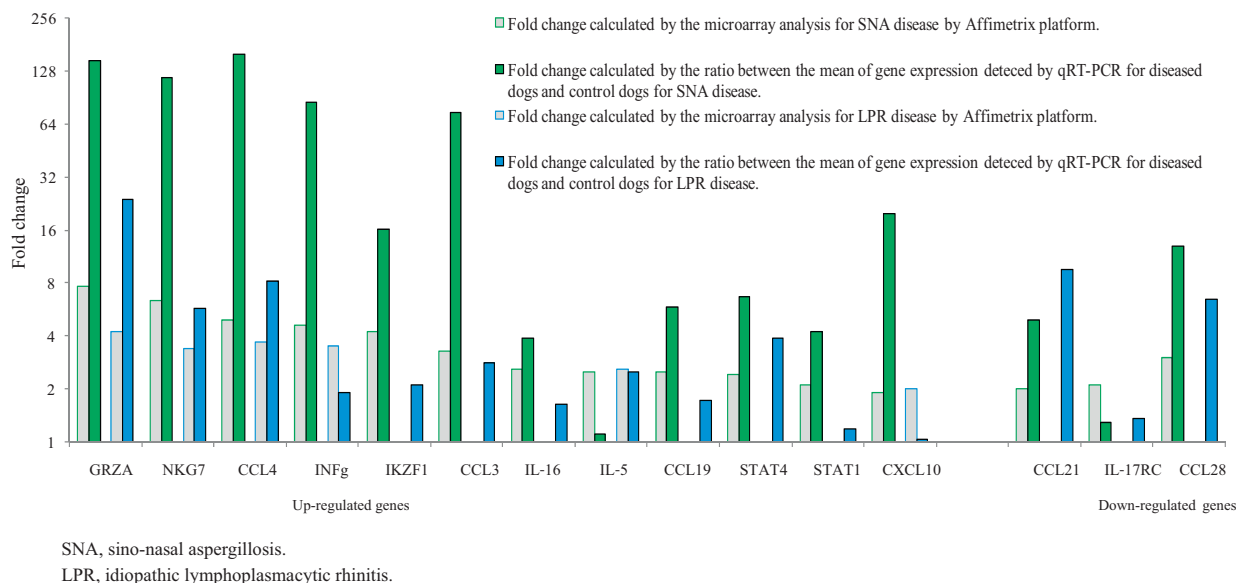


Fig. 2. fold change associated to the selected up- or down regulated genes in nasal mucosa of dogs with sino-nasal aspergillosis or idiopathic lymphoplasmacytic rhinitis. SNA, sino-nasal aspergillosis. LPR, idiopathic lymphoplasmacytic rhinitis.

one dog in the SNA group had measurable expression of IL-5 mRNA.

4. Discussion

This paper reports the first gene expression study using microarray technology in the nasal mucosa of dogs. As an insufficient amount of mRNA was obtained from each individual dog to allow microarray analysis and subsequent qRT-PCR validation, a pool of mRNA was created for each of the groups of dogs for microarray analysis, with a proportion of mRNA from each individual dog kept for subsequent qRT-PCR analyses. Pooled mRNA samples are considered advantageous as they reinforce weak signals, decrease the biological variation and reduce cost through global analysis, as only one microarray chip is used for each group. However, a disproportionate level of expression of a gene in one of the samples in the pool can produce expression artifacts, reinforcing the importance of qRT-PCR confirmation of microarray results (Kendzierski et al., 2003; Zhang et al., 2007).

A significant difference in expression of IL-5 in the nasal mucosa of dogs with SNA as compared to control dogs was revealed by the microarray analysis, but this result was not confirmed by qRT-PCR; in fact, only one dog with SNA had measurable amounts of IL-5 mRNA in the nasal mucosa; this result probably had a disproportionate effect on the pooled sample used for the microarray. This likely represents the range of expression variation among dogs with SNA.

Previous studies (Peeters et al., 2007) have concluded that SNA and LPR involve different patterns of cytokine mRNA expression, and this was confirmed by the present study. The biological annotations over-expressed in SNA included those related to inflammatory and immune processes (e.g. immune response, cytokine

activity, chemokine signaling pathways, lymphocyte activation) whereas over-expressed annotations in LPR included only those involved in inflammatory responses.

The immune response of dogs with SNA has been previously associated with a potent Th1 immune reaction (Peeters et al., 2007). In the present study, a strong increase of mRNA encoding IFN- γ was shown in the nasal mucosa of dogs with SNA. STAT1 and STAT4 play pivotal roles in Th1 differentiation (Kaplan et al., 1996; Szabo et al., 2000; Thierfelder et al., 1996). The results obtained with the array showed an up-regulation of the expression of both transcription factors while a significant up-regulation was demonstrated by qRT-PCR only for STAT4.

In the present study, there was an increased IL-16 expression in the nasal mucosa of dogs with SNA. To our knowledge, the potential role of IL-16 in immunity against fungi has not been reported, and the expression of IL-16 in dogs has not yet been studied. The implication of IL-16 in Th1/Th2 balance has been documented in humans. Interleukin-16 has a chemoattractant activity for several cell types, with preferential effect on the Th1 subset of CD4⁺ T cells (Kaser et al., 1999; Lynch et al., 2003). It has also been suggested that IL-16 is capable of recruiting and expanding the FoxP3⁺ T regulatory cells (McFadden et al., 2007). The local over-expression of IL-16 mRNA in canine SNA may suggest: (1) a beneficial effect of this cytokine through Th1 cell recruitment to the site of infection or (2) a detrimental effect of IL-16 through the recruitment of T regulatory cells producing TGF- β and IL-10. The role of IL-16 in the pathogenesis of canine SNA warrants further investigation.

The local immune response mounted in canine LPR is different from that found in SNA as it is characterized by increased IL-5 mRNA expression without up-regulation of other Th2 cytokines (Peeters et al., 2007). The results of the present study confirmed the increased IL-5 mRNA

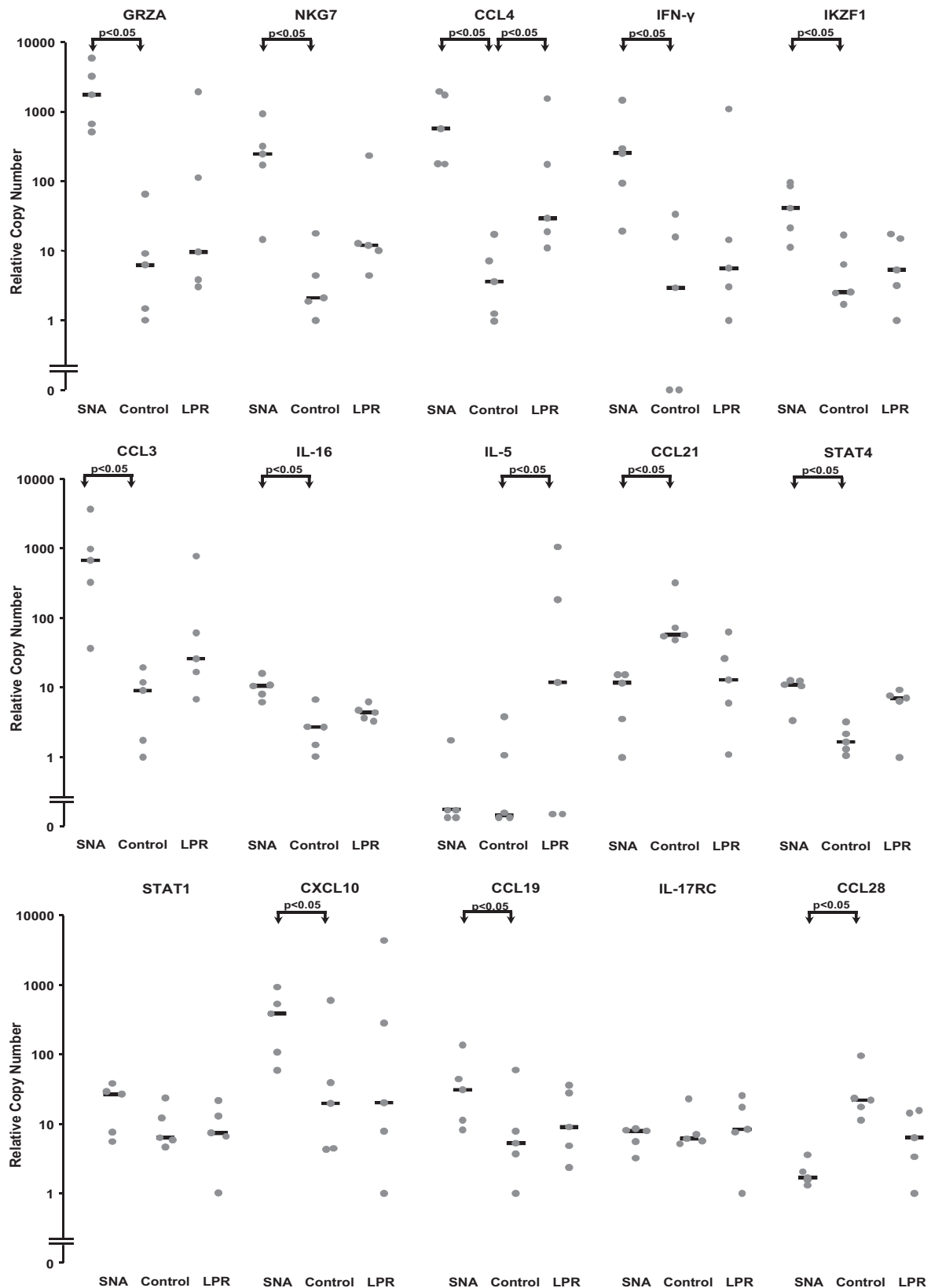


Fig. 3. relative copy numbers obtained by quantitative RT-PCR assays for the fifteen selected genes. The horizontal line corresponds to the median value for that group.

expression; however, the IL-4 and IL-13 probe sets were not called on the microarray.

In the present study, the nasal mucosa from dogs with SNA had significantly increased expression of NKG7, GZMA and IKZF1. In humans, the NKG7 gene is involved in Th cell differentiation and is highly expressed in Th1 polarized cells compared to Th2 cells (Lund et al., 2005; Nagai et al., 2001). GZMA is a cytotoxic serine protease playing a key role in the killing of cells infected by intracellular organisms (Chowdhury and Lieberman, 2008). To our knowledge, no study has been published regarding the potential roles of NKG7 or GZMA in anti-fungal immunity. Nevertheless, the over-expression of these proteins (at the mRNA level) in the nasal mucosa of dogs with SNA suggests their involvement in the pathogenesis of the disease.

IKZF1 is the gene encoding for Ikaros, a zinc finger DNA-binding protein essential for normal lymphocyte development (Wang et al., 1996). This protein has been categorized as an activator of IL-10 and suppressor of IFN- γ in cells in both naïve and differentiated states (Umetsu and Winandy, 2009). The over-expression of IKZF1 mRNA in the nasal mucosa of dogs with SNA could be one of the immune mechanisms triggering the activation of local IL-10 production in this disease.

An increased amount of mRNA encoding CCL3, CCL4, CXCL10 and CCL19 has been found in the nasal mucosa of dogs with SNA as compared to that found in control dogs. In humans, *A. fumigatus* infection results in CCL3, CCL4 and CXCL10 production which induces the migration of effector memory Th1 cells (Gafa et al., 2007). The role of these chemokines in the pathogenesis of canine SNA warrants further investigation.

5. Conclusion

The present study has highlighted potential new avenues of research into understanding the pathogenesis of canine SNA. SNA has been previously associated with a Th1 immune response biased by an over production of IL-10. The results presented here support that hypothesis and indicate that several molecules may be involved in this process.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

MV processed the biopsies, analyzed and interpreted the microarray study, conceived and carried out the real-time PCR analysis and drafted the manuscript. FB contributed to the conception and the design of the study and participated to the analysis and the interpretation of the data. IRP contributed to the conceived of real-time assay, the interpretation of the data and revisited actively the material and method section. MJD contributed to the conception and the design of the study. CC carried out the rhinoscopies and took the biopsies. DP conceived the study, participated to the analysis and the interpretation of

the data and implicated in revising the manuscript. All the authors read and approved the final manuscript.

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