RESEARCH ARTICLE

Variants in the interleukin-1 alpha and beta genes, and the risk for periodontal disease in dogs

C. ALBUQUERQUE[1,2,*], F. MORINHA[1,3], J. MAGALHÃES[1], J. REQUICHA[2], I. DIAS[2,3,4], H. GUEDES-PINTO[1], E. BASTOS[1] and C. VIEGAS[2,3,4]

[1]*Department of Genetics and Biotechnology,* [2]*Department of Veterinary Sciences, and* [3]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes e Alto Douro (UTAD), Quinta de Prados, P. O. Box 1013, 5000-801 Vila Real, Portugal*

[4]*3B's Research Group–Biomaterials, Biodegradables and Biomimetics, ICVS/3B's–PT Government Associate Laboratory, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Zona Industrial da Gandra, 4805-017 Barco GMR, Portugal*

Abstract

Elevated levels of interleukin-1 (IL-1) have been shown to amplify the inflammatory response against periodontopathogenic bacteria. In humans, polymorphisms in the *IL1A* and *IL1B* genes are the most well-studied genetic polymorphisms associated with periodontal disease (PD). In contrast to human, there is a lack of knowledge on the genetic basis of canine PD. A case–control study was conducted in which a molecular analysis of dog *IL1A* and *IL1B* genes was performed. Of the eight genetic variants identified, seven in *IL1A* gene and one in *IL1B* gene, *IL1A*/1_g.388A>C and *IL1A*/1_g.521T>A showed statistically significant differences between groups (adjusted OR (95% CI): 0.15 (0.03–0.76), $P = 0.022$; 5.76 (1.03–32.1), $P = 0.046$, respectively). It suggests that in the studied population the *IL1A*/1_g.388C allele is associated with a decreased PD risk, whereas the *IL1A*/1_g.521A allele can confer an increased risk. Additionally, the *IL1A*/2_g.515G>T variation resulted in a change of amino acid, i.e. glycine to valine. *In silico* analysis suggests that this change can alter protein structure and function, predicting it to be deleterious or damaging. This work suggests that *IL1* genetic variants may be important in PD susceptibility in canines.

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Introduction

Periodontal disease (PD) is a highly prevalent infection-induced inflammatory disease that affects both dogs and humans, resulting in the destruction of alveolar bone and other tooth-supporting tissues (Albuquerque *et al.* 2012). Gingivitis is the earliest and reversible stage of PD that, if not treated properly, may lead to a more destructive disease stage called periodontitis in susceptible individuals (Pihlstrom *et al.* 2005). Besides local periodontium destruction, PD has several systemic health implications, being associated with various systemic diseases, especially cardiovascular disease and diabetes in humans (Cullinan and Seymour 2013), and renal, hepatic and cardiac disorders in dogs (Pavlica *et al.* 2008).

PD is a complex and multifactorial disease in which genetic and environmental risk factors are essential to determine the individual susceptibility and to explain the marked clinical variability (Stabholz *et al.* 2010). Bacterial biofilms play an essential role in the oetiology of PD, but persistent host inflammatory response against the bacterial aggression is more responsible for the tissue destruction (Genco 1992; Liu *et al.* 2010). Thus, the emphasis must be placed on the interaction between periodontopathogenic bacteria and the host's immune response (Amano 2010).

The interleukin-1 (IL-1) cytokine family plays a key regulator role in acute and chronic inflammation, presenting pleiotropic effects being involved in the pathogenesis of many infectious, autoimmune and inflammatory diseases (Dinarello 2011; Karimbux *et al.* 2012). IL-1α and IL-1β are the two principal forms of IL-1 with agonist proinflammatory activity, and their increased levels have been associated

*For correspondence. E-mail: cmralb@hotmail.com.

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with periodontitis severity (Masada *et al.* 1990; Preiss and Meyle 1994). These mediators lead to amplification of the inflammatory response, the induction of adhesion molecules and enzymes that degrade extracellular matrix, osteoclastic bone resorption and can inhibit osteoblast differentiation (Shirodaria *et al.* 2000; Graves and Cochran 2003). The treatment of PD leads to the reduction of IL-1 levels, and the therapeutic value of IL-1 inhibitors was also demonstrated (Shirodaria *et al.* 2000; Delima *et al.* 2002).

In humans, several studies have shown the importance of genetic polymorphisms to the periodontitis susceptibility and severity, mainly in genes implied in the regulation of inflammatory and immune responses (Kinane *et al.* 2005; Yoshie *et al.* 2007). Periodontitis in adults was estimated to have ~50% heritability (Michalowicz *et al.* 2000). The host response to periodontopathogenic bacteria is strongly influenced by genetic variants at multiple loci acting synergistically (Yoshie *et al.* 2007). In this context, polymorphisms in the genes coding for IL-1α and IL-1β (*IL1A* and *IL1B*) are the most well studied in PD (Shirodaria *et al.* 2000; Taylor *et al.* 2004). The first study was reported by Kornman *et al.* (1997) who found a 'composite' *IL1* genotype as a strong indicator of susceptibility to severe periodontitis. This composite genotype means at least one copy of the rare allele at both *IL1A* (−889) and *IL1B* (+3953) loci. A third locus, *IL1A* (+4845), is in complete linkage disequilibrium with *IL1A* (−889), and most recent studies have genotyped this locus and not *IL1A* (–889) because it is technically easier (Cullinan *et al*. 2001; Papapanou *et al*. 2001). Elevated levels of IL-1α and IL-1β in the gingival crevicular fluid are found in periodontitis patients positive for the 'composite' genotype (Engebretson *et al*. 1999; Shirodaria *et al*. 2000). This overproduction of IL-1 results in a higher inflammatory response to bacterial stimulus resulting in more severe disease presentation as well as an unfavourable response to treatments (Kornman *et al.* 1997; McGuire and Nunn 1999). A systemic hyperinflammatory state shown by increased levels of C-reactive protein is also associated with this 'composite' genotype (Berger *et al*. 2002).

More than 125 studies have evaluated the association between *IL1* genetic variants and PD in humans (Karimbux *et al.* 2012). The results are heterogeneous but recent systematic reviews and metaanalysis reinforce that *IL1A* and *IL1B* genetic polymorphisms are significantly associated with PD (Nikolopoulos *et al.* 2008; Karimbux *et al.* 2012). Additionally, variations in these genes are also correlated with other diseases, including osteoarthritis (Moxley *et al.* 2010), inflammatory bowel disease (Bioque *et al.* 1995), atherosclerotic cardiovascular disease (Brown *et al.* 2010), Alzheimer's disease (Nicoll *et al.* 2000) and gastric cancer (El-Omar *et al.* 2000).

In veterinary medicine, the specific role of IL-1 in the pathogenesis of inflammatory diseases has not been extensively studied, but increasing evidence shows that the involvement of the IL-1 in dog and human diseases is quite similar (Gibson *et al.* 2004). Additionally, in contrast to human PD, the genomic basis of dog PD has been understudied to date, and only now the first investigations are emerging (Morinha *et al.* 2011, 2012; Albuquerque *et al.* 2014). In this context, we hypothesized that in canine PD, similar to human PD, a dysregulated IL-1 production resulting from genetic variation can be part of the explanation of the differences in disease susceptibility between individuals. A case–control study was delineated in which a molecular analysis of dog *IL1A* and *IL1B* genes was performed to identify genetic variations and evaluate its possible association with PD.

Material and methods

Study population

Forty dogs with PD and 50 healthy dogs were included in this case–control study. The owners of all participant dogs gave their informed consent, and all dogs were submitted to a general clinical examination assessing the individual health condition. Following all the animal welfare guidelines, the dogs were sedated by an intramuscular administration of butorphanol (Torbugesic 1%; Fort Dodge, The Netherlands) and acepromazine (Vetranquil; CEVA Sante Animal, France). Anaesthesia was performed by an intravenous administration of diazepam (Diazepam MG; Labesfal, Portugal), ketamine (Imalgene 1000; Merial, France) and propofol (Lipuro 2%; Braun, Germany) balanced anaesthesia and was maintained using isoflurane (IsoFlo; Abbott Animal Health, USA) administered in oxygen through an endotracheal tube. The clinical assessment included a systematic odonto-stomatological examination to determine the presence or absence of PD and classify the correct PD stage, following the American Veterinary Dental College recommendations (AVDC 2009, AVDC nomenclature committee, Recommendations adopted by the AVDC board, Periodontal disease classification, http://www.avdc.org/nomenclature.html#periostages, accessed 15 April 2014).

During the selection process, the criteria included mesocephalic dogs with similar diet patterns (mix of home-prepared rations and commercial pet foods). Selected animals did not have any history of previous dental treatments or preventive measures (e.g. tooth brushing, dental diets). All dogs included were unrelated/unfamiliar individuals, mixed-breed with body weight ranging from 9 to 18 kg and aged between 2 and 8 years. A more detailed characterization of the population is presented in table 1.

Sample collection and DNA isolation

Blood samples were collected from each dog into EDTA tubes and genomic DNA was isolated from buffy coats obtained after centrifugation at 2500 rpm for 15 min at room temperature. DNA extraction was performed according to the Fujifilm QG-Mini 80 equipment following the instructions of

Table 1. Main characteristics of the population under study.

PD stage	Frequency, *n* (%)	Age (years)	Weight (kg)	Sex (males/females)
PD0 (healthy periodontium)	50 (55.6)	3.9 (2–5)	12.2	19/31
PD1 (gingivitis)	5 (5.6)	3.4 (2–4)	11.7	2/3
PD2 (early periodontitis)	15 (16.6)	4.1 (3–6)	10.5	6/9
PD3 (moderate periodontitis)	11 (12.2)	4.2 (3–8)	11.3	5/6
PD4 (advanced periodontitis)	9 (10.0)	5.4 (4–8)	11.2	4/5
Control group	50 (55.6)	3.9 (2–5)	12.2	19/31
Cases group	40 (44.4)	4.3 (2–8)	11.1	17/23

QuickGene DNA whole blood kit S (DB-S) (Fujifilm, Tokyo, Japan). An initial volume of 250 μL was used and a final volume of 200 μL elution buffer (CDT) was obtained. The integrity of the genomic DNA was verified by 1% agarose gel electrophoresis. The NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to determine the concentration and quality of the DNA.

PCR conditions and genotyping

For *IL1A* gene, two fragments were studied: fragment 1 (includes exon 2 and partial intron 1) was obtained with the forward primer 5′-TCTTGTCTGTGCCTGGTTTG-3′ and the reverse primer 5′-TCTGGCTCCATGCTAGTTACTG-3′; and fragment 2 (includes exon 5) was obtained with the forward primer 5′-TCCTCAGTAACAAATATAAGTC-3′ and the reverse primer 5′-TACAAGTTGAAGGGTCTT-3′. For *IL1B* gene one single fragment was analysed (includes exons 4 and 5) and it was obtained with the forward primer 5′-CCCTTCAGCCTCTCTGATGT-3′ and the reverse primer 5′-TCCATAATTCGCCTTATTGTTC-3′.

These target regions were defined according to data available in genome browsers for human and dog *IL1A* and *IL1B* genes. Two of the three studied fragments correspond to the homologous regions that contain polymorphisms associated with PD in human genes (*IL1A* (+4845) and *IL1B* (+3953)). Additionally, for *IL1A* gene, the intron 1 and exon 2 were selected because in this gene region a genetic variation was already identified in dog and corresponds to the homologous sequence of human *IL1A* gene which contains polymorphisms related with other inflammatory diseases, namely ankylosing spondylitis (Maksymowych *et al.* 2006) and rheumatoid arthritis (Johnsen *et al.* 2008).

All the PCR amplifications were optimized by using the DreamTaq PCR Master Mix kit (Fermentas, Amherst, USA) in a reaction mixture containing 12.5 μL of DreamTaq PCR Master Mix (2×), 1 μL of each primer (16 pmol), 1 μL of genomic DNA (100 ng) and 9.5 μL of H_2O. For all the fragments, the amplification reaction was performed with a initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. All amplified fragments were purified with ExoSAP-IT (USB Corporation, Cleveland, USA) following the manufacturer's protocol, and subsequently bidirectionally sequenced at Stab Vida (Lisbon, Portugal). Sequences were aligned with ClustalW2 tool, edited and analysed with GeneDoc software (ver. 2.6.003).

Statistical analysis

The genotype and allele frequencies for each genetic variation were calculated by direct counting. The genotype frequencies of both groups were evaluated for deviation from Hardy–Weinberg equilibrium (HWE). Chi-square test (χ^2) or Fisher's exact test were used to analyse the distribution of genotype and allele frequencies between groups. All *P*-values were evaluated in a two-sided model, and a *P*-value of <0.05 was accepted as statistically significant. To increase statistical power of the less frequent variant, the rare homozygotes were combined with the heterozygotes assuming a dominant effect. Odds ratios (OR) and 95% confidence interval (CI) were estimated. Adjusted ORs (controlling for age, weight and gender variables) were calculated with a logistic regression model. The association between gene variants and sequential PD stages was tested by trend χ^2 test (linear by linear association). Statistical analysis was performed using the software SPSS (Statistical Package for Social Sciences) ver. 17.0. RunGC programe (Curtis *et al.* 2006) was used to estimate haplotype frequencies in both cases and controls, and to test for significant differences between groups using a likelihood-ratio test.

Results

The amplified fragments of the two genes were sequenced and genotyped (GeneBank ID: KF477322 and KF477323). A total of eight genetic variations were identified, seven in *IL1A* gene and one in *IL1B* gene (table 2). For *IL1A* gene (figure 1), we analysed two fragments, namely, fragment 1 (*IL1A/1*) includes exon 2 and partial intron 1, and fragment 2 (*IL1A/2*) includes exon 5. For *IL1B* gene (figure 2), we analysed one fragment which includes exons 4 and 5. The distribution of the genotypes for all the identified genetic variations satisfied HWE in both groups. The distribution and analysis of genotypes and allele frequencies are summarized in table 3.

Table 2. Detected single nucleotide variations in *IL1A* and *IL1B* genes.

Genetic variation	Location	Codon	Position	Amino acid
IL1A				
IL1A/1_g.110A>G	Intron 1	–	–	–
IL1A/1_g.113C>A	Intron 1	–	–	–
IL1A/1_g.129G>A	Intron 1	–	–	–
IL1A/1_g.388A>C	Intron 1	–	–	–
IL1A/1_g.521T>A	Intron 1	–	–	–
IL1A/2_g.153T>A	Intron 4	–	–	–
IL1A/2_g.515G>T	Exon 5	GGA/GTA	146	Gly-G/Val-V
IL1B				
*IL1B*_g.525G>A	Exon 5	GGG/GGA	146	Gly-G/Gly-G

For *IL1A* gene, the analysis of fragment 1 (*IL1A/1*) allowed the identification of five single-nucleotide variations, all located in intron 1, among which one was already described (dbSNP ID: rs22530526) and the other four are new genetic variations. A transversion of cytosine to adenine (*IL1A*/1_g.113C>A) was identified only in one dog (2.5%) of the PD group; whereas a transition of guanine to adenine (*IL1A*/1_g.129G>A) was found in two dogs (4.0%) of the control group. These two variations were not included in the statistical analysis due to its low frequencies. A transition of adenine to guanine (*IL1A*/1_g.110A>G) was found in both groups (PD dogs group with 2.5% AG versus control group with 12% AG); as well as a tranversion of thymine to adenine (*IL1A*/1_g.521T>A) which appeared in 12.5% of individuals in the PD dogs group and in 4% of dogs in the control group. For these two variations, the unadjusted OR analysis revealed no statistically significant differences among groups. However, after adjusting for confounding variables, the OR of the *IL1A*/1_g.521T>A allelic comparison was statistically significant (OR: 5.76 (95% CI: 1.03–32.1), $P = 0.046$). Finally, a transversion of adenine to cytosine (*IL1A*/1_g.388A>C) was found in 5% of individuals in the PD dogs group and in 20% of dogs in the control group, with two homozygous individuals for the rare allele. The statistical analysis of this variation revealed significant differences in the allelic frequencies between groups. The unadjusted and adjusted OR values confirm the significant differences in allele frequencies between groups (unadjusted OR: 0.19 (95% CI: 0.04–0.87), $P = 0.018$; adjusted OR: 0.15 (95% CI: 0.03–0.76), $P = 0.022$). This result shows that C allele is more frequent in the control group, meaning that in the studied population the C allele may contribute to the decreased risk of PD.

Considering fragment 2 of *IL1A* gene (*IL1A/2*), we identified two single-nucleotide variations, one already described (dbSNP ID: rs22530525) in intron 4, and a new variation in exon 5. A transversion of thymine to adenine (*IL1A*/2_g.153T>A) was found in both groups (PD dogs group with 5% TA versus control group with 10% TA), but

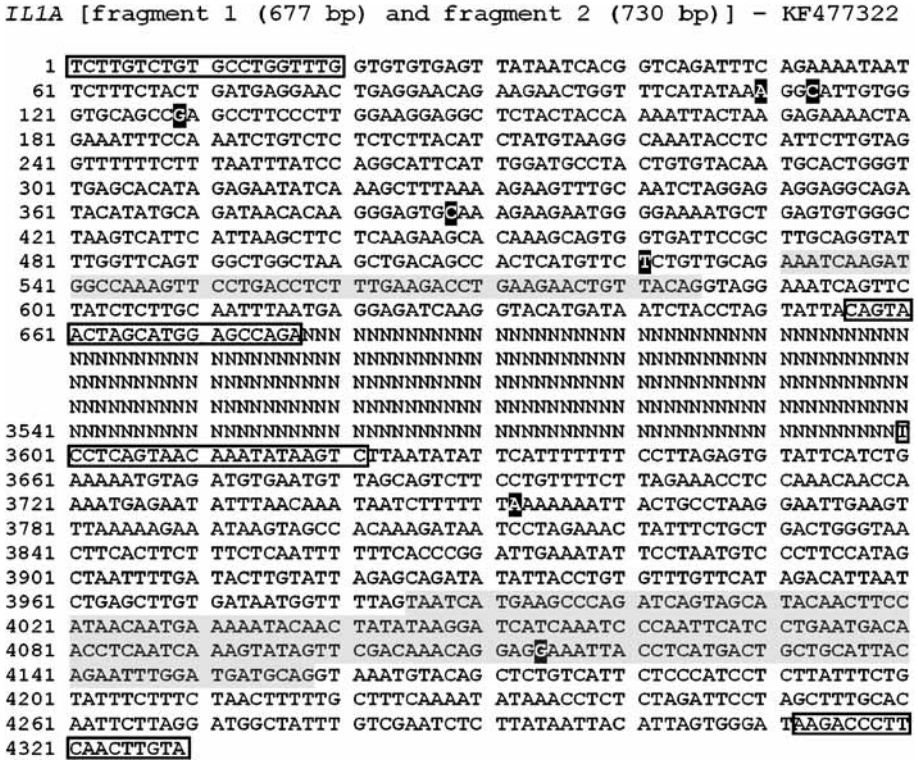

Figure 1. Nucleotide sequence of the *IL1A* gene fragments analysed in this study. Primers are in unshaded boxes, exons 2 and 5 are in gray-shaded boxes and genetic variations are highlighted by black-shaded boxes. The gap separating the two analysed fragments is represented by NNNs.

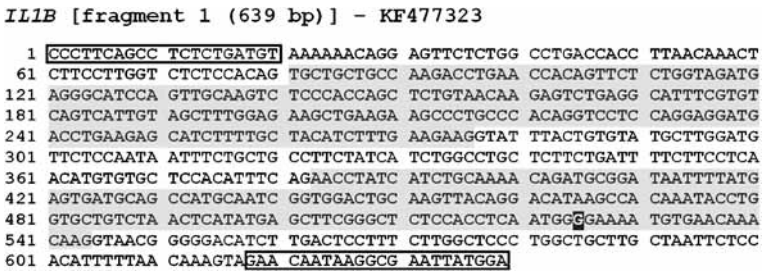

Figure 2. Nucleotide sequence of the *IL1B* gene fragment analysed in this study. Primers are in unshaded boxes, exon 5 is in a gray-shaded box and genetic variation is highlighted by one black-shaded box.

without statistical significant differences. A transversion of guanine to thymine (*IL1A*/2_g.515G>T) located in exon 5 was identified only in one dog (2%) of the control group. This genetic variation results in a change of amino acid, glycine (Gly-G) to valine (Val-V).

For *IL1B* gene, we identified a transition of guanine to adenine (*IL1B*_g.525G>A) located in exon 5. This single-nucleotide variation was found in 5% of individuals in the PD dogs group and in 2% of dogs in the control group. No statistically significant differences were found between groups, prior and after adjustment for age, weight and gender variables. Additionally, this genetic variation does not change the encoded amino acid (glycine (Gly-G) in both cases).

The haplotype combinations between identified *IL1* variations were analysed, giving estimated frequencies for controls and PD cases. This analysis showed the discrimination of 11 haplotypes. Four haplotypes were present only in the control group, among which the highest Likelihood-Ratio test (LRT) value was obtained for the haplotype ACAATTGG. The haplotype AAGATTGG was present only

Table 3. Genotype frequencies and allele frequencies of the *IL1A* and *IL1B* genes variations.

Genetic variation	Genotype/allele	PD dog *n* (%)	Control *n* (%)	OR (95% CI)	*P* value*	Adjusted OR† (95% CI)	*P* value*
IL1A/1_g.110A>G	A/A	39 (97.5)	44 (88.0)				
	A/G	1 (2.5)	6 (12.0)				
	G/G	–	–				
	A/G + G/G	1 (2.5)	6 (12.0)	0.19 (0.02–1.63)	0.127	0.13 (0.01–1.38)	0.091
	A	79 (98.8)	94 (94.0)				
	G	1 (1.2)	6 (6.0)	0.20 (0.02–1.68)	0.101	0.15 (0.02–1.42)	0.098
IL1A/1_g.388A>C	A/A	38 (95.0)	40 (80.0)				
	A/C	2 (5.0)	8 (16.0)				
	C/C	–	2 (4.0)				
	A/C + C/C	2 (5.0)	10 (20.0)	0.21 (0.04–1.02)	0.059	0.20 (0.04–1.09)	0.063
	A	78 (97.5)	88 (88.0)				
	C	2 (2.5)	12 (12.0)	0.19 (0.04–0.87)	0.018	0.15 (0.03–0.76)	0.022
IL1A/1_g.521T>A	T/T	35 (87.5)	48 (96.0)				
	T/A	4 (10.0)	2 (4.0)				
	A/A	1 (2.5)	–				
	T/A + A/A	5 (12.5)	2 (4.0)	3.43 (0.63–18.7)	0.235	4.85 (0.77–30.2)	0.091
	T	74 (92.5)	98 (98.0)				
	A	6 (7.5)	2 (2.0)	3.97 (0.78–20.2)	0.075	5.76 (1.03–32.1)	0.046
IL1A/2_g.153T>A	T/T	38 (95.0)	45 (90.0)				
	T/A	2 (5.0)	5 (10.0)				
	A/A	–	–				
	T/A + A/A	2 (5.0)	5 (10.0)	0.47 (0.09–2.58)	0.456	0.38 (0.06–2.22)	0.281
	T	78 (97.5)	95 (95.0)				
	A	2 (2.5)	5 (5.0)	0.49 (0.09–2.58)	0.389	0.40 (0.07–2.23)	0.293
IL1B/1_g.525G>A	G/G	38 (95.0)	49 (98.0)				
	G/A	2 (5.0)	1 (2.0)				
	A/A	–	–				
	G/A + A/A	2 (5.0)	1 (2.0)	2.58 (0.23–29.5)	0.583	1.44 (0.12–17.6)	0.777
	G	78 (97.5)	99 (99.0)				
	A	2 (2.5)	1 (1.0)	2.54 (0.23–28.5)	0.435	1.42 (0.12–16.6)	0.782

*Chi-square test or Fisher's two-tailed exact test, when applicable.
†OR adjusted for age, weight and gender variables.

Table 4. *IL1* haplotype estimated frequencies (analysed by RunGC software) in controls ($n = 50$) and PD cases ($n = 40$).

Allele combination								Estimated frequencies		LRT mean
IL1A/1_g. 110A>G	*IL1A*/1_g. 113C>A	*IL1A*/1_g. 129G>A	*IL1A*/1_g. 388A>C	*IL1A*/1_g. 521T>A	*IL1A*/2_g. 153T>A	*IL1A*/2_g. 515G>T	*IL1B*_g. 525G>A	Control	PD case	PD cases versus controls
A	C	G	A	T	T	G	G	0.7377	0.8250	0.38
A	C	G	C	T	T	G	G	0.0923	0.0250	3.13
A	C	G	A	A	T	G	G	0.0200	0.0625	1.81
A	A	G	A	T	T	G	G	0.0000	0.0250	2.81
A	C	A	A	T	T	G	G	0.0200	0.0000	2.06
G	C	G	C	T	T	G	G	0.0177	0.0000	1.83

in the PD dogs group. The haplotype ACGCTTGG was detected in both groups and also presented a high LRT test value. The six haplotypes with higher frequency in our population are shown in table 4.

Discussion

In the present study, a candidate gene approach was followed with a selection of genes taking into account previous association of specific polymorphisms with PD in humans. This kind of study design is currently used in other diseases shared by dogs and humans, such as rheumatoid arthritis (Ollier *et al.* 2001; Clements *et al.* 2010) and diabetes mellitus (Short *et al.* 2007). Moreover, the structure and organization of *IL1A* and *IL1B* genes are highly conserved among humans and dogs, as occurs with the structure of the respective proteins (Soller *et al.* 2007). This homology supports the availability of knowledge transfer between species, since the essential genetic and protein properties are comparable. In this context, the locations of the polymorphisms associated with PD in human genes were identified (*IL1A* (+4845) and *IL1B* (+3953)), as well as other important polymorphisms and the homologous regions of the dog genes were studied with identification of eight single-nucleotide variations.

For the *IL1A*/1_g.388A>C variation, the results of this study showed statistically significant differences between groups in allele frequencies (A versus C). The results suggest that in the studied population, dogs carrying the C allele may have a slight decrease in PD susceptibility (unadjusted OR: 0.19 (95% CI: 0.04–0.87), $P = 0.018$; adjusted OR: 0.15 (95% CI: 0.03–0.76), $P = 0.022$). For the *IL1A*/1_g.521T>A variation, although no statistically significant differences on the unadjusted analysis, the adjusted OR calculation showed significant differences in allele frequencies (OR: 5.76 (95% CI: 1.03–32.1), $P = 0.046$), meaning that in this population, the A allele may results in a slight increase in PD susceptibility.

The detection of genetic variations in the dog *IL1A* gene potentially associated with PD susceptibility is consistent with what is described in humans, since the great number of reports confirms *IL1A* polymorphisms as putative risk factors in PD, mainly influencing the IL-1 production (Nikolopoulos *et al*. 2008; Karimbux *et al*. 2012). The location of these variations in an intronic region does not reduce its importance, because introns contain a multiplicity of functional elements, including intron splice enhancers and silencers that regulate alternative splicing, *trans*-splicing elements and other regulatory elements. In addition to pathological mutations, introns also contain functional polymorphisms that can influence the gene expression (Cooper 2010). Regulation of IL-1 production is a critical point of immune response mainly because, although IL-1 is essential in response to infection, an inappropriate production can lead to damaging effects on the host (Kornman and di Giovine 1998; Taylor *et al.* 2004). Elevated IL-1 levels were described in the gingival crevicular fluid of diseased teeth compared with periodontally healthy individuals (Shirodaria *et al.* 2000). This overproduction of IL-1 leads to an enhanced reaction of the host to periodontal pathogens, inducing a higher severity of PD (Graves and Cochran 2003). Asthma, congestive heart failure, arthritis, septic shock and preterm labour are some examples of other conditions which pathogenesis includes alterations in IL-1 levels (Graves and Cochran 2003).

Although much less studied than in the human species, similar involvement of IL-1 in the pathogenesis of canine inflammatory diseases has also been described (Gibson *et al.* 2004). IL-1 has been identified as a marker of joint inflammation associated with osteoarthritis in both humans and dogs (Carter *et al.* 1999; Punzi *et al.* 2002; Maccoux *et al.* 2007), as well as in inflammatory bowel disease (Casini-Raggi *et al*. 1995; Maeda *et al*. 2012). Considering the major role of cytokines in the pathogenesis of human and canine malignant histiocytosis, various genetic variations in the coding sequences which led to missense mutations within the protein sequences of IL-1α and IL-1β have already been reported (Soller *et al.* 2006).

Two genetic variations identified are located in exons (*IL1A*/2_g.515G>T and *IL1B*_g.525G>A), which correspond exactly to the same exons where the polymorphisms associated with PD are located (*IL1A* (+4845) G>T and *IL1B* (+3954) C>T) in the human *IL1A* and *IL1B* genes (Karimbux *et al.* 2012). The *IL1B*_g.525G>A genetic variation was found in both groups, without statistically significant differences. Further, the encoded amino acid, glycine (Gly-G) does not change with this variation. The *IL1A*/2_g.515G>T genetic variation was found in low frequency in our population, being identified in only one dog

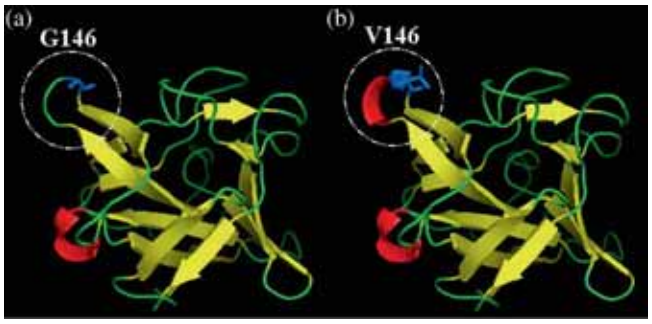

Figure 3. The predicted tertiary structures of IL-1α protein, evidencing the G146V amino acid variation. (a) IL-1α protein with glycine at position 146. (b) IL-1α protein with valine at position 146. The dissimilitude between the two proteins is enclosed in circles with broken lines, and the amino acid interchange (blue) seems to alter the protein structure converting a coil region (green) in a helical region (red).

of the control group. Nevertheless, this variation alters the encoded amino acid (glycine (Gly-G) to valine (Val-V)). The amino acid change may alter protein structure and function, and to predict the possible impact of this variation we used different computational tools, namely PROVEAN, PolyPhen-2 and SIFT (Kumar *et al.* 2009; Adzhubei *et al.* 2010; Choi *et al.* 2012). All the results agreed that this amino acid change is predicted to be deleterious or probably damaging. Using the classic Grantham matrix, which quantify the chemical distance between amino acids, an exchange Gly→Val represents a Grantham score difference of 109 which means a significant chemical dissimilarity (moderately radical change) (Grantham 1974). Recurring to the SWISS-MODEL, an automated protein modelling server (Arnold *et al*. 2006), models of the IL-1α protein; with and without the amino acid exchange were constructed (figure 3). In the predicted model, the change of amino acid Gly-G to Val-V seems to modify the protein structure converting a coil region in a helical region. This alteration in the protein structure may be important for its functionality and interactions with other proteins. However, functional studies are needed to address the possible role of these genetic variations in the PD pathogenesis.

The other four genetic variations (*IL1A*/1_g.110A>G, *IL1A*/1_g.113C>A, *IL1A*/1_g.129G>A and *IL1A*/2_g.153T >A) are all located in intronic regions and did not present statistically significant differences in this case–control study.

Comparing our data with nucleotide sequences of *IL1A* canine gene displayed in NCBI (GeneBank ID: NM_001003157) and Ensembl (ENSCAFG00000007245) genome browsers, we found that the displayed sequences present in the allele C at position of the variation *IL1A*/1_g.388A>C and in allele A at position of the variation *IL1A*/2_g.153T>A. In both cases this corresponds to the less frequent allele in the population under study.

In human periodontology, the *IL1* composite genotype or the *IL1* gene cluster polymorphism are more relevant than each individual polymorphism. In our study, the haplotype analysis revealed several combinations, but we can highlight three haplotypes (ACAATTGG, ACGCTTGG and AAGATTGG) which present higher LRT values. The first two haplotypes may confer a protective action against PD, whereas the last one may increase the PD risk. However, these findings should be seen with prudence because the low frequencies of some haplotypes in our population may influence the asymptotic distribution for LRT (Curtis *et al.* 2006).

Finally, to evidence IL-1α and IL-1β interactions and associations with other proteins, we used the STRING database (Szklarczyk *et al.* 2011). IL-1α and IL-1β are functionally closely linked inflammatory mediators, assuming a central role in multiple interactions and regulatory networks (figure 4).

The relevance of this study arises from the fact that canine PD, besides its huge importance in veterinary practice, always represented an undoubted model contributing for the achievements of the actual knowledge in periodontology. The canine and human PD share most of the oetiological, clinical and pathological characteristics (Albuquerque *et al.* 2012). Additionally, dogs are also recognized as a powerful model to study and understand the genetics background of various complex diseases homologous between the two species (Derrien *et al.* 2012). The present study is a contribution to the characterization of the genetic basis of canine PD,

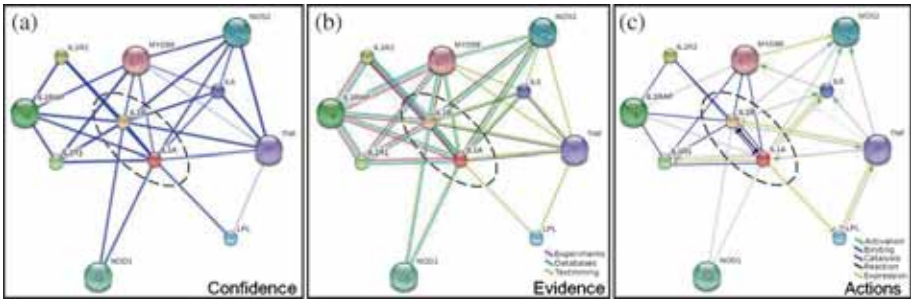

Figure 4. Interactions of the canine IL-1α and IL-1β proteins, according to STRING database predictions. (a) Confidence view (stronger associations are represented by thicker lines). (b) Evidence view (types of evidence for the association). (c) Actions view (different modes of action). IL-1α and IL-1β proteins are highlighted by dotted lines.

mainly considering that PD is a complex disease whose susceptibility results from the interplay between various genetic variations and other risk factors. Further studies are mandatory to clarify the biological relevance of our outcomes; as well as other investigations with different candidate genes. But, it is undeniable that advances in this area are fundamental to improve the clinical management of PD, particularly with the development of novel strategies of risk assessment.

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