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Gingival Toll-like receptor and cytokine mRNA levels in equine periodontitis and oral health

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Summary

Reasons for performing study: Equine periodontitis is a common and painful condition. Despite this, the disease often goes unnoticed by owners and is thus a major welfare concern. The aetiopathogenesis of the condition remains poorly understood with few recent studies performed. The innate immune system is known to play an important role in human periodontitis, but its role in equine periodontitis has not been examined.

Objectives: To quantify the mRNA levels of Toll-like receptors (TLRs) and cytokines in gingival tissue from orally healthy horses and those affected by periodontitis.

Study design: Observational study.

Methods: Gingival tissue samples were taken *post mortem* from 13 horses with no clinical signs of oral disease and 20 horses with periodontitis. mRNA levels of TLR2, TLR4 and TLR9 and cytokines IL-1β, TNFα, IL-4, IL-6, IL-10, IL-12, IL-17 and IFN-ɣ were determined using quantitative real-time PCR. Statistical significance of results was assessed using appropriate t-tests.

Results: mRNA levels of all TLRs and cytokines were up-regulated in equine periodontitis. Significant increases in mRNA levels of TLR2, TLR9, IL-4, IL-10, IL-12 ($p \leq 0.05$) and IFN-ɣ ($p \leq 0.01$) were observed for both un-weighted and age-weighted analyses of diseased gingival tissue samples compared to healthy gingival samples. When comparing periodontitis lesions to healthy gingival control samples from the same horse, significant increases in mRNA levels of TLR4, TLR9, IL-10, IFN-ɣ ($p \leq 0.05$), TLR2, IL-1β and IL-12p35 ($p \leq 0.01$) were observed.

Conclusions: This study has provided an initial insight into the involvement of the immune system in equine periodontitis. Increased mRNA levels of TLR2, TLR4 and TLR9 indicate substantial microbial challenge in diseased gingival tissue. A mixed Th1/Th2/Th17 cytokine response is produced in equine periodontitis. Further studies are required to more fully characterise the role of the innate immune system in this disease.

Introduction

Periodontal disease (gingivitis and periodontitis) is an important and common oral disease of the horse which causes significant pain and distress to affected animals [1]. As in humans, gingivitis is reversible and causes bleeding and inflammation of the gingivae. Periodontitis is characterised by inflammation which damages the periodontal ligament and alveolar bone surrounding the teeth, potentially resulting in tooth loss in affected horses. The condition is common and prevalence increases with advancing age. Almost 60% of horses over the age of 15 years [2] and 75% of horses aged 30 years and older are affected [3]. Gingival inflammation with formation of periodontal pockets usually impacted with feed are commonly found upon clinical dental examination. The most frequently reported clinical sign is quidding with eventual weight loss [4,5]. However, the disease often goes undetected by horse owners, making it a disease of significant welfare concern.

The innate and adaptive immune systems play an important role in the pathogenesis of periodontal disease and periodontitis has been extensively studied in humans. Several pathogens have been identified and the host response to infection, particularly the role of neutrophils, B cells and T cell subsets (e.g. Th1, Th2 and Th17) and their products, has been investigated in detail [6]. This response can be destructive to the host [7], resulting in loss of support around the tooth in deep periodontal pockets that support the growth of anaerobic bacteria, further exacerbating inflammatory responses, tissue damage and tooth loss. While microbes associated with equine periodontitis can be identified by culture or molecular methods, their role in periodontitis remains to be confirmed. The extent to which host responses are involved in the aetiopathogenesis of equine periodontitis also remains to be elucidated. Host immune cells recognise pathogen-associated molecular patterns via specific pattern recognition receptors, including Toll-like receptors (TLRs). Bacterial and viral classes produce distinct pathogen-associated molecular patterns which bind and activate specific TLRs, allowing the host to gain information on the nature of the invading pathogen with subsequent induction of discrete activation pathways that modulate expression of TLRs,

cytokines and other inflammatory mediators [8]. Altered expression of TLR and cytokines in tissue informs on the nature of pathogens and host responses involved in disease aetiopathogenesis [8]. Histopathological examination of diseased equine periodontium has revealed neutrophilic and eosinophilic inflammation, gingival hyperplasia, degradation or ulceration alongside erosion and inflammation of the periodontal ligament [9]. To date, there have been no studies of innate immunity in the equine oral cavity or of its possible involvement in the pathogenesis of equine periodontal disease.

The aim of this study was to investigate the role of the equine innate immune system and aspects of adaptive immunity in equine periodontitis by quantifying the expression of TLR and cytokine genes in normal and diseased gingival tissue.

Materials and Methods

Sample collection

Samples were collected during *post mortem* examination of horses humanely euthanatised for reasons other than dental disease. Horses that had received any systemic medication in the prior four weeks were excluded from the study. A full dental examination was performed and results recorded on a dental chart (BEVA: https://www.beva.org.uk/_uploads/documents/beva-dental-chart-fillable.pdf).

Periodontal disease was assessed using a grading system based on periodontal pocket depth [9] ranging from normal (grade 0) to severe periodontal disease (grade 3). Tissue biopsies (4 mm x 4 mm) were collected from the gingival margin of mandibular premolar teeth 306/7 (Triadan numbering system [10]) of 13 orally healthy horses and from the gingivae of 20 horses with periodontitis. Control samples of healthy gingival tissue were also collected from 8 horses with localised periodontitis. All gingival biopsies were collected within 60 minutes of death and placed in 0.5 ml of RNAlater®[a] and stored at -20°C until RNA extraction and cDNA synthesis were performed.

Tissue processing

A 25 mg section of each gingival biopsy was selected for homogenisation. After adding 300 µl of Buffer RLT[b] a motorised pellet pestle[a] was used to macerate the tissue.

RNA extraction

The RNeasy Fibrous Tissue Mini Kit[b] was used to extract RNA from the gingival tissue samples in accordance with the manufacturer's instructions.

cDNA synthesis

cDNA was synthesised from RNA samples using a High Capacity cDNA Reverse Transcription Kit[c]. 10 µl of sample RNA was added to pre-prepared 2x RT Master Mix, comprised of 10x RT Buffer (2 µl), 25x dNTP mix (0.8µl), 10x RT Random Primers (1 µl), nuclease-free water (6.2 µl) in a total reaction volume of 20 µl. Reactions were conducted on a thermal cycler as follows: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C before termination at 4°C.

Primer design and efficiency

The National Centre for Biotechnology Information (NCBI) online tool at http://www.ncbi.nlm.nih.gov/tools/primer-blast/ was used to design and validate primers for the following equine genes: TLR2, TLR4, TLR9, IL-1β, IL-4, IL-6, IL-10, IL-12 (subunits p35 and p40), IL-17, IFN-ɣ and TNF-α (Supplementary Item 1) utilising a BLAST search on the full equine genome. The primers were synthesised commercially by Invitrogen[d]. Comparative Critical Threshold (Ct) measurements were used to calculate mRNA levels in gingival tissue and, in order to calculate primer efficiency, Ct values were plotted against $\log_{10}$ of the sample volume using the following formula: Efficiency = $-1+10^{(-1/\text{slope})}$. Primer efficiencies of between 0.85 and 1.1 were considered acceptable. In addition, agarose gel electrophoresis and melt curve analysis was performed for each primer to ensure that a

single product of the correct size and denatured at the predicted melt temperature was produced during the reaction.

Quantitative PCR

Quantitative PCR was performed using a MX300P™ real time PCR system[e]. SYBR® Select Mastermix[c] was used with reactions performed in triplicate. Each 25 µl reaction comprised 12 µl SYBR® Select Mastermix, 11.5 µl RNAse free water, 0.5 µl cDNA, 0.5 µl of forward primer and 0.5 µl of reverse primer. PCR was carried out as follows: denaturation at 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C (TLR2, IL-1, IL-6, IL-12, 18S rRNA, GAPDH) or 60°C (TLR4, TLR9, IL-4, IL-10, IL-17, TNF-α, IFN-ɣ) for 1 min and primer extension at 72°C for 1 min. GAPDH and 18S rRNA were used as housekeeping genes.

Data analysis

Ct values obtained for equine TLR and cytokines genes were adjusted using the geometric mean of values obtained for housekeeping genes GAPDH and 18S rRNA and the 2-Delta Delta Ct method [11]. To visualise the results, data was normalised to the control IL-12p35 mRNA levels. Fold change was also calculated.

An *a priori* power calculation indicated that with alpha set at (0.05) for two tailed t-test a minimum of 33 animals would be split between the two groups (16 and 17, respectively) - this number could be reduced in a one-tailed statistical test (alpha = 1.0) n = 27, which would have given at least 80% statistical power with an effect size = 1.

Cross tabulation (Chi-squared test) was used to determine whether there was a statistically significant difference in the prevalence of periodontitis with the breed of horse and to determine whether there was a correlation between the presence or incidence of periodontitis with the age of the animals. The paired Students t-test was used for comparisons of healthy and diseased sites in the same animal. For independent group comparisons of parameters measured in healthy and diseased horses, the variance of the

data was assessed by Levene's, the results of which were used to determine which independent sample test (t-test or the Welch's test) was used. These parametric statistical tests were also utilised to analyse data that was weighted by the age of the horses, a potential confounding factor in the analysis. The association between clinical severity of periodontitis and gene expression was measured using Bivariate Spearman correlation and age-adjusted correlation analysis. Data graphics were constructed using Microsoft Excel 2013 and Minitab® Version 17. Significance was set at $P \leq 0.05$.

Results

Sample demographics

Twenty-four tissue samples originated from the School of Veterinary Medicine at the University of Glasgow and 9 samples from the Royal (Dick) School of Veterinary Studies, University of Edinburgh. Twenty horses were euthanatised due to chronic disease: chronic musculoskeletal disease (n = 10), poor condition/chronic weight loss (n = 6), chronic ocular disease (n = 2), chronic hepatic disease (n = 1) and chronic gastrointestinal disease (n = 1). The remaining 13 horses were euthanatised due to congenital disease (n = 5), dangerous behaviour (n = 4), neoplasia (n = 2), poor performance (n = 1) and neurological disease (n = 1). The age and disease status of horses sampled are shown in Fig 1. The mean age of horses with periodontitis was 16.8 years (range 1-27 years), including 60% mares, 35% geldings and 5% stallions. The mean age of orally healthy horses was 6 years (range 1-16 years) and included 58% geldings, 25% mares and 17% stallions. The animals affected with disease were significantly older than healthy animals ($P = 0.007$). The proportion of horses with periodontitis correlated with the age of the animals ($\rho = 0.586$, $P<0.001$,Fig 1).

Breed and disease status of the horses was as follows: 16 native ponies (7 healthy, 9 disease), 8 Thoroughbred (TB) or TB crosses (2 healthy, 6 disease), 3 Irish Sport Horses (one healthy, 2 disease), 2 Icelandic horses (both disease), and one each of Shire, Selle Français, Standardbred and Irish Draught which were grouped together into a miscellaneous

breed category (3 healthy, one disease). The breed did not appear to significantly influence the presence of periodontitis.

Grading of periodontal disease

The average Periodontal Disease Grade [9] in horses with periodontitis was 2.2 (moderate). Nine horses had severe (Grade 3 periodontal pocket depth i.e. >15 mm) periodontal disease, six had moderate disease (Grade 2, periodontal pocket depth i.e. 10-14 mm) and four had mild disease (Grade 1 periodontal pocket depth i.e. 5-9 mm).

TLR mRNA expression

Expression of mRNA encoding TLR2, TLR4 and TLR9 was increased in tissue samples from periodontitis lesions in comparison to healthy gingivae (Fig 2). In an age-weighted analysis, the levels of TLR4 mRNA, TLR2 mRNA and TLR9 mRNA were significantly greater in biopsies of horses with periodontitis ($P<0.001$, $P<0.001$ and $P = 0.013$, respectively; Table 1). Levels of TLR2 mRNA showed a particularly large 389-fold increase in periodontitis samples compared to healthy gingivae. In comparison, levels of TLR4 mRNA were only increased 2-fold and levels of TLR9 mRNA increased 3.8-fold in diseased samples. Control samples of healthy gingival tissue from horses with periodontitis lesions at other sites were used to provide site comparisons. When comparing healthy and diseased sites from the same animals, levels of TLR2 mRNA ($P = 0.005$), TLR4 mRNA ($P = 0.03$) and TLR9 mRNA ($P = 0.02$) were significantly increased at diseased as compared to control sites (Fig 3). TLR2 mRNA levels showed a smaller fold increase between these sample groups, being increased 3.5-fold in diseased tissue. TLR4 mRNA was increased 17-fold and TLR9 mRNA was increased 16-fold in diseased tissue.

Cytokine mRNA expression

Expression of mRNA encoding for all measured cytokines was increased in diseased as compared to healthy gingival tissue samples (Fig 2). In an age-weighted analysis, the levels of *TNF-α, IL-4, IL-10, IL-12p35, IL-12p40,* and *IFN-γ* gene transcripts were significantly greater in the periodontitis samples (P<0.001; Table 1). Transcripts were increased as follows: IL-1β mRNA (2-fold), IL-10 mRNA (9-fold), IFN-γ mRNA (11-fold), TNF-α mRNA (12-fold), IL-12p35 mRNA (18-fold), IL-12p40 (18-fold). When comparing cytokine mRNA expression in healthy gingival tissue with periodontitis lesions from the same animal, mRNA expression of all cytokines measured was increased in diseased tissue with statistically significant increases observed for the following genes: *IL-1β* (P = 0.004), *IL-4* (P = 0.009), *IL-10* (P = 0.02), *IL-12p35* (P = 0.00007) and *IFN-γ* (P = 0.02) (Fig 3). When comparing diseased tissue with healthy tissue from the same animal, large fold increases were observed for transcripts of the following genes: *IL-1β* (25-fold), *IL-10* (52-fold*), IL-12p35* (65-fold), *IL-12p40* (22-fold), *IL-4* (23-fold), *TNF-α* (65-fold) and *IFN-γ* (30-fold).

Clinical severity and TLR and cytokine expression

Periodontitis cases were grouped according to disease grade [9]: mild, moderate and severe. TLR mRNA expression and clinical severity of periodontal disease showed no statistically significant correlations, except for TLR2 mRNA in the age-adjusted analysis (P<0.001). Statistically significant positive correlations existed between disease severity and expression, in an age-adjusted analysis, for IL-1β, IL-6, IL-10, IL-12p40, IL-17 and TNF-α (all P<0.01).

Discussion

Periodontal disease is a painful and common equine disorder [1]. An intense, localised inflammatory response has been found histologically in tissue sections from diseased equine periodontal pockets [9] with a marked neutrophil transmigration into the periodontal tissues,

and this was significantly associated with increased severity of periodontitis. Gingival epithelial erosion and ulceration as well as infiltration of mononuclear cells into the periodontal ligament was also noted [9]. Stimulation of the innate immune response by periodontopathic bacteria is known to play an instrumental role in the aetiopathogenesis of periodontitis in other species by causing inflammation and degradation of periodontal tissue.

The role of the innate immune system in equine periodontitis has not previously been investigated. In the current study, the level of mRNA encoding TLR2, TLR4, TLR9 and cytokines (IL-1β, IL-4, TNF-α, IFN-ɣ, IL-10, IL-12p35, IL-12p40, IL-17) were increased in diseased samples. Correlation between pro-inflammatory, and between anti-inflammatory cytokines with disease severity was also seen. Differences in the pattern of cytokine and TLR expression between health and periodontitis are probably due in part to dynamic changes in the cell population in tissues as well as induction of genes by TLR activation. When comparing changes in the mRNA levels of periodontally diseased tissue with both healthy control sites and tissue from unaffected animals, although some similarities existed, significant increases in the mRNA encoding TLR2, TLR9, IL-12, IL-10 and IFN-ɣ were recorded for the disease group in both analyses. A limitation of our study is that we did not perform histochemistry to ascertain the difference in the cell population. However, with inference to what is seen in other species certain conjectures can be made about the inflammatory cell infiltrate.

TLR2 is located on the plasma membrane of cells and responds to a wide variety of pathogen-associated molecular patterns from fungal, bacterial and mycobacterial pathogens. While TLR2 is mainly activated by lipotechioic acid, lipoproteins and lipopeptides from Gram-positive bacterial species [12], the lipopolysaccharide (LPS) of Gram-negative periodontopathogenic micro-organisms activate TLR2 more efficiently than TLR4 [13]. Increased levels of TLR2 expression occur in gingival tissue of human periodontitis patients [14] and human gingival epithelial cells have been shown to recognise bacteria through activation of TLR2 [6,15]. TLR4 is also located on the surface of cells and mainly detects

LPS of Gram-negative bacteria [16]. Expression of TLR4 has been shown to be up-regulated in human periodontal disease [14]. TLR9 responds to unmethylated cytosine-phosphate-guanine of bacterial, fungal and viral DNA [17]. Other studies have shown increased expression of TLR9 in diseased human gingival tissue [18]. It is likely that increased expression of TLR2, TLR4 and TLR9 in equine periodontitis is indicative of high bacterial challenge in the periodontal pocket and surrounding tissue. Bacteria play a causative role in the pathogenesis of human, canine and feline periodontal disease and thus their potential importance in equine periodontitis is to be expected. High numbers of bacteria have been observed in equine periodontal pockets, including spirochetes in the gingival epithelium [9]. *Porphyromonas gingivalis*, *Tannerella* and *Treponema* species have been more commonly isolated from periodontal pockets of horses with clinically overt Equine Odontoclastic Tooth Resorption and Hypercementosis than from horses with no clinical signs of disease in the mouth [19].

Binding of Toll-like receptors to their microbial ligands results in activation of second messenger systems which subsequently alter expression of a variety of immune and inflammatory genes [8]. In order to further characterise the response of the innate immune system in equine periodontitis, expression of Th1, Th2 and Th17 cytokines were evaluated in the current study and a mixed Th1/Th2 response was seen in equine periodontitis. It has been suggested that the stimulation of Th1 cytokines in response to periodontopathogenic bacteria leads to the destruction of periodontal tissue [20]. When comparing healthy control sites with periodontitis lesions in the same horses, significant increases were observed for mRNA levels of IL-1β, IL-12p35 and IFN-ɣ in periodontitis. A positive correlation between disease severity and IL-1β mRNA levels was observed. IL-1β plays a pivotal role in periodontal tissue destruction [21] by enhancing bone resorption and inducing synthesis of matrix metalloproteinases (MMPs) which may lead to further tissue destruction. However, collagen breakdown in the periodontal ligament induced by MMP-1 may be central to the normal physiology of the healthy equine periodontium due to the long-term dental eruption and periodontal tissue remodelling occurring in hypsodont dentition [22]. It has been

suggested that expression of TNF-α in gingival tissue, alongside IL-1β, plays an instrumental role in periodontal tissue destruction by stimulating osteoclast development and loss of alveolar bone [20] in addition to enhancing synthesis of collagenase by fibroblasts [23,24]. In the current study, the TNF-α mRNA level was greater in periodontitis lesions and correlated with disease severity. The mRNA levels of *IL-12p35* and *IL-12p40* were increased when comparing both unaffected healthy and periodontitis groups, and healthy control sites and periodontitis lesions. IL-12 activates natural killer cells and induces CD4 T cell differentiation into IFN-ɣ-producing Th1-like cells [25]. The increased IL-12 mRNA level observed in equine periodontitis may explain the significant increase in IFN-ɣ mRNA seen in periodontitis when compared to both horses with no clinical signs of oral disease and healthy control sites of horses with periodontitis. IFN-ɣ stimulates phagocytosis by macrophages and Th1 responses, increasing IL-12 responsiveness [26]. Significantly higher levels of IFN-ɣ expression have been noted in active human periodontitis lesions [27]. IL-17 is a pro-inflammatory cytokine which supports Th1 responses [28] and plays a key role in recruiting and activating neutrophils at inflammatory sites [29]. The Th2 response regulates the inflammatory response and counteracts the effects of the pro-inflammatory Th1 response. IL-4 mRNA levels were significantly increased in equine periodontitis. IL-4 stimulates B-cell proliferation and subsequent immunoglobulin production [30] whilst inhibiting phagocytosis and production of periodontally destructive cytokines such as IL-1β and TNF-α [7]. In the current study, significant increases in the levels of IL-10 were recorded in diseased periodontal tissue when compared to both horses with no clinical signs of oral disease and healthy control sites of horses with periodontitis. In addition, IL-10 levels were 9-fold higher in equine periodontitis. IL-10 also down regulates the pro-inflammatory response, having a protective role against disease progression in human periodontitis [31]. IL-10-producing cells are widely distributed in periodontitis granulation tissue [32].

Although the aetiopathogenesis of equine periodontitis differs to that in brachydont species, similarities in expression of components of the innate immune system exist. A mixed Th1/Th2/Th17 response was observed in gingival tissue samples from equine periodontitis cases with statistically significant increases in mRNA levels of both destructive pro-inflammatory and protective anti-inflammatory cytokines. It is likely that increased expression of these cytokine genes is a result of stimulation of TLR2, TLR4 and TLR9 that recognise bacterial receptors. Our results suggest that these receptors are also increased in equine periodontitis, although this was only demonstrated at the gene transcript level. In other species, recognition of periodontopathogenic bacteria by TLRs in gingival and periodontal tissue initiates cytokine production, often causing a destructive inflammatory response and thereby facilitating disease progression [7]. The results of the current study suggests a similar aetiopathogenesis is likely in the horse. However, the innate immune response of equine periodontitis is complex and further studies would be useful, in particular to investigate the interaction between oral bacteria and innate immunity.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The study was approved by the University of Glasgow School of Veterinary Medicine Ethics and Research Committee and by the University of Edinburgh Veterinary Ethical Review Committee. Tissues were collected with the informed consent of the horse owners.

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Authorship

M.P. Riggio and D. Lappin were involved in all aspects. P.M. Dixon was involved in study design, study execution and final approval of the manuscript. D. Bennett was involved in study design, study execution and final approval of the manuscript. R. Kennedy was involved in study execution, data analysis and interpretation, preparation of the manuscript and final approval of the manuscript.

Manufacturers' addresses

[a]Sigma-Aldrich, Poole, UK.

[b]Qiagen, Manchester, UK.

[c]Life Technologies, Paisley, UK.

[d]Invitrogen, Paisley, UK.

[e]Stratagene, Cambridge, UK.

Table 1: Age-adjusted mRNA levels of the listed target gene products as a proportion of house-keeping genes mRNA in horses without oral disease and horses with periodontitis

Target	Diagnosis	Mean	SE	s.d.	P= *
TLR2	Healthy	0.0007[t]	0.0006	0.0023	<0.0001
	Periodontitis	0.0378	0.0086	0.0386	
TLR4	Healthy	0.0003	0.0002	0.0005	<0.0001
	Periodontitis	0.0023	0.0004	0.0017	

TLR9	Healthy	0.0223	0.0070	0.0253	0.01
	Periodontitis	0.0844	0.0080	0.0359	
IL1	Healthy	0.0112	0.0054	0.0195	0.2
	Periodontitis	0.0239	0.0033	0.0149	
IL4	Healthy	0.0016	0.0006	0.0022	0.0009
	Periodontitis	0.0051	0.0006	0.0026	
IL6	Healthy	0.0028	0.0009	0.0032	0.7
	Periodontitis	0.0026	0.0002	0.0011	
IL10	Healthy	0.0016	0.0007	0.0026	0.0008
	Periodontitis	0.0055	0.0007	0.0033	
IL12P35	Healthy	0.0001	0.0000	0.0001	<0.0001
	Periodontitis	0.0003	0.0000	0.0002	
IL12p40	Healthy	0.0004	0.0002	0.0006	0.0001
	Periodontitis	0.0016	0.0002	0.0008	
IL17A	Healthy	0.0007	0.0003	0.0009	0.09
	Periodontitis	0.0013	0.0004	0.0017	
IFN	Healthy	0.0015	0.0006	0.0022	0.0003
	Periodontitis	0.0055	0.0006	0.0029	
TNF	Healthy	0.0022	0.0011	0.0040	0.0009
	Periodontitis	0.0097	0.0014	0.0062	

* Statistical analysis was performed on the Age weighted ΔΔCt values.

ǂ Age weighted results are expressed as mean, standard error (SE) and standard deviation. (s.d.) of the target mRNA levels relative to house-keeping mRNA levels.

Figure Legends

Fig 1: Age and disease status of 13 horses with no clinical signs of oral disease and 20 horses with periodontitis.

Fig 2: Gingival TLR and cytokine mRNA expression in 13 horses with no clinical signs of oral disease and 20 horses with periodontitis. Levels of mRNAs were adjusted to the housekeeping genes (18S rRNA and GAPDH) and are shown

relative to control IL12p35 mRNA levels.

*Statistically significant difference ($p<0.05$) between health and disease.

**Statistically significant difference ($p<0.01$) between health and disease.

***Statistically significant difference ($p<0.001$) between health and disease.

Fig 3: Comparison of TLR and cytokine mRNA expression in healthy gingival tissue and periodontitis lesions in the same animal. Levels of mRNAs were adjusted to the housekeeping genes (18S rRNA and GAPDH) and are shown relative to control IL12p35 mRNA levels.

*Statistically significant difference ($p<0.05$) between health and disease.

**Statistically significant difference ($p<0.01$) between health and disease.

Supplementary Information

Supplementary Item 1: Primers used for amplification of equine TLR and cytokine genes.

References

1. Dixon, P.M., Barakzai, S., Collins, N. and Yates, J. (2008) Treatment of equine cheek teeth by mechanical widening of diastemata in 60 horses (2000-2006). *Equine Vet. J.* **40**, 22-28.
2. Baker, G.J. (1970) Some aspects of equine dental disease. *Equine Vet. J.* **2**, 105-110.
3. Ireland, J.L., McGowan, C.M., Clegg, P.D., Chandler, K.J. and Pinchbeck, G.L. (2012) A survey of health care and disease in geriatric horses aged 30 years or older. *Vet. J.* **192**, 57-64.
4. Dixon, P.M., Ceen, S., Barnett, T., O'Leary, J.M., Parkin, T.D. and Barakzai, S. (2014) A

long-term study on the clinical effects of mechanical widening of cheek teeth diastemata for treatment of periodontitis in 202 horses (2008-2011). *Equine Vet. J*. **46**, 76-80.

5. Lane, J.G. (1994) A review of dental disorders of the horse, their treatment and possible fresh approaches to management. *Equine Vet. Educ.* **6**, 13-21.
6. Teng, Y-T.A. (2003) The role of acquired immunity and periodontal disease progression. *Crit. Rev. Oral Biol. Med*. **14**, 237-252.
7. Gemmell, E., Marshall, R.I. and Seymour, G.J. (1997) Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease. *Periodontol*. *2000* **14**, 112-143.
8. Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. *Nature Rev. Immunol.* **4**, 499-511.
9. Cox, A., Dixon, P. and Smith, S. (2012) Histopathological lesions associated with equine periodontal disease. *Vet J*. **194**, 386-391.
10. Floyd, M.R. (1991) The modified Triadan system: nomenclature for veterinary dentistry. *J. Vet. Dent.* **8**, 18-19.
11. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402-408.
12. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* **11**, 443-451.
13. Lappin, D.F., Sherrabeh, S. and Erridge, C. (2011) Stimulants of Toll-like receptors 2 and 4 are elevated in saliva of periodontitis patients compared with healthy subjects. *J. Clin. Periodontol*. **38**, 318-325.
14. Sarah, S.M, Tamilselvan, S., Kamatchiammal, S. and Suresh, R. (2006) Expression of Toll-like receptors 2 and 4 in gingivitis and chronic periodontitis. *Ind. J. Dent. Res.* **17**, 114-116.
15. Asai, Y., Ohyama, Y., Gen, K. and Ogawa, T. (2001) Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. *Infect.*

Immun. **69**, 7387-7395.

16. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol*. **162**, 3749-3752.
17. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740-745.
18. Beklen, A., Hukkanen, M., Richardson, R. and Konttinen, Y.T. (2008) Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. *Oral Microbiol. immunol*. **23**, 425-431.
19. Sykora, S., Pieber, K., Simhofer, H., Hackl, V., Brodesser, D. and Brandt, S. (2014) Isolation of *Treponema* and *Tannerella* spp. from equine odontoclastic tooth resorption and hypercementosis related periodontal disease. *Equine Vet. J*. **46**, 358-363.
20. Graves, D.T. and Cochran, D. (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J. Periodontol*. **74**, 391-401.
21. Liu, C.M., Hou, L.T., Wong, M.Y. and Rossomando, E.F. (1996) Relationships between clinical parameters, interleukin 1B and histopathologic findings of gingival tissue in periodontitis patients. *Cytokine* **8**, 161-167.
22. Warhonowicz, M., Staszyk C., Rohn K. and Gasse, H. (2006) The equine periodontium as a continuously remodeling system: Morphometrical analysis of cell proliferation. *Arch. Oral Biol*. **51**, 1141-1149.
23. Okada, H. and Murakami, S. (1998) Cytokine expression in periodontal health and disease. *Crit. Rev. Oral Biol. Med.* **9**, 248-266.
24. Meikle, M.C., Atkinson, S.J., Ward, R.V., Murphy, G. and Reynolds, J.J. (1989) Gingival fibroblasts degrade type I collagen films when stimulated with tumor necrosis factor and interleukin 1: Evidence that breakdown is mediated by metalloproteinases. *J. Periodont*. *Res.* **24**, 207-213.

25. Honda, T., Aoki, Y., Takahashi, N., Maekawa, T., Nakajima, T., Ito, H., Tabeta, K., Okui, T., Kajita, K., Domon, H. and Yamazaki, K. (2008) Elevated expression of IL-17 and IL-12 genes in chronic inflammatory periodontal disease. *Clin. Chim. Acta.* **395**, 137-141.

26. Mullen, A.C., High, F.A., Hutchins, A.S., Lee, H.W., Villarino, A.V., Livingston, D.M., Kung, A.L., Cereb, N., Yao, T.P., Yang, S.Y. and Reiner, S.L. (2001) Role of T-bet in commitment of T_H1 cells before IL-12-dependent selection. *Science* **292**, 1907-1910.

27. Dutzan, N., Vernal, R., Hernandez, M., Dezerega, A., Rivera, O., Silva, N., Aguillon, J. C., Puente, J., Pozo, P. and Gamonal, J. (2009) Levels of interferon-gamma and transcription factor T-bet in progressive periodontal lesions in patients with chronic periodontitis. *J. Periodontol*. **80**, 290-296.

28. Takahashi, K., Azuma, T., Motohira, H., Kinane, D.F. and Kitetsu, S. (2005) The potential role of interleukin-17 in the immunopathology of periodontal disease. *J. Clin. Periodontol*. **32**, 369-374.

29. Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J. and Gurney, A.L. (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* **278**, 1910-1914.

30. Dohmann, K., Wagner, B., Horohov, D.W. and Leibold, W. (2000) Expression and characterisation of equine interleukin 2 and interleukin 4. *Vet. Immunol. Immunopathol*. **77**, 243-256.

31. Bozkurt, F.Y., Ay, Z.Y., Berker, E., Tepe, E. and Akkuş, S. (2006) Anti-inflammatory cytokines in gingival crevicular fluid in patients with periodontitis and rheumatoid arthritis: A preliminary report. *Cytokine* **35**, 180-185.

32. Lappin, D.F., Macleod, C.P., Kerr, A., Mitchell, T. and Kinane, D.F. (2001) Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. *Clin. Exp. Immunol.* **123**, 294-300.