Studies on the aetiopathogenesis of feline chronic gingivostomatitis

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Abstract

Feline chronic gingivostomatitis (FCGS) is an inflammatory disease of the oral cavity that causes severe pain and distress. No specific treatment methods are available and little is known about its aetiology. The aims of this study were:- 1) to identify the bacterial flora, including uncultivable and potentially novel species, in healthy cats and those with FCGS, using 16S rRNA gene sequencing in combination with conventional culture methods; 2) to investigate the viral status of cats with and without FCGS; 3) to assess the immune response by investigating the expression of cytokine and Toll-like receptor (TLR) genes in tissue biopsies from normal cats and those with FCGS; 4) to investigate the histopathological changes in tissue biopsies from normal cats and those with FCGS, 5) to assess putative risk factors for FCGS by the use of a questionnaire-based study.

Oral swabs, mucosal biopsies and blood were collected and the location of the oral lesions was recorded. A total of 32 cats with FCGS and 16 normal cats were included in the study. Bacteria were identified from swabs by use of 16S rRNA gene sequencing and by conventional culture methods. Blood samples and swabs were used for diagnosis of infection with feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV), feline herpes virus 1 (FHV-1), feline calicivirus (FCV) and for blood biochemistry and haematology. Gene expression levels for TLR2, TLR3, TLR4, TLR7 and TLR9, and cytokines IL-1β, IL-4, IL-6, IL-10, TNF-α and IFN-γ mRNA were determined using quantitative PCR in biopsy samples from healthy cats and cats with FCGS. Histopathological examination of the tissue biopsies was done using hematoxylin and eosin (H&E) staining.

In the healthy group, 16S rRNA gene sequencing demonstrated that the most prevalent bacteria were part of the *Proteobacteria* and *Bacteroidetes* phyla, plus a group of uncultured bacteria. The most prevalent species in the healthy group were *Xanthomonadaceae* bacterium (6.2 % of clones analysed), *Capnocytophaga canimorsus* (5.4%), *Capnocytophaga cynodegmi* (4.8%), *Bergeyella* species (4.5%) and *Pasteurella multocida* subspecies *septica* (4.4%). Uncultured bacteria accounted for 29% of the clones analysed. In the FCGS group most of the identified species were part of the phylum *Proteobacteria*. The most prevalent species in the FCGS group were *P. multocida* subsp. *multocida* (14.1%) *P. multocida* subsp. *septica* (11.5%), *Pseudomonas* sp. (7.3%), *Tannerella forsythia* (6.6%) and *Porphyromonas circumdentaria* (5.6%). A variety of uncultured bacteria represented 7.7% of all analysed FCGS clones. The culture data showed the most prevalent bacteria in the healthy group were

P. multocida subsp. *septica* (9.9%), and uncultured bacteria (30.5%). In the FCGS group the most prevalent isolates were *P. multocida* subsp *septica* and *P. multocida* subsp. *multocida* (both 9.9%). Uncultured bacteria accounted for 21.7% of all isolates.

FCV was detected in 71% of cats with FCGS and in 13.3% of normal cats. FeLV antigen was detected in 33.3% of normal cats but not in any cats with FCGS. FIV antibodies were detected in 3.4% of cats with FCGS and in 33.3% of normal cats. FHV-1 was detected in 6.9% of cats with FCGS, but was not detected any of the normal cats.

In the FCGS group a significant increase was seen in the expression of TLR2 and TLR7 genes as well as TNF-α, IFN-γ, IL-1β and IL-6 cytokine genes. The healthy cats and cats with FCGS in the study that were found to harbour *T. forsythia* and *P. circumdentaria* showed an increase in the expression of several TLR and cytokine genes when compared to the group of cats in which these bacterial species were absent.

The most severely inflamed sites in the oral cavity of cats with FCGS included the tissue lateral to the palatoglossal folds and the maxillary attached gingiva. Histopathological analysis of the tissue from the palatoglossal folds showed two types of infiltrates:- 1) a combination of lymphocytes and plasma cells, most often seen in the milder inflamed tissue samples; 2) a predominantly plasmacytic infiltration, most often seen in the severely inflamed tissue samples.

Preliminary data from a questionnaire-based epidemiological study showed that the presence of potential environmental stress factors such as no ability to roam outdoors and the presence of more than one cat in the household is significantly higher in cats with FCGS when compared to normal cats.

This study highlights the possibility of a multifactorial aetiology for FCGS in which FCV, specific bacteria and stress factors may play an important role. Although species from the *Bacteroidetes* phylum appeared to be capable of eliciting an immune response, these were not the most prevalent species in the FCGS group. A shift could be seen in the composition of the bacterial flora when healthy cats and those with FCGS were compared.

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Author's Declaration

This thesis is the original work of the author, except where explicit reference is made to the contribution of others. The thesis or parts from it have not been submitted for any other degree at the University of Glasgow or any other institution.

Sanne Maria Johanna Dolieslager, June 2012

List of abbreviations

Abbreviation	Meaning
A	Adenine
ACD	Anaemia of chronic disease
AID	Anaemia of inflammatory disease
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
APC	Antigen presenting cell
AST	Aspertate aminotransferase
ATM	Anaerobic transport medium
AVDC	American veterinary dental college
Bluo-Gal	5-Bromo-3-indolyl-ß-D-Galactopyranoside
C	Cytosine
CBA	Colombia blood agar
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleid acid
CF	*Chlamydophila felis*
CFU	Colony forming units
CPE	Cytopathic effect
CpG	Cytosine-phospodiester bond-Guanine
DC	Dendritic cell
DLH	Domestic longhair
DMH	Domestic medium hair
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DSH	Domestic shorthair
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
F	Female
FAA	Fastidious anaerobe agar
FAB	Fastidious anaerobe broth
FCGS	Feline chronic gingivostomatitis
FCV	Feline calicivirus
FEA cells	feline embryonic fibroblast
FeLV	Feline leukaemia virus
FHV-1	Feline herpes virus 1
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
FN	Female neutered
FORL	Feline odontoclastic resorption lesions
FPV	Feline panleukopenia virus
FV	Fibrovascular
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGT	Gamma-glutamyl transferase
H&E	Haematoxylin and eosin
H0	Null hypothesis
Hb	Haemoglobin
HCT	Haematocrit
HSP	Heat shock protein
IC	Immunochromatography

IFA	Immunofluorescence antibody
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin 1 receptor
L1	Leucocyte antigen 1
LB	Lysogeny broth
LPS	Lipopolysaccharide
M	Male
MAL	MyD88-adaptor-like protein
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MHC	Major histocompatibility complex
MN	Male neutered
mRNA	Messenger ribonucleid acid
MyD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear factor kappa B
NK	Natural killer
NSAID	Non steroidal anti-inflammatory drug
OD	Optic density
PAMPs	Pathogen associated molecular patterns
PC	Plasma cell
PCR	Polymerase chain reaction
PIND-ORF	Paramunity inducer from inactivated parapox ovis virus
PPV	Positive predictive value
RBCC	Red blood cell count
RCW	Red cell distribution width
rFeIFN	Feline recombinant interferon
RFLPs	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleid acid
SD	Standard deviation
SPF	Specific pathogen free
ssRNA	Single stranded RNA
T	Thymine
TBE	10x Tris/Borate/EDTA
TEMED	N,N,N',N' tetramethylethyl enediamine
TGF	Transforming growth factor
Th	T helper
Th0	Naive T-helper
TIR	Toll/IL-1R
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TR	Tooth resorption
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing interferon-ß
U	Uracil
URTD	Upper respiratory tract disease
UV	Ultra violet
v/v	Volume to volume ratio
VTM	Viral transport medium
WBCC	White blood cell count

Chapter 1 General introduction

1.1 Preface

Feline chronic gingivostomatitis (FCGS) is a severe inflammatory disease of the feline oral cavity that causes pain and distress and can be serious enough to lead to euthanasia of affected cats. More insight into the aetiopathogenesis is needed to make progress in the development of treatments for this disease. Many different bacterial species associated with human periodontal disease have been implicated in the feline disease and there is a strong suggestion that viruses play an important role. FCGS is the most challenging of all the oral inflammatory diseases to treat. No standard treatment or preventive measures showing consistent results are available in first opinion practice. Previous research in FCGS has been relatively limited and focused on three main aspects: bacterial culture, determination of the viral status of cats and nature of the immune response to infection. To fully understand the complex aetiopathogenesis of this disease there is a need to study all three aspects together and try to identify links between them. This study was based on this approach. Molecular microbiological methods were combined with conventional microbiological culture for bacterial identification. The cellular immune response to infection was investigated by determining Toll-like receptor (TLR) and cytokine gene expression. The prevalence of feline viruses in affected cats was determined and a questionnaire based epidemiological study was included to explore the putative risk factors in FCGS.

1.2 Feline chronic gingivostomatitis

1.2.1 Definition of FCGS

1.2.1.1 Nomenclature

FCGS is a severe inflammation of the oral cavity. The syndrome is referred to in the literature by various names including feline gingivitis-stomatitis pharyngitis, lymphocytic plasmacytic gingivitis stomatitis, plasma cell stomatitis pharyngitis and chronic stomatitis. These names either refer to location of the lesions in the oral cavity or reflect the type of inflammation found in the histopathological evaluation of biopsies from the oral cavity (Knowles et al., 1991; White et al., 1992; Diehl and Rosychuk, 1993; Lommer and Verstraete, 2003; Healey et al., 2007). FCGS is the name that is commonly referred to in the recent literature but the described clinical signs may vary (Table 1.1).

1.2.1.2 Clinical appearance

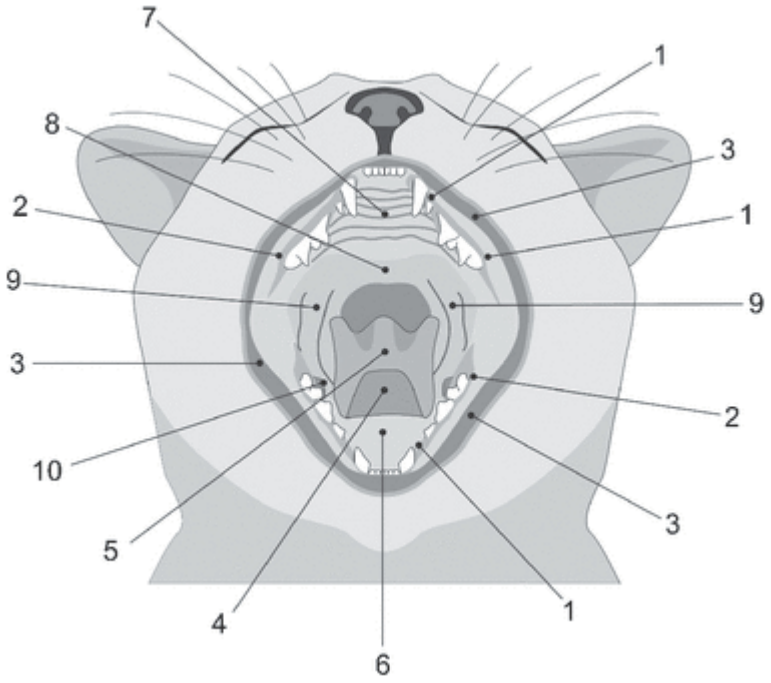
The different names used for FCGS in the literature often refer to a variety of clinical signs (Table 1.1) and the terms used, have generally been defined differently as far as the location of the lesions within the oral cavity is concerned. The research carried out to date is difficult to compare because of such inconsistencies. FCGS is described as a proliferative and ulcerative inflammation of the oral cavity that can affect a variety of sites (White et al., 1992). The most severe presentation, that is most challenging to treat, involves inflammation at two main sites; the tissue lateral to the palatoglossal folds, often referred to as the fauces (Figure 1.1, site 9) and the mucosa overlying the premolar/molar area extending to the buccal mucosa (Figure 1.1, sites 2 and 3) (Southerden, 2010; Hennet et al., 2011). Other areas that can be affected are the pharynx, tongue and lips. Inflammation of the palate can also be seen and periodontal disease is often identified (Hennet, 1997). The pain that is caused by the inflammatory changes is the main cause of the variety of symptoms seen in affected animals. Symptoms include dysphagia, weight loss, loss of grooming behaviour, excessive saliva, pawing at the mouth and halitosis. Additionally these severe symptoms can cause a change the cats' demeanour. Affected animals may seek seclusion and can become more aggressive when handled (Bonello, 2007; Southerden and Gorrel, 2007; Bellei et al., 2008).

Table 1.1: Nomenclature for feline chronic gingivostomatitis

Described location of lesions	Name given to syndrome	Reference
Premolar/molar gingiva with or without palatoglossal arches, pharynx, hard palate, tongue, periodontal disease	Gingivitis stomatitis	Frost and Williams (1986)
Gingiva with or without palatoglossal folds, pharynx, hard palate, tongue	Plasma cell gingivitis-pharyngitis	Sims et al. (1990)
Gingiva or palatoglossal folds, pharyngeal walls, tongue, palate, lips, buccal mucosa	Plasma cell stomatitis pharyngitis	White et al. (1992)
Gingiva or adjacent mucosa or palatoglossal folds with or without cheeks, tongue, lips	Feline gingivitis stomatitis pharyngitis complex	Diehl and Rosychuk (1993)
Fauces with or without gingiva and tongue	Gingivostomatitis	Hawkins (1999)
Isolated or a combination of gingivitis, stomatitis, periontal disease, glossitis, palatitis, buccostomatitis, faucitis, ostitis, osteomyelitis	Gingivitis-stomatitis-oropharyngitis (GSO)	Mihaljevic (2003)
Gingivitis-stomatitis: premolar/molar gingiva/bucca with or without palatoglossal folds and lingual area Stomatitis-gingivitis: Palatoglossal folds and gingival/buccal mucosa with or without hard palate, tongue	FCGS	Gorrel (2004)
Gingiva, caudal parts, lips, ventral tongue	Lymphoplasmacytic gingivitis	Baird (2005)
Caudal parts extending to buccal and gingival mucosa with or without pharynx, palate, tongue	FCGS	Healey et al. (2007)
Palatoglossal folds or gums or palate or buccal mucosa or tongue	FCGS	Bellei et al. (2008)
Palatoglossal folds, premolar/molar gingival and buccal mucosa	FCGS	Southerden and Gorrel (2007) Southerden (2010) Arzi et al. (2010b) Hennet et al. (2011)

A selection of names for FCGS taken from the literature and the described location of the lesions

Figure 1.1: Anatomy of the feline oral cavity



Reproduced from Arzi et. al. (2010a)

1. Attached gingiva
2. Alveolar mucosa
3. Buccal mucosa
4. Ventral surface of the tongue
5. Dorsal surface of the tongue
6. Floor of the mouth
7. Hard palate
8. Soft palate
9. Palatoglossal folds
10. Molar lingual salivary gland

1.2.1.3 Histopathological appearance

Histopathologically, two patterns can be seen in the inflammatory cell infiltrates (Barker et al., 1992). The first pattern (Group 1) is described as either feline ulcerative stomatitis and glossitis or lymphocytic plasmacytic stomatitis. This is described as an ulcerative chronic active inflammation of the mucosa and palatoglossal folds. The predominant cells in mucosa and submucosa are lymphocytes and plasma cells. The second pattern (Group 2) is feline plasma cell gingivitis-pharyngitis or FCGS, which is described as proliferative lesions, mainly at the glossopalatine arches. The inflammatory reaction in the submucosa consists predominantly of plasma cells with smaller numbers of lymphocytes, neutrophils and histiocytes. Mott cells, which are plasma cells containing immunoglobulin (Ig), also named Russell bodies, and multi-nuclear plasma cells are also seen (Johnessee and Hurvitz, 1983; Barker et al., 1992; White et al., 1992; Diehl and Rosychuk, 1993; Lyon, 2005; Wiggs, 2007). Approximately 70% of cases demonstrate a lympho-plasmacytic infiltrate (Group 1) and 30% of cases show a predominant plasmacytic infiltrate (Group 2) (White et al., 1992; Diehl and Rosychuk, 1993).

1.2.2 Prevalence

Feline oral diseases that are most commonly seen in practice include periodontal disease and feline tooth resorption (TR) lesions, previously referred to by other names such as feline odontoclastic resorption lesions (FORL) and neck lesions (Ingham et al., 2001; Girard et al., 2009). In the United States a study showed that 13% of cats visiting veterinary practices were diagnosed with gingivitis (Lund et al., 1999) but no record was made of FCGS despite the fact that it is a well known disease in veterinary practice. A survey from the American Dental Society found that 62% of veterinarians were seeing at least one case of gingivostomatitis per week (Frost and Williams, 1986). In a study from the north-west of England a FCGS prevalence of 0.7% was seen in a population of 4858 cats visiting veterinary practices. Forty-four percent of these were new cases (Healey et al., 2007).

1.3 Treatment and management of FCGS

1.3.1 Preventive measures

The unknown aetiology of FCGS makes preventive measures difficult. Any oral preventive medicine starts with hygiene. The focus should therefore be a reduction of plaque accumulation and mechanical disruption of the bacterial biofilm to reduce the risk of periodontal disease that often accompanies the caudal stomatitis.

1.3.1.1 Dietary management

A variety of dental care diets is available. These diets are designed to reduce plaque accumulation and consequently are used as an oral hygiene product. The diet is presented as kibbles that are larger than ordinary cat food and have a tougher structure to mechanically clean the teeth during eating. This is based on the observation that dried foods have a positive effect on periodontal health when compared with moist food (Studer and Stapley, 1973). Ordinary moist cat food does not represent the natural diet of felids, where natural diets would normally involve considerable chewing. A different pattern of dental disease is seen in wild felidae with more tooth fractures than infection. This is possibly related to the diet and the greater chance of trauma during hunting (Longley et al., 2007; Longley, 2010). One study on the prevalence of TR showed a lower prevalence in wild cats when compared to domestic cats (Roux et al., 2009; Longley, 2010). In addition to dental care ‘main’ diets for domestic cats, there are chew treats available with the same purpose of removing plaque during mastication. Research on the effect of these diets and treats on oral status has shown a decrease in calculus and plaque accumulation when used regularly (Ingham et al., 2002; Vrieling et al., 2005). Changing the shape of the kibble and increasing the surface area and volume results in a reduction in plaque accumulation (Clarke et al., 2010).

As well as presentation of the diet, the ingredients may also play a role in oral health. In a single case study of FCGS, the cat was given symptomatic treatment and the diet was changed to a natural cat diet without artificial ingredients (Addie et al., 2003). The cat showed improvement after the change in diet. In human dentistry, food additives are seen as a possible factor in the aetiology of stomatitis. In a retrospective study of 1252 patients with stomatitis and 100 healthy controls, the stomatitis patients were significantly more likely to have hypersensitivity towards

food additives (Wray et al., 2000). No large scale research has been done in cats to support this suggestion.

1.3.1.2 Dental hygiene

The modern cat diets, which are soft and easy to consume, have created the need for home dental hygiene measures in cats. A wide range of products is available from pet shops and veterinary practices and include toothpaste, mouthwash, toothbrushes and hygienic wipes. Dedication by the owner is mandatory for this form of preventive medicine to be effective but calculus formation is decreased significantly when cats teeth are brushed once or twice weekly (Richardson, 1965).

1.3.2 Symptomatic treatment

1.3.2.1 Hygienic treatment and surgical measurements

Initial treatment in cats diagnosed with FCGS is professional oral hygiene treatment including dental scaling, periodontal debridement and polishing. TR lesions require restoration or removal (Wiggs, 2007). Studies recording TR lesions in combination with FCGS showed the presence of minimal one TR lesion in 41% (Bellei et al., 2008) and 66% (Hennet, 1997) of the cases. Antibiotic treatment and advice on home hygienic measurements, including brushing and chlorhexidine gel are important (Frost and Williams, 1986; Harvey, 1991). When the response to initial treatment is unsatisfactory, further treatment is likely to include extracting the teeth most closely located to the mucosal inflammation or removing all premolars and molars (Gorrel, 2004; Baird, 2005; Bellei et al., 2008). In areas with hyperplasia of the gingiva, gingivoplasty may be indicated (Wiggs, 2007). Dental extraction requires a thorough approach since any retained roots or parts of roots can cause persistence of the clinical signs. Studies on the success rate of elective surgical extraction (removing all premolars and molars), show that approximately 50-60% of cases will require no further treatment, 20-40% of cases show improvement and 10-20% of the cats will show no improvement after the procedure (Hennet, 1997; Girard and Hennet, 2005; Bellei et al., 2008). In the study by Hennet (1997) these results were shown after removing all teeth in 6.6% of cases, leaving one to five premolar teeth in 13.4% of cases and all premolar and molar teeth were removed in the remaining cases.

1.3.2.2 Antibiotic drug therapy

Antibiotic therapy alone provides only temporary improvement in FCGS cases (Harvey, 1991; Lyon, 2005). No standardised clinical trials of antibiotic therapy have been reported. In a small study, improvement was noted in 30% of cats treated with different antibiotic regimes (White et al., 1992). No treatment period was recorded and no details of the scale of improvement or of any additional treatments were provided. Antibiotics that are often recommended in combination with surgical methods include clindamycin, amoxicillin, amoxicillin/clavulanic acid, metronidazole and doxycycline (Frost and Williams, 1986; Harvey, 1991; Harvey, 1994; Lyon, 2005; Wiggs, 2007). Administration of antibiotic treatment ranging from a week to months is suggested and a combination of topical and systemic treatment is sometimes advised.

1.3.2.3 Anti-inflammatory and immunosuppressive drug therapy

Corticosteroid use in FCGS cases is advocated for its immunosuppressive and anti-inflammatory actions (Frost and Williams, 1986; Diehl and Rosychuk, 1993; Lyon, 2005; Wiggs, 2007). In a study where a number of corticosteroids was tested, improvement was seen in 50% to 80% of the cases (White et al., 1992). In many cases, the effect of the drug became less reliable over a period of months. The co-administration of other drugs was not recorded in this study and there was no standardised treatment period. A more recent study evaluated the use of a tapering anti-inflammatory dose of prednisolone for three weeks in cats with persistent clinical signs at least two months after dental extractions and antibiotic therapy (Hennet et al., 2011). The cats were treated for three weeks. Eight percent of cats were completely cured, improvement was seen in 46% of cases and no response or worsening of symptoms was seen in 46% of cases. Pain scores decreased during the first 30 days, although not significantly, and increased subsequently from day 30 to 90. A scoring system was used to score the caudal stomatitis intensity and area affected and the presence of alveolar and buccal mucositis; these scores decreased over a 90 day period although not significantly past 30 days.

Other immunosuppressive drugs that have been used are azathioprine, chlorambucil and cyclophosphamide (Diehl and Rosychuk, 1993; Wiggs, 2007). Non-steroidal anti-inflammatory drugs (NSAIDs) are also prescribed in FCGS often in combination with antibiotic therapy (Mihaljevic, 2003; Hennet et al., 2011). However, no studies have shown effectiveness of these treatment methods.

1.3.2.4 Interferon treatment

Type I recombinant interferons (IFNs) have been used for their action against viruses in cats (Jameson and Essex, 1983). Human recombinant INF-α was the first to be commercially available and has been used against feline retrovirus infections (Kociba et al., 1995; Domenech et al., 2011). To overcome problems with the production of neutralising antibodies, species-specific feline recombinant IFN (rFeINF) α has been produced (Ueda et al., 1993). rFeIFN-α has been described as having *in vitro* anti-viral effects (Wonderling et al., 2002) and rFeIFN-ω has been described as having antiviral effects against feline and canine viruses, including feline calicivirus (FCV) and feline herpesvirus-1 (FHV-1) (Truyen et al., 2002). rFeIFN-ω is now commercially available and registered for veterinary use in the UK. In the past few years several studies have been conducted on the effect of rFeIFN-ω on FCV-positive cats with FCGS (Mihaljevic, 2003; Southerden and Gorrel, 2007; Hennet et al., 2011). Cats that do not respond to the initial surgical treatment as described previously (Section 1.3.2.1) have been treated with positive results. A non-controlled clinical study on 20 cats showed an improvement in all cats and seven of these were described as clinically cured according to veterinary evaluation after a treatment period of three to six months (Mihaljevic, 2003). A single case report described a successful result after six weeks of treatment (Southerden and Gorrel, 2007). A double-blind study on 39 cats compared the results of rFeIFN-ω with prednisolone treatment (Hennet et al., 2011). A statistically significant improvement was seen in the scores for clinical lesions and pain after 90 days of treatment with rFeIFN-ω. A significant difference between the groups was found with the pain scores at day 60 and day 90, the group treated with rFeIFN-ω having lower pain scores.

1.3.2.5 Other treatments

Oral administration of bovine lactoferrin, an antimicrobial peptide, provided improvement in pain, salivation, appetite and oral inflammation scores of cats with FCGS (Sato et al., 1996). None of the cats was totally cleared of signs and some cats showed no improvement. It has been proposed that treatment with bovine lactoferrin should be in combination with antibiotics and glucocorticoids.

A paramunity inducer containing an inactivated parapox ovis virus (PIND-ORF) (Buttner, 1993), has been tested as a treatment for FCGS. Thirty-three cats with FCGS were treated with PIND-ORF and compared to 39 cats with FCGS treated with conventional methods. According to this study 42% of the cats did not need other treatment after PIND-ORF, compared to 13% in the conventionally treated group

(Mayr et al., 1991). When tested on 17 cats with FCGS, of which 15 were FCV positive, a significant improvement was seen in all the clinical scores of the treated cats (Zetner et al., 2006).

Administration of megoestrol acetate is also reported to have positive results in cats with FCGS (Johnessee and Hurvitz, 1983; White et al., 1992). Of a total of five cats that were treated with megoestrol acetate four showed improvement but due to side-effects the drug was discontinued in two of these four cats (White et al., 1992). Due to the high risk of side effects this treatment is not recommended.

Anti-inflammatory chrysotherapy, or 'gold salts', has been utilised in human medicine for years (Forestier, 1932; Stuhlmeier, 2007). One study has been published on the use of weekly injections with aurothioglucose in 17 cats with FCGS. 82% of these cats showed improvement but no information on improvement scales was given (White et al., 1992).

Laser therapy has been used in FCGS to reduce proliferative tissue and create fibrous tissue to reduce the inflammatory reaction (Harvey, 1991; Lyon, 2005; Lewis et al., 2007). No studies have shown any effectiveness of the treatment on its own, although one single case study showed a cat free of caudal stomatitis after treatment with CO_2-laser therapy in combination with other treatment methods including a full dental extraction (Lewis et al., 2007).

Other approaches that have been advised but not supported by clinical trials are food supplements such as vitamins A, B and C and shark cartilage (Gaskell and Gruffydd-Jones, 1976; Wiggs, 2007) and chemical cautery (Gaskell and Gruffydd-Jones, 1976).

1.4 **Bacteriology**

1.4.1 The healthy bacterial oral flora in cats

From studies on the bacteria of the human oral cavity we have learnt that there is a diverse microbiome with thousands of different species growing in complex biofilms. Initial colonisers of oral bacterial biofilms are predominantly gram-positive aerobes such as *Actinomyces* species and *Streptococcus* species. Secondary colonisers of the biofilm are mostly gram-negative bacteria including pathogenic bacteria like *Tannerella* species (Kolenbrander, 2000; Li et al., 2004).

The healthy oral flora of the cat has been poorly studied. Most studies to date have used cultivable methods and from studies on the human oral microflora it is estimated that approximately 50% of the bacteria are uncultivable (Socransky et al., 1963); a similar situation could be expected in the feline oral cavity. A summary of the oral bacteria that have been identified in cats is shown in Table 1.2

In a microbiological study on plaque bacteria in five healthy cats and five cats with gingivitis, *Bacteroides* species, *Fusobacterium nucleatum*, and *Actinomyces* species were isolated from both healthy cats and cats with periodontal disease (Tannock et al., 1988). Gram staining showed a combination of gram-positive and gram-negative bacteria and spiral-shaped forms (spirochaetes) in both healthy cats and cats with gingivitis.

In another study the gingival margin of 14 healthy cats between six and 12 month of age which were free of gingivitis or tartar build up was investigated (Love et al., 1990). From these cats 150 isolates were obtained. The most common obligate anaerobes identified belonged to the genera *Bacteroides* (presently partly classified as *Porphyromonas* or *Prevotella*) and *Fusobacterium*. The most common facultative anaerobe species were *Actinomyces*, *Pasteurella multocida* and *Propionibacterium*. *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* are common organisms in the oral cavity of the cat and are often isolated (Baldrias et al., 1988; Ganiere et al., 1993).

Studies on *Bacteroides* sp. showed that *Bacteroides tectum* was the most prevalent *Bacteroides* species in the feline oral cavity (Love et al., 1987; 1989).

Table 1.2: Bacteria found in the healthy feline oral cavity.

Gram-positive		**Gram-negative**	
Aerobes/facultative anaerobes	Anaerobes	Aerobes/facultative anaerobes	Anaerobes
Actinomyces sp.	*Bifidobacterium* sp.	*Actinobaccilus* sp.	*Campylobacter* sp.
Lactobacillus sp.	*Clostridium* sp.	*Branhamella* sp.	*Bacteroides* sp.
Micrococcus sp	*Peptostreptococcus* sp.	*Campylobacter* sp.	*Fusobacterium* sp.
Nocardia sp.	*Propionibacterium* sp.	*Capnocytophaga* sp.	*Porphyromonas* sp.
Streptococcus sp.		Coliforms	*Prevotella* sp.
		Eikenella sp.	Spirochaetes sp.
		Haemophilus sp.	*Veilonella* sp.
		Neisseria sp.	*Wolinella* sp.
		Pasteurella sp.	

Adapted from Bonello (2007)

Studies on the oral microflora of cats are often focused on potential human pathogens. Bacteria such as *P. multocida* and *Capnocythophaga canimorsus* are of particular interest in human medicine and can cause local abscessation or severe illness, respectively, following cat bites (Blanche et al., 1998; Love et al., 2000; Gaastra and Lipman, 2009). Studies on cat bite infections have shown that a variety of bacteria can be transferred during cat bites which implies their presence in the oral cavity (Talan et al., 1999; Love et al., 2000). A summary of the types of bacteria identified from cat bite infections is shown in Table 1.3 (Talan et al., 1999).

1.4.2 The oral flora in the inflamed feline oral cavity

Studies of the bacterial oral flora in diseased cats have mostly focused on periodontal disease and therefore isolated bacteria are often from plaque samples (Mallonee et al., 1988; Norris and Love, 1999a).

Culture-dependent methods in cats with gingivitis showed that the prevalence of *Bacteroides, Provetella* and *Porphyromonas* species is higher when compared to the healthy gingival margin and increased with severity of disease (Mallonee et al., 1988; Love et al., 1989). *Porphyromonas* species are known to be of importance in human periodontal disease and are often described in combination with *Actinobacillus actinomycetemcomitans* and *Tannerella forsythia* (Slots et al., 1986; Bragd et al., 1987; Socransky et al., 1998). In cats a positive correlation between the presence of *Porphyromonas* species and the grade of periodontal disease has been observed (Norris and Love, 1999a). *T. forsythia* has been identified in plaque samples from cats with and without periodontal disease. The proportion of *T. forsythia* was higher in cats with periodontitis (Booij-Vrieling et al., 2010).

Peptostreptococcus species were found to be more predominant in cats with increasingly severe periodontal disease (Mallonee et al., 1988). *P. multocida* was often isolated but numbers seemed to decrease with increasing severity of periodontal disease (Mallonee et al., 1988). Another study demonstrated the presence of significantly higher numbers of *P. multocida* in cats with inflamed gingiva compared to normal gingiva and with mildly inflamed gingiva showing the largest increase (Mihaljevic and Klein, 1998). In a study on gingivitis *P. multocida* could be isolated and accounted for 5% of all isolates (Harvey et al., 1995). *Fusobacterium nucleatum* is detected more frequently in plaque from cats with gingivitis when compared to healthy cats (Tannock et al., 1988).

When investigating the oral cavity of the cat, several sources of bacteria must be taken into account. Grooming and hunting are natural habits and consequently will be adding to the bacterial diversity in the oral cavity. The possibility of not only colonisation of a variety of bacteria but also the presence of bacterial DNA from other sources needs to be considered.

Table 1.3: Bacteria isolated from cat bite infections.

Gram-positive		**Gram-negative**	
Aerobes/facultative anaerobes	**Anaerobes**	**Aerobes/facultative anaerobes**	**Anaerobes**
Actinomyces sp.	*Clostridium sordelli*	*Acinetobacter* sp.	*Bacteroides* sp.
Bacillus sp.	*Eubacterium* sp.	*Actinobaccilus* sp.	*Fusobacterium* sp.
Brevibacterium sp.	*Filifactor Villosus*	*Aeromonas hydrophila*	*Porphyromonas* sp.
Corynebacterium sp.	*Peptostreptococcus* sp.	*Alcaligenes* sp.	*Prevotella* sp.
Enterococcus sp.	*Propionibacterium* sp.	*Capnocytophaga* sp.	*Veilonella* sp.
Erysipelothrix rhusiopathiae		*EF4B bacteria*	
Gemella morbillorum		*Eikenella corrodens*	
Lactobacillus sp.		*Enterobacter cloacae*	
Rhodococcus sp.		*Klebsiella oxytoca*	
Rothia dentocariosa		*Moraxella* sp.	
Staphylococcus sp.		*Neisseria* sp.	
Streptococcus sp.		*Pantoea agglomerans*	
Streptomyces sp.		*Pasteurella* sp.	
		Pseudomonas sp.	
		Reimerella anatipestifer	
		Weeksella sp.	

Adapted from Talan et al. (1999)

In a study on periodontal disease, *Bacteroides* sp. *Porphyromonas* sp. and Gram negative bacillus were found to be the most predominant anaerobes and *Neisseria* and *Staphylococcus epidermidis* were the main aerobic bacteria in calculus samples (Samsar et al., 2003)

Studies that report bacteria relating to FCGS specifically are relatively rare. In a study of affected and normal cats where serum antibodies against a variety of human and cat gram-negative anaerobes and lipopolysaccharide (LPS) from these bacteria were analysed, cats with FCGS had significantly increased titres when compared to control cats for all bacteria and most of the LPS tested (Sims et al., 1990). The bacteria tested included *A. Actinomycetemcomitans* and *P. gingivalis*. Two single FCGS case studies showed large numbers *P. multocida* in both cats (Reindel et al., 1987; Addie et al., 2003).

1.4.3 *Bartonella henselae*

Bartonella species are intracellular gram-negative bacteria that can cause a persistent intra-erythrocytic bacteraemia in the host. Transfer of *Bartonella* species can be mediated by bloodsucking arthropodes (Chomel et al., 2009; Breitschwerdt, 2011). The precise pathogenesis of *Bartonella* sp. still needs to be unravelled. So far two important pathogenicity factors of *B. henselae* have been described: *Bartonella* adhesion A which allows binding to the host cells and the VirB/VirD4 type IV secretion system which modulates the host cell function, for example, by inhibion of apoptosis of infected cells (Franz and Kempf, 2011). The first authors researching the possible connection between the presence of *Bartonella* sp. in cats and clinical disease showed a higher prevalence of gingivitis in cats coinfected with FIV and *B. henselae* (Ueno et al., 1996). Several studies have been done on the possible relationship between *Bartonella henselae* and other feline oral diseases but without any consistent results. A study compared illness and health in cats seropositive and seronegative for *B. henselae* (Glaus et al., 1997). There was no higher prevalence of disease in seropositive cats but a higher frequency of stomatitis was seen in the seropositive group. When a study combined blood culture, polymerase chain reaction (PCR) on oral swabs and antibody tests and compared these results to the presence of oral lesions, no correlation was found between cats with a bacteraemia and having oral lesions (Namekata et al., 2010). A higher but not significant prevalence of oral lesions was seen in cats where PCR showed the presence of *Bartonella* sp. However cats with antibodies against both, *B. clarridgelae* and *B. henselae* were 3-4 times more likely to suffer from oral lesions. Another study found opposing results when blood culture and serological tests were used to assess any

correlation with a number of clinical illnesses (Sykes et al., 2010). In that study an association between the presence of FCGS and isolation of *Bartonella* sp. was seen, but not between FCGS and seropositivity for *Bartonella* sp. Some studies have found no correlation at all between FCGS and *Bartonella* sp (Quimby et al., 2008). In a group of nine cats with FCGS and 39 healthy cats, enzyme linked immunosorbent assay (ELISA) and Western blot immunoassays on blood samples showed no correlation between seropositivity for *Bartonella* sp. and FCGS. Also, a study on 52 cats with FCGS and 50 healthy controls failed to show a correlation between FCGS and *B. henselae* (Belgard et al., 2010). When blood from a total of 70 FCGS and 61 healthy cats was tested for deoxyribonucleic acid (DNA) of *Bartonella* species by PCR and for antibodies by ELISA and Western blot, the prevalence of *Bartonella* sp. did not differ between the two groups (Dowers et al., 2010). Additionally, PCR on oral biopsies from 42 cats with FCGS and 19 healthy cats did not show a correlation between FCGS and *Bartonella* species. At this point no evidence of a correlation between the presence of *Bartonella* species and FCGS can be found in any large scale studies that have investigated the aetiology of FCGS.

1.5 Feline viruses and FCGS

1.5.1 Feline leukaemia virus

Feline leukaemia virus (FeLV) is a retrovirus first discovered in 1964 (Jarrett et al., 1964; Kawakami et al., 1967) Between 1-5% of healthy cats in European countries are believed to be infected with FeLV (Hosie et al., 1989; Levy, 2005; de Lange, 2008; Gleich et al., 2009). Young cats that spend time outdoors are more likely to be infected than older cats that remain mostly indoors (Hosie et al., 1989). The most common transmission route is oro-nasal spread through saliva from viraemic cats (Hardy et al., 1973; Francis et al., 1977). Infection with FeLV can have several outcomes. The acute infection can follow one of three courses: it can develop into a persistent viraemia, an inactive latent form or, if the immune system eliminates the virus, the cat can become immune (Hardy et al., 1976; Rezanka et al., 1992). It is difficult to differentiate between an inactive latent form and an infection that has been eliminated. The path that is followed differs and depends on factors such as age, dose and duration of exposure. In 60% of cases the immune system eliminates the virus and approximately 30% of the cats develop a persistent viraemia (Dunham and Graham, 2008). In case of a persistent viraemia most cats develop signs including lymphoma and leukaemia, within three years. Clinical symptoms that are often observed are weight loss, fever, conjunctivitis, dehydration and rhinitis. In 15% of cases, oral inflammation is seen (Sparkes, 1997; Levy, 2005).

Often commercial immunochromatography (IC) tests in the veterinary practice or laboratory ELISAs are used to screen for FeLV. Both tests detect the FeLV p27 capsid protein. The positive predictive value (PPV; the proportion of correctly diagnosed positives in a population) for these tests is variable and a positive screening, especially in healthy, low risk cats, needs to be confirmed with viral isolation, PCR or immunofluorescence (Hartmann et al., 2007; Dunham and Graham, 2008). Whole virus, viral nucleic acids and protein p27 respectively are detected in these tests (Levy, 2005).

A possible relationship between FeLV and FCGS has been described in the literature based on the fact that oral inflammation is sometimes seen with FeLV infection (Cotter et al., 1975; Frost and Williams, 1986). It has not been possible to show a consistent relationship between the presence of FeLV and FCGS. In three small case studies all cats with FCGS tested negative for FeLV (Johnessee and Hurvitz, 1983; Thompson et al., 1984; Quimby et al., 2008). In a study that tested 36 cats suffering from plasma cell stomatitis pharyngitis, 16.6% tested positive for FeLV (White et al., 1992). In a study of

23 cats with FCGS, none were found positive for the virus (Hennet, 1997). Several single case reports have shown that cats with FCGS were negative for FeLV (Addie et al., 2003; Baird, 2005; Southerden and Gorrel, 2007).

1.5.2 Feline immunodeficiency virus

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus that was first isolated in a cattery in California (Pedersen et al., 1987). Its prevalence varies around the world and depends on age, lifestyle, health status and gender. Adult cats that spend time outdoors are more likely to be infected than younger and indoor cats (Crawford and Levy, 2007; Dunham and Graham, 2008; Gleich et al., 2009). The most common way of transmission is through bite-wounds. Vertical transmission and infection through sexual contact has been shown in experimental studies (Ueland and Nesse, 1992; O'Neil et al., 1995; Jordan et al., 1998). FIV infection occurs in several phases. The acute infection, which is associated with clinical signs such as anorexia, depression and pyrexia, is followed by a persistent viraemic stage in which the cat appears healthy (Callanan et al., 1992; Dunham and Graham, 2008). The length of the viraemic stage is variable and may last for years. The outcome of the viraemic stage is variable and in most cats this is followed by the terminal phase. In this phase the immune system is not able to prevent the plasma viral load from increasing and this leads to immunodeficiency. The most common clinical signs of infection are secondary bacterial infections of the upper respiratory tract and oral cavity (Hartmann, 2005). Other symptoms include chronic enteritis, neurologic disease and neoplasia.

For diagnosis of FIV, commercial ELISA based screening tests that test the presence of anti-FIV antibodies are available (Crawford and Levy, 2007; Dunham and Graham, 2008). Due to a higher prevalence of FIV when compared to FeLV, the PPV for the screening tests is also higher; however confirmation by another method is recommended after initial screening (Hartmann et al., 2007; Dunham and Graham, 2008). Methods that can be used to confirm an initial screening test are Western blot, immunofluorescence assays, virus isolation and PCR. Western blot and the immunofluorescence assay detect anti-FIV antibodies, virus isolation detects whole virus and viral or proviral DNA will be detected with the PCR assay. However, the PCR assays currently available do give varying results (Bienzle et al., 2004; Hartmann, 2005; Crawford and Levy, 2007; Dunham and Graham, 2008).

FIV is often seen in cats with chronic forms of oral ulceration. The severity of oral lesions can increase in cats positive for FIV, especially when co-infections with other

viruses such as FCV and FeLV occur (Dawson et al., 1991; Tenorio et al., 1991; Waters et al., 1993). In a UK and USA study, 75% and 81% of cats with FCGS in the UK tested positive for FIV compared to only 16% in the control group. 54% of the cats with FCGS in the USA tested positive for FIV compared to 50% in the control group (Knowles et al., 1989). Other studies have shown lower levels of FIV infection (13-28%) in FCGS cases (White et al., 1992; Hennet, 1997; Bellei et al., 2008; Quimby et al., 2008). Several single case reports have shown cats with FCGS that were negative for FIV (Addie et al., 2003; Baird, 2005; Southerden and Gorrel, 2007)

1.5.3 Feline calicivirus

FCV is a non-enveloped Ribonucleic acid (RNA) virus that causes upper respiratory tract disease in cats. FCV belongs to the family of *Caliciviridae*. The prevalence of the virus is variable, and is considered to be approximately 10% in the UK for cats that live in small groups or alone (Wardley et al., 1974; Gaskell, 2005; Radford et al., 2007). Larger groups such as shelter cats or catteries have a much higher prevalence of up to 40%. FCV is transmitted via nasal, oral or conjunctival mucous membranes (Poulet et al., 2000). Clinical signs that are associated with FCV infection are variable but oral ulceration is the most common sign. Signs typical of acute upper respiratory tract disease (URTD) caused by FCV include serous or mucopurulent nasal and ocular discharge, sneezing, conjunctivitis, depression, anorexia and pyrexia. Occasionally pneumonia and coughing develop and some strains of the virus can induce lameness (Dawson et al., 1994; Poulet et al., 2000). In 1998, a very highly contagious strain was described for the first time (Pedersen et al., 2000). This strain causes in addition to the typical URTD symptoms, ulcerative facial dermatitis, cutaneous oedema, severe pyrexia, vomiting and diarrhoea. Mortality in this strain is high at around 50%. Diagnosis of FCV is by viral isolation from nasal, conjunctival or oropharyngeal swabs (Hurley and Sykes, 2003; Gaskell, 2005). Immunofluorescence antibody test (IFA) has been used but is considered less sensitive than viral isolation, as is PCR.

FCV and FCGS have been linked in many studies (Gaskell and Gruffydd-Jones, 1976; Povey, 1976). A high percentage of cats have been found to be positive for FCV especially when cats with caudal stomatitis are examined (Reubel et al., 1992). In one of the first studies in which ten cats with chronic stomatitis were tested for FCV, eight tested positive (Thompson et al., 1984). In 78 British cats tested in three different locations 79 to 92% of cats with chronic stomatitis were positive for FCV compared with 19% for control cats (Knowles et al., 1989). In the same study, in cats from the USA, 50% of cats with chronic stomatitis tested positive for FCV, compared to 0% for controls. A

study on co-infection with FCV and FHV-1 showed that in 25 cats with FCGS, 88% shed both viruses compared to a control group of 24 cats with periodontal disease where only 21% shed these viruses (Lommer and Verstraete, 2003). Only two out of 25 cats were positive for FCV alone and one for FHV-1 alone. When a group of 32 cats with FCGS were tested, 66.6% were positive for FCV (Bellei et al., 2008). In one study all of the cats were negative for FCV by PCR, serological tests showed 100% positive for both, control and FCGS groups (Quimby et al., 2008). A later study from the same research group showed that 40.5% of cats in the FCGS group were positive for FCV compared to 0% in the control group (Dowers et al., 2010).

Most studies have demonstrated a significantly higher prevalence of FCV in cats with FCGS when compared to a control group but in almost every study a proportion of the cats with FCGS tested negative for the presence of FCV. Cats that were infected with FCV did not show a higher prevalence or a greater severity of oral lesions compared to virus-free cats (Tenorio et al., 1991). Also, infection of specific pathogen-free (SPF) cats with FCV induced signs of acute faucitis and oral ulceration but no signs of chronic oral inflammation were observed (Knowles et al., 1991; Reubel et al., 1992). When FCV was introduced into a colony of cats some of which had been infected with FIV, chronic gingivitis was observed in nine cats, six positive for FCV and FIV, one only positive for FIV and two positive only for FCV. Gingivitis was more severe in cats positive for both viruses (Waters et al., 1993).

1.5.4 Feline herpes virus 1

FHV-1 is a member of the subfamily of *alphaherpesvirinae* (Davison et al., 2009). FHV-1 is a common virus among cats with respiratory problems and is also known as feline rhinotracheitis virus. Studies have been conducted to investigate the prevalence of the disease. A study in the UK of 622 cats showed that 11% of cats with respiratory problems were positive for the virus and 1% of clinically healthy cats were also positive (Binns et al., 2000). In Korea the prevalence in clinically healthy cats in an animal shelter was found to be as high as 63% (Kang and Park, 2008). The virus is transmitted by direct contact through nasal, oral and conjunctival mucous membranes (Binns et al., 2000; Gaskell, 2005; Gaskell et al., 2007). The most important sources of viral spread are acutely infected cats but carrier cats can also spread the virus. An infection with FHV-1 is most common in young cats and cats from multi-cat households. Following an incubation period of two to six days, clinical signs start to develop. Sneezing, pyrexia and depression are the most common changes in the early stages of the disease (Povey, 1976; Gaskell et al., 2007). The signs quickly worsen to include ocular and nasal

discharge and sometimes severe mucopurulent conjunctivitis, dyspnoea and coughing will occur. Oral ulceration can also occur but is not as common as in other viral infections (Povey, 1979; Gaskell, 2005; Gaskell et al., 2007). After recovery most cats will become latently infected. Reactivation of the virus is likely in times of stress, after corticosteroid therapy and during lactation. At such times the cat can develop mild signs and will shed virus. Diagnosis of FHV-1 is usually done by viral isolation from saliva (Gaskell, 2005; Maggs, 2005; Gaskell et al., 2007). Another technique is immunofluorescence from conjunctival smears (Maggs, 2005; Gaskell et al., 2007). PCR to detect viral DNA is now used in many laboratories and is significantly more sensitive than viral isolation, although variation in sensitivity between different assays is significant (Hara et al., 1996; Gaskell, 2005; Maggs, 2005; Maggs and Clarke, 2005; Gaskell et al., 2007).

FHV-1 in combination with FCV, was present in 88% of cats with FCGS compared to 21% in cats with periodontal disease (Lommer and Verstraete, 2003). Other studies have failed to show a correlation between FHV-1 and FCGS (Quimby et al., 2008; Dowers et al., 2010).

1.6 The immune response

1.6.1 Defence mechanism in the oral cavity

1.6.1.1 Mucous membrane

The first defence mechanism in the oral cavity is the mucous membrane. The oral cavity is exposed to a variety of antigens and the epithelium needs to protect the host against mechanical trauma and micro-organisms, either commensal or pathogenic (Orsini and Hennet, 1992). The mucosa consists of a stratified squamous epithelium and connective tissue divided into lamina propria and submucosa (Garant, 2003a; Eubanks, 2007). Three different types of mucosa are seen in the in the oral cavity: 1) Lining mucosa which is elastic and loosely bound to the connective tissue that is rich in elastin. Lining mucosa covers the moving structures in the mouth such as the bucca, floor of the mouth and soft palate. 2) Masticatory mucosa which lines the gingivae and hard palate; it consists of tough keratinised epithelium with dense connective tissue. 3) Specialised mucosa found on the dorsum of the tongue that comprises cornified, stratified squamous epithelium (Orsini and Hennet, 1992; Garant, 2003a)

1.6.1.2 Saliva

Saliva has several functions in the oral cavity including digestion, lubrication and protection against microorganisms (Lehner, 1992; Amerongen and Veerman, 2002; Garant, 2003b). The protective function of saliva is provided by a variety of proteins and peptides secreted in the saliva. Both innate and adaptive responses play a role in the defence mechanisms of saliva.

The nature of the immunoglobulins in the cat saliva, has been studied. The levels of IgM, IgG and IgA were determined and IgA was the most predominant class in the saliva from healthy cats (Harley et al., 1998). When cats with FCGS were tested, salivary immunoglobulins changed from predominantly IgA to predominantly IgG and IgM (Harley et al., 2003b).

1.6.1.3 Mucosal cellular immune response

The immune cell population of the oral cavity includes aggregates of, as well as individual lymphocytes and dendritic cells (DC), macrophages and mast cells (Arzi et al., 2010a). The immune cell population has been investigated in a variety of locations

within the healthy oral cavity of the cat. T-lymphocytes, oral mucosal Langerhans cells, mast cells and macrophages are present in the different tissues (Arzi et al., 2010a). In the epithelium of the palatoglossal folds, Cluster of differentiation (CD) 3^+ cells were observed with $CD8^+$ cells being detected in higher numbers than $CD4^+$ cells (Harley et al., 2003a). The tissue from the palatoglossal folds contained mostly mast cells in the lamina propria and submucosa and also included $CD3^+$ T-lymphocytes and an equal division of $CD4^+$/$CD8^+$ cells. Least often detected were $CD79^+$ cells and leucocyte antigen 1 ($L1^+$) cells. The plasma cells that were detected were either IgG^+ or IgA^+. Aggregation of T-lymphocytes and antigen presenting cells (APCs) were seen in the lamina propria and may represent lymphoid follicles (Harley et al., 2003a; Arzi et al., 2010a). No B-lymphocytes were detected in the oral mucosa of the SPF cats tested (Arzi et al., 2010a).

The immune cells in the oral mucosa of cats with FCGS has also been assessed and the most prevalent cells in the mucosa were $CD79a^+$, IgG^+ and $L1^+$ (Harley et al., 2011). $CD3^+$ cells were also detected with $CD8^+$ cells being more prevalent than $CD4^+$ cells. Mast cells accounted for a small proportion of the cells but were higher when compared to previous studies in the healthy cat (Harley et al., 2003a). Another study showed an increase in the numbers of mast cells in the oral mucosa of cats with FCGS when compared to SPF cats (Arzi et al., 2010b).

1.6.2 Toll-like receptors

When pathogens invade through the anatomical barrier, the immune system reacts through the leucocytes of the innate immune system. Phagocytes (macrophages, dendritic cells and neutrophils) recognise pathogens and initiate phagocytosis, cytokine excretion and present antigen (Paltrinieri, 2008). Inflammation is the response that follows the excretion of the pro-inflammatory cytokines Interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α by activated macrophages. These cytokines organise the acute phase reaction in which fever, acute phase proteins, phagocytosis and the first stimulation of the adaptive immune system play a role (Gabay, 1999; Paltrinieri, 2008). On the surface of the leucocytes and of some epithelial cells, Toll-like receptors (TLRs) are found (Akira and Hoshino, 2003). They recognise different pathogens and when activated will stimulate the production of cytokines (Janeway and Medzhitov, 2002). TLRs activate the innate and the adaptive immune response.

TLRs are pattern recognition receptors that recognise pathogens and differentiate them from the host by the pathogen-associated molecular patterns (PAMPs) that are found on

the microorganisms. The process of TLR signalling is reviewed by Akira and Takeda (2004) and Kawai and Akira (2006). TLRs are found on the cell surface or in the cell compartment of a variety of leucocytes and epithelial cells. TLRs are part of a superfamily that includes interleukin-1 receptors (IL-1R) based on the similarity found in the cytoplasmatic region designated the Toll/IL-1R (TIR) domain (Akira and Takeda, 2004). This domain is essential for the signalling pathway. After binding to the ligand, the TLRs activate different adapter molecules. These adapter molecules are the start of the intracellular pathway that ultimately releases nuclear factor kappa B (NF-κB) to induce expression of genes within the nucleus. Myeloid differentiation primary response protein 88 (MyD88) is the adapter that is shared by almost all TLRs. The MyD88-dependent pathway controls inflammatory responses by inflammatory cytokine production. TLR2 and TLR4 require an additional adapter namely the TIR domain-containing adapter protein (TIRAP)/MyD88-adaptor-like protein (MAL), to induce the inflammatory response through MyD88-dependent signalling (Kawai and Akira, 2006). TIR-domain-containing adaptor protein inducing IFN-β (TRIF) is the adaptor that is activated after ligand binding to TLR3 and, in some cases, TLR4. This is known as the MyD88-independent pathway. TRIF-related adaptor molecule (TRAM) is another adapter. TRAM plays a role in the TLR4 MyD88-independent pathway in combination with TRIF. TRAM is not found in the TLR3 MyD88-independent pathway (Figure 1.2).

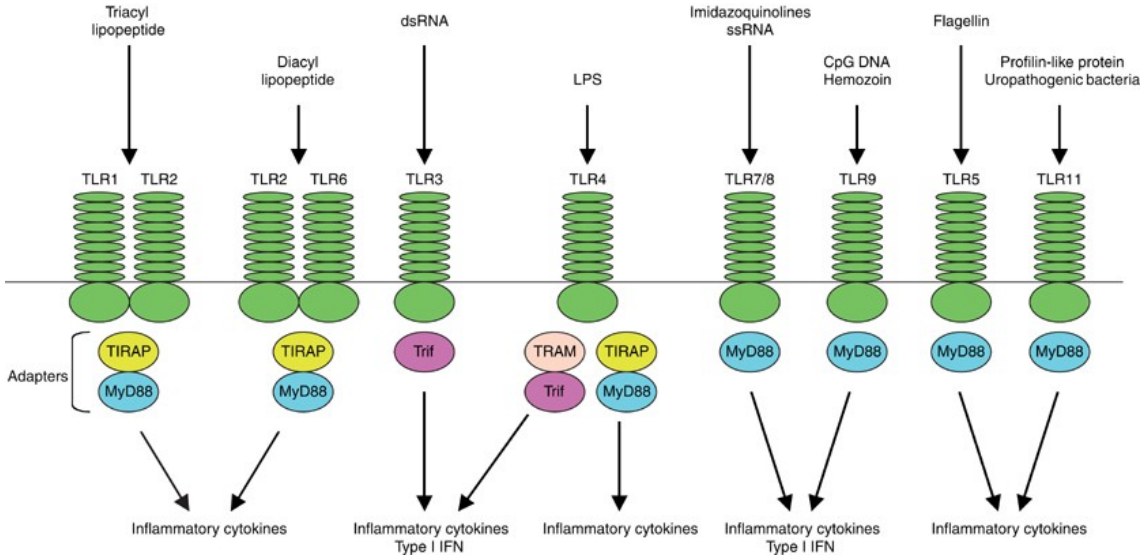
1.6.2.1 Feline TLR mRNA sequences

References which provide the cloned sequences of feline TLR messenger RNA (mRNA) are summarised in Table 1.4.

1.6.2.2 Toll like receptor 1, Toll like receptor 2 and Toll like receptor 6

TLR1, TLR2 and TLR6 are found on the cell surface. They combine together as a TLR1/2 dimer and as a combination of TLR2 and TLR6 (Figure 1.2). Each TLR in the dimer has different ligands but as a dimer they can discriminate between triacyl (TLR1/2) and diacyl (TLR2/6) lipopeptide. The adapter molecules that are activated after binding those combinations are TIRAP and MyD88, which mediate the production of inflammatory cytokines (Akira and Takeda, 2004; Ignacio et al., 2005; Kawai and Akira, 2006).

Figure 1.2: TLR-mediated immune responses



Reproduced from Kawai and Akira (2006)

Table 1.4: References which provide the cloned sequences of feline TLRs and cytokines

Gene	Reference
TLR1	Ignacio et al. (2005)
TLR2	Ignacio et al. (2005)
TLR3	Ignacio et al. (2005); Astakhova et al. (2009)
TLR4	Asahina et al. (2003)
TLR5	Ignacio et al. (2005)
TLR6	Ignacio et al. (2005)
TLR7	Ignacio et al. (2005); Astakhova et al. (2009)
TLR8	Ignacio et al. (2005); Astakhova et al. (2009)
TLR9	Griebel et al. (2009)
IL-1β	Daniel et al. (1992)
IL-4	Genbank accession number X87408
IL-6	Bradley et al. (1993); Ohashi et al. (1993)
IL-10	Genbank accession number U39569
IL-12 P35	Fehr et al. (1997)
IL-12 P40	Schijns et al. (1997)
IFN-γ	Argyle et al. (1995); Schijns et al. (1995)
TNF-α	McGraw et al. (1990)

1.6.2.3 Toll like receptor 3

TLR3 is found in the cell compartment and recognises double stranded RNA. TRIF is activated in a MyD88 independent pathway and leads to the production of inflammatory cytokines and type 1 IFN in particular IFN-β (Figure 1.2) (Ignacio et al., 2005; Kawai and Akira, 2006; Astakhova et al., 2009).

1.6.2.4 Toll like receptor 4

TLR4 is found on the cell surface, recognises bacterial LPS and stimulates production of inflammatory cytokines and type 1 IFN, IFN-β in particular (Figure 1.2). TLR4 utilises adapters TRAM, TRIF, TIRAP/MAL and MyD88 (Asahina et al., 2003; Kawai and Akira, 2006).

1.6.2.5 Toll like receptor 5

TLR5 is found on the cell surface and recognises bacterial flagellin. The MyD88 pathway is then activated which stimulates production of inflammatory cytokines (Figure 1.2) (Ignacio et al., 2005; Kawai and Akira, 2006).

1.6.2.6 Toll like receptor 7 and Toll like receptor 8

Both TLR7 and TLR8 recognise small synthetic compounds and single stranded RNA. They are found in the cell compartment. Binding of the ligand to these TLRs activates MyD88 and inflammatory cytokines and type 1 IFN are produced (Figure 1.2) (Ignacio et al., 2005; Kawai and Akira, 2006; Astakhova et al., 2009).

1.6.2.7 Toll like receptor 9

Cytosine-guanine bond (CpG) DNA of viruses and bacteria is recognised by TLR9. It is situated in the cell compartment and activates MyD88 after binding to the ligand. Inflammatory cytokines and type 1 IFN are produced after activation (Figure 1.2) (Griebel et al., 2005; Kawai and Akira, 2006).

1.6.2.8 TLR10 and TLR11

TLR10 and TLR11 have been discovered but the pathways in which they are involved are not yet fully understood. TLR11 recognises uropathogenic bacteria and activates MyD88

but the exact ligand is unknown (Akira and Hoshino, 2003; Akira and Takeda, 2004; Hasan et al., 2005; Kawai and Akira, 2006)

1.6.2.9 Feline Toll-like receptors in infection

Little research has been conducted on the function of feline TLRs. Full length cDNA cloning of the sequences for feline TLR3, TLR4, TLR7, TLR8 and TLR9 has been carried out (Asahina et al., 2003; Griebel et al., 2005; Astakhova et al., 2009). Feline TLR expression was determined in lymphoid tissue of the cat by Ignacio et al. (2005). TLR expression was tested in the spleen, thymus, intestinal intra-epithelial lymphocytes, lymphocytes from the lamina propria and retropharyngeal and mesenteric lymph nodes. TLR1 was only detected in the spleen. TLR6 expression was not detectable in any lymphoid tissue. All other TLRs were detected in the different lymphoid tissues investigated. Infection of a macrophage cell line with FIV resulted in an increase in TLR3 and TLR6 expression, whereas TLR9 expression was increased when an epithelial cell line was similarly infected. A down-regulation in TLRs was found in infected T-cell lines (Ignacio et al., 2005). When TLR expression was investigated in bone marrow dendritic cells during FIV infection, no significant changes were detected in the expression of TLR2, TLR3, TLR4, TLR7 and TLR9 (Lehman et al., 2009).

1.6.3 Cytokines

Cytokines are small proteins with a range of regulating functions in the immune system. They are released from various cells after a stimulus and activate cells of the immune system in an autocrine, paracrine or endocrine manner (Janeway et al., 2001). The different cytokines evaluated in this study are described. The references for the cloned feline cytokines are summarised in Table 1.4.

1.6.3.1 Interleukin-1

IL-1 consists of two proteins, IL-1-α and IL-1-ß. Il-1 is synthesised by macrophages, monocytes and dendritic cells and is a mediator of inflammation. Together with IL-6 and TNF-α, IL-1 induces inflammatory responses during the initiation of an infection. IL-1 plays a part in activating B lymphocyte growth and differentiation and $CD4^+$ T cell proliferation (Daniel et al., 1992; Paltrinieri, 2008; Cruse and Lewis, 2010).

1.6.3.2 Interleukin-4

IL-4 is produced by Th2 and mast cells. IL-4 function has mostly been studied in mice and includes the activation of B-cells, B-cell class switching to IgE, up-regulation of MHC class II production and differentiation of naive helper T cells (Th0 cells) into Th2 cells (Cruse and Lewis, 2010).

1.6.3.3 Interleukin-6

IL-6 is a pro-inflammatory cytokine produced by fibroblasts, mononuclear phagocytes, activated-lymphocytes and vascular endothelial cells. A variety of neoplasms are known to produce IL-6. IL-6 activates immune responses after tissue damage and is secreted in response to IL-1 and TNF-α stimulation. Hepatocytes are induced to form acute-phase proteins and B-lymphocytes are activated (Bradley et al., 1993; Ohashi et al., 1993; Cruse and Lewis, 2010).

1.6.3.4 Interleukin-10

IL-10 is expressed by $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, keratinocytes, monocytes and macrophages. IL-10 down regulates Th1 cytokine expression, blocks antigen presentation and IFN-γ formation. IL-10 inhibits macrophage production of IL-1, IL-6 and TNF-α and macrophage presentation of antigen. IL-10 is likely to contribute to the regulation and proliferation of B cells and mast cells and is known to stimulate B cells *in vitro* (Moore et al., 1993; Cruse and Lewis, 2010).

1.6.3.5 Interleukin-12

IL-12 stimulates natural killer (NK) cells and is a growth factor for $CD4^+$ and $CD8^+$ T lymphocytes. IL-12 stimulates the production of IFN-γ by NK cells and T cells. IL-12 is a heterodimeric molecule and consists of two subunits of 35-kDa (P35 or IL-12A) and 40-kDa (P40 or IL-12B) (Fehr et al., 1997; Schijns et al., 1997; Cruse and Lewis, 2010).

1.6.3.6 Interferon-γ

IFN-γ is produced by activated CD4 Th1 cells, CD8 T cells and NK cells. IFN-γ-stimulated NK cells activate mononuclear phagocytes and can stimulate expression of class I and class II MHC and the differentiation of both B- and T-lymphocytes. IFN-γ is known to decrease in several diseases such as chronic lymphocytic leukemia, lymphoma and

infections with rubella, Epstein-Barr virus and cytomegalovirus (Argyle et al., 1995; Schijns et al., 1995; Schroder et al., 2004; Cruse and Lewis, 2010).

1.6.3.7 Tumor necrosis factor-α

TNF-α is produced by cells such as macrophages, monocytes, T and B lymphocytes and NK cells once stimulated by pathogens. TNF-α facilitates wound healing. Vascular endothelial cells are stimulated to express adhesion molecules and secretion of chemokines by macrophages and endothelial cells is induced by TNF-α (McGraw et al., 1990; Cruse and Lewis, 2010).

1.6.3.8 T helper 1/T helper 2/T helper 17 subgroups

Helper T lymphocytes (Th cells) are subsets of CD4 cells that can be separated into functional groups. Naive T cells (Th0 cells) are differentiated into certain groups to facilitate the immune system by producing a distinct pattern of cytokines (Cruse and Lewis, 2010). Th1 cells are known to produce IFN-γ, IL-2 and TNF-ß and are responsible for activation of NK cells, macrophages and $CD8^+$ cells and for the delayed type hypersensitivity responses. Th2 cells produce a combination of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Their main function is stimulation of B-cell, IgE and IgG humoral responses (Cruse and Lewis, 2010). The recently identified Th17 subgroup consists of cells that produce IL-17, which is an important cytokine in inflammatory and autoimmune diseases. IL-23, transforming growth factor (TGF) ß, IL-6 and IL-7 appear to play an important role in Th17 production (Gaffen and Hajishengallis, 2008; Cruse and Lewis, 2010).

In cats with FCGS, the expression of IL-2, IL-4, IL-6, IL-10, IL-12 and IFN-γ was shown to be increased when compared to healthy cats (Harley et al., 1999). The profile in healthy cats is of a Th-1 type and in cats with FCGS there is a combined Th1/Th2 profile.

1.7 Risk factors in FCGS

Few studies have investigated the epidemiology of FCGS. Possible risk factors such as breed and age have been suggested (Frost and Williams, 1986; Tenorio et al., 1991; Diehl and Rosychuk, 1993).

1.7.1 Age

Gingivitis stomatitis pharyngitis has been described as most commonly seen in cats under the age of two and is gradually progressive (Diehl and Rosychuk, 1993). Age distribution among cats with FCGS has been assessed in a study in the UK (Healey et al., 2007). In this study of 4858 cats visiting the veterinary practice, 34 were suffering from FCGS and two age peaks were seen in the cats with FCGS - one to five years and 10 to 13 years. Also, the mean age of the cats with FCGS was higher (9 years) than the mean age of the non-FCGS cats (6.7 years) but this difference was not significant. In numerous studies a mean age of 6.9-8.3 years with a minimal age of 0.8 years and a maximum of 15 years has been reported (Johnessee and Hurvitz, 1983; Hennet, 1997; Hennet et al., 2011).

1.7.2 Breed

The proportion of different cat breeds suffering with FCGS has been described. Breeds that have been cited are Siamese, Himalayan, Burmese, Abyssinian and Persian (Frost and Williams, 1986; Diehl and Rosychuk, 1993). In the study of 4858 cats by Healey et al. (2007), no breed could be identified that appeared predisposed to FCGS. Of 34 cats, two were pedigree animals, a Siamese and a Persian. Out of the total population of cats the two most common pedigrees were also Siamese and Persian.

1.7.3 Sex

Sex has never been a suggested risk factor in FCGS and studies demonstrate an approximately equal distribution of male and female in cats with FCGS. In the 4858 cat population studied in the UK, 50% were female, 49% male and 1% unknown (Healey et al., 2007). Within the FCGS population 47% were female, 50% were male and 3% were unknown. This concurs with other studies which show a similar distribution (Johnessee and Hurvitz, 1983; White et al., 1992).

1.8 Aims

The aims and objectives of the study were as follows:

1. Identify the bacteria associated with a healthy oral cavity and FCGS in cats using conventional aerobic and anaerobic microbiological culture

2. Identify the bacteria associated with a healthy oral cavity and FCGS in cats using 16S ribosomal RNA (rRNA) gene sequencing and determine if fastidious

3. Determine the prevalence of FCV, FIV, FHV and FeLV in each cohort

4. Quantify the expression patterns of TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 and cytokines IL-1β, TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10 and IL-12 in oral tissue using quantitative PCR (qPCR)

5. Using matched data arising from Aims 2 and 4, establish if associations exist between the presence and quantity of bacterial species and TLR expression

6. Elucidate if bacterial/viral load and TLR expression levels correlate with the pattern of cytokine expression

7. Explore the association of putative risk factors for FCGS in cats

Chapter 2 Materials and methods

2.1 Ethical approval

Ethical approval was obtained from the Local Research Ethics Committee to collect biopsies, swabs and blood samples from cats suffering from FCGS. For the healthy control samples, approval was given to collect saliva swabs. Biopsies and blood samples were collected from cats euthanatised for reasons unrelated to the oral cavity but that presented with a healthy oral cavity.

2.2 Sample collection

Samples were collected from cats with a clinical appearance and history of FCGS as described in Section 1.2.1.2. To exclude other oral diseases such as juvenile stomatitis and non-chronic forms of stomatitis (Williams and Aller, 1992), all cats were over 12 months of age and the time since onset of the disease was at least two months. FCGS samples (32) were collected by two veterinary dentistry specialists from cats brought to the following veterinary clinics: Dental Vets in North Berwick (F2-F19) and Mulberry Court Veterinary Surgery in Sudbury (F20-F32) between March 2009 and December 2011. Sample F1 was collected at the School of Veterinary Medicine, University of Glasgow. Healthy control samples (16) were collected from cats at the School of Veterinary Medicine, University of Glasgow. Control swabs from healthy cats were also collected at a local veterinary practice.

From each cat, three swab samples were collected; one sterile swab was placed into viral transport medium (VTM) (University of Glasgow, School of Veterinary Medicine, Glasgow, UK) and two sterile swabs were placed into anaerobic transport medium (ATM) (Barloworld Scientific, Staffs, UK). Each swab was taken from the palatoglossal folds of the oral cavity (Figure 1.1). Blood samples were collected from the cats following a standard protocol. A total of 3 ml blood was collected, from which 1 ml was transferred into a tube containing ethylenediaminetetra-acetic acid (EDTA) (Teklab, Durham, UK) and 2 ml into a tube containing heparin (Teklab).

Tissue samples were collected from the caudal mucosa of the cats' oral cavity. Two biopsies with a minimum size of 2 x 2 mm were collected. One of the biopsies was placed into RNAlater (Sigma-Aldrich, Irvine, UK) and one into 4% buffered paraformaldehyde.

From 18 cats with FCGS (F2–F19), swab, blood and tissue samples were obtained; from three cats (F20-22) blood and tissue samples were collected, one VTM and one ATM swab were obtained; from one cat with FCGS (F1) one ATM swab sample was collected; from seven cats with FCGS (F23-F28) complete blood samples, tissue biopsies and viral swabs were collected; from one cat (F29) VTM swabs, heparin blood and tissue biopsies were collected; from two cats with FCGS (F30, F31) tissue biopsies and VTM swabs were collected; from one cat (F32) complete blood samples and tissue biopsies were collected (Table 2.1).

From one healthy cat (H1) one ATM swab sample was collected; from two healthy cats (H2, H5) blood and tissue samples were collected, one VTM and one ATM swab were obtained; from two healthy cats (H3, H15) one VTM swab, one ATM swab, tissue biopsies and heparin blood were collected; from three healthy cats (H4, H6, H14) one VTM swab, one ATM swab and tissue biopsies were collected; from seven healthy cats (H7-H13) two swabs, one ATM and one VTM were collected; from one healthy cat (H16), one VTM swab, tissue biopsies and blood samples were collected (Table 2.1).

2.3 **Sample processing**

The blood, one formalin sample, one ATM swab and the VTM swab were sent for diagnostic evaluation to the School of Veterinary Medicine, University of Glasgow. One ATM swab and the biopsy stored in RNAlater were sent to Glasgow Dental Hospital and School for laboratory analysis.

Each ATM swab was immersed in 2 ml Fastidious Anaerobe Broth (FAB) (Bioconnections, Leeds, UK) and mixed for 30 s to remove bacteria. 1 ml of the mixed material was plated immediately, as described in Section 5.2.1.1, and 1 ml was stored at -20ºC until needed for DNA extraction. Each biopsy sample in RNAlater was stored at 4ºC until required for RNA extraction.

2.4 **Statistical analysis**

Throughout the thesis statistical analysis was performed using GraphPad Prism for Windows, version 5 (GraphPad Software Inc., San Diego, California, USA). For the comparison of two unpaired samples, continuous data with a normal distribution was analysed with a 2-sample t-test, the Mann-Whitney U test was used as a non-parametric test for continues data. All ordered data was analysed by a Mann-Whitney U test. For nominal data, a X^2 test was used and binary data was also analysed by a X^2 test,

however, when category numbers were below 5 in binary data, a Fisher's exact test was used. In a few occasions, when more than 2 groups were compared, the non-parametric Kruskal-Wallis test was used with the Dunn's comparison test as the post-hoc analysis.

Table 2.1: Sample collection from 32 cats with FCGS (A) and 16 healthy cats (B)

A	ATM swab	VTM swab	Biopsies	EDTA	Heparin
F1	■				
F2	■	■	■	■	■
F3	■	■	■	■	■
F4	■	■	■	■	■
F5	■	■	■	■	■
F6	■	■	■	■	■
F7	■	■	■	■	■
F8	■	■	■	■	■
F9	■	■	■	■	■
F10	■	■	■	■	■
F11	■	■	■	■	■
F12	■	■	■	■	■
F13	■	■	■	■	■
F14	■	■	■	■	■
F15	■	■	■	■	■
F16	■	■	■	■	■
F17	■	■	■	■	■
F18	■	■	■	■	■
F19	■	■	■	■	■
F20	■	■	■	■	■
F21	■	■	■	■	■
F22	■	■	■	■	■
F23		■	■	■	■
F24		■	■	■	■
F25		■	■	■	■
F26		■	■	■	■
F27		■	■	■	■
F28		■	■	■	■
F29		■	■		■
F30		■	■		
F31		■	■		
F32			■	■	■

B	ATM swab	VTM swab	Biopsies	EDTA	Heparin
H1	■				
H2	■	■	■	■	■
H3	■	■	■		■
H4	■	■	■		
H5	■	■	■	■	■
H6	■	■	■		
H7	■	■			
H8	■	■			
H9	■	■			
H10	■	■			
H11	■	■			
H12	■	■			
H13	■	■			
H14		■	■		
H15		■	■		■
F16		■	■	■	■

2.5 List of suppliers

Suppliers	Product
Affymetrix/USB, High Wycombe, UK	Rapid Gel-XL-40% concentrate
Agilent technologies, Cheshire, UK	StrataClone™ PCR cloning kit
BioConnections, Leeds, UK	Fastidious anaerobe broth
Cambio ltd, Cambridge, UK	SequiTherm EXCEL™ II DNA
E&O laboratories ltd, Bonnybridge, UK	Defibrinated horse blood
Fermentas Life Sciences, York, UK	*Rsa*I, *Mnl*I restriction enzymes
Fisher Scientific, Loughborough, UK	Dimethyl sulfoxide (DMSO) Ethanol Sodium hydroxide (NaOH) Phosphate buffered saline (PBS)
Invitrogen, Paisley, UK	SuperScript™ first-Strand Synthesis System QPCR primers SYBR-Green Mastermix
Lab M limited, Bury, UK	Fastidious anaerobe agar
MWG biotech, Milton Keynes, UK	357f IRD800 labelled primer
New England Biolabs, Hitchin, UK	100 bp DNA marker dNTPs
Promega, Southampton, UK	1 x *GoTaq*® PCR buffer 1.5mM $MgCl_2$ *GoTaq*® polymerase
Qiagen, Crawley, UK	Qiaquick PCR Purification Kit RNeasy® RNA extraction kit
Roche, Burgess Hill, UK	Agarose
Sigma Genosys, Cambridge, UK	Primers 63F/1387R
Sigma-Aldrich, Irvine, UK	Ammonium persulphate Ampicillin Colombia agar Ethidium bromide Glycerol L-agar L-broth N,N,N',N' tetramethylethyl enediamine (TEMED) Urea
Technical Service Consultants LTD, Heywood, UK	PROTECT cryopreservation vials
Thermoscientific, Epsom , UK	Reddymix
VWR, Lutterworth, UK	0.5 M Hydrogen chloride (HCl)

2.6 General stock solutions and buffers

Columbia Blood Agar:	42 g powder dissolved in 1 litre dH_2O. Sterilise by autoclaving. Cool to 50°C and add 7.5% defibrinated horse blood.
Ethidium bromide (10 mg/ml):	10 g ethidium bromide, dH_2O to 1 litre. Store away from light.
Fastidious anaerobe blood agar:	46 g powder dissolved in 1 litre dH_2O by swirling and mixing. Sterilise by autoclaving. Cool to 50°C and add 7.5% defibrinated horse blood.
Fastidious anaerobe broth :	29.7 g powder dissolved in 1 litre dH_2O. Sterilise by autoclaving.
L agar:	35 g powder dissolved in 1 litre dH_2O. Sterilise by autoclaving.
L Broth Medium :	25 g powder dissolved in 1 litre dH_2O. Sterilise by autoclaving.
4% Paraformaldehyde:	4 g paraformaldehyde dissolved at 70-80°C in 100 ml 1 x PBS. Add 1 ml of 1M NaOH pH 7.2-7.4
5M NaOH:	200g NaOH, dH_2O to 1 litre sterilise by filtration.
Phosphate buffered saline (PBS):	Dissolve one tablet in 100 ml dH_2O. Sterilise by autoclaving
Proteinase-K:	Dissolve 100 mg powder in 5 ml nuclease-free water. Aliquot and store at -20°C.
10x Tris/Borate/EDTA (TBE):	108 g Tris base 55 g Boric Acid 50 ml 0.2M EDTA (pH 8.0) dH_2O to 1 litre

Chapter 3 Clinical signs and laboratory diagnostic evaluation

3.1 Introduction

FCGS is a poorly described disease. The clinical signs that are seen in cats with FCGS are mostly a direct result of the pain caused by the oral inflammation and include a poor appetite, dysphagia, weight loss, loss of grooming behaviour, excessive salivation, halitosis and pawing at the mouth (Southerden and Gorrel, 2007). Other oral pathologies are often seen in combination with FCGS, these include periodontal disease with calculus accumulation and feline odontoclastic resorption lesions (Hennet, 1997). The signalment of cats with FCGS has been sparsely studied with no evidence of sex, age or breed predisposition (Healey et al., 2007).

The description of the oral lesions in FCGS has not been standardised and a variety of lesions can be seen. As described in Section 1.2.1.2, the most recent literature refers to the most severe and hard to treat chronic form of FCGS as having inflammation at two main sites; the tissue lateral to the palatoglossal folds and the attached mucosa at the premolar and molar area, extending to the buccal mucosa (Southerden, 2010; Hennet et al., 2011). Other sites in the oral cavity, such as the tongue and pharynx, can be affected but lesions in these areas are seen in less than a quarter of the patients (Johnessee and Hurvitz, 1983; White et al., 1992; Hennet, 1997; Healey et al., 2007; Bellei et al., 2008).

The blood biochemistry and haematology data that has been described in cats with FCGS show features that can be expected in cases of chronic inflammation. Increases in the total protein and globulin concentration in the blood of cats with FCGS have been demonstrated (Johnessee and Hurvitz, 1983; White et al., 1992; Mihaljevic, 2003). Haematology often shows an elevated white blood cell count (WBCC) (Johnessee and Hurvitz, 1983; White et al., 1992). The differential WBCC in FCGS generally show an absolute lymphopenia and a neutrophilia with a mild left shift (Johnessee and Hurvitz, 1983).

In this chapter the signalment and the clinical signs of the cats with FCGS are described. The distribution of the inflammatory lesions in the oral cavity of the cats is reported and all biochemistry and haematology results of the cats with FCGS are evaluated.

3.2 Materials and methods

3.2.1 History and clinical signs

For each cat, where possible, detailed records were collected in the form of a questionnaire (Appendix). Signalment (age, sex and breed) and vaccination status were recorded and the cats' owner together with the veterinarian and/or veterinary nurse completed the questionnaire.

3.2.2 Oral examination

An oral examination was performed under general anaesthesia. Concurrent oral diseases were recorded and full mouth radiographs were taken. The severity of plaque and calculus accumulation was recorded and the grade of periodontal disease assessed. Plaque accumulation was assessed according to the plaque index by Loë (1967):

- Grade 0: No plaque in the gingival area.

- Grade 1: Mild; a film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may only be recognized by running a probe across the tooth surface.

- Grade 2: Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye.

- Grade 3: Severe; abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface.

Dental calculus was scored according to the following categories:

- Grade 0: No dental calculus.

- Grade 1: Supragingival with slight subgingival calculus accumulation.

- Grade 2: Moderate subgingival calculus accumulation.

- Grade 3: Abundant supragingival and/or subgingival calculus accumulation.

The scoring used for periodontal disease was as advised by the American Veterinary Dental College (AVDC) Nomenclature committee (http://avdc.org/nomenclature.html) (Wolf et al., 2005) and divided as follows:

- Grade 0: Clinically normal.
- Grade 1: Gingivitis only, without attachment loss.
- Grade 2: Initial periodontitis, less than 25% attachment loss.
- Grade 3: Moderate periodontitis, 25-50% attachment loss.
- Grade 4: Severe periodontitis, more than 50% attachment loss.

Tonsillitis was scored using the range of none, mild, moderate and severe tonsillitis according to visual signs of inflammation, ranging from mild swelling and redness to exudate and necrotic foci. The degree of mucosal inflammation in the oral cavity was scored according to a modified version of the gingival index (Loe, 1967):

- Grade 0: No inflammation; pale pink to pink mucosal tissue.
- Grade 1: Mild inflammation; reddened, may include slight oedema.
- Grade 2: Moderate inflammation; severe redness, oedema.
- Grade 3: Severe inflammation; severe redness and oedema, ulceration and hypertrophy.

The degree of mucosal inflammation was scored for each of the following anatomical sites in the feline oral cavity (Figure 1.1):

- Maxillary buccal mucosa.
- Mandibular buccal mucosa.
- Maxillary attached gingiva.
- Mandibular attached gingiva.
- Mucosa lateral to the palatoglossal folds.
- Molar salivary gland.

- Oropharynx.
- Lingual/sublingual mucosa.

3.2.3 Blood biochemistry and haematology

Blood biochemistry values and haematology results, including blood smears, were assessed by the Veterinary Diagnostics Service, School of Veterinary Medicine, University of Glasgow. The tested profiles and the reference range can be found in Tables 3.1 and 3.2.

3.2.4 Statistics

Graphical representations and statistics of clinical signs and signalment were prepared in GraphPad Prism for Windows, version 5 (GraphPad Software Inc., San Diego, California, USA).

Table 3.1: Biochemistry profile used by the Veterinary Diagnostic Service

Biochemistry	Abbreviation	Reference range	Unit
Sodium		145-160	mmol/l
Potassium		2.6-5.2	mmol/l
Sodium:Potassium ratio		27-	
Chloride		94-120	mmol/l
Calcium		1.6-2.65	mmol/l
Phosphate		1.29-2.84	mmol/l
Urea		2.7-9.2	mmol/l
Creatinine		91-180	µmol/l
Cholesterol		1.8-5.2	mmol/l
Triglyceride		-0.6	mmol/l
Total Bilirubin		-10	µmol/l
Alkaline Phosphatase	ALK phos	-100	U/l
Aspertate aminotransferase	AST	-30	U/l
Alanine aminotransferase	ALT	-35	U/l
Gamma-glutamyl transferase	GGT	-15	U.l
Total Protein		60-85	g/l
Albumin		26-36	g/l
Globulin		27-45	g/l
Albumin:Globulin ratio		0.6-1.5	

Table 3.2: Haematology profile used by the Veterinary Diagnostic Service

Heamatology	Abbreviation	Reference range	Unit
Red blood cell count	RBCC	5.0-10.0	x10 E12/l
Haemoglobin	Hb	10.0-15.0	g/dl
Haematocrit	HCT	30-45	%
Mean corpuscular volume	MCV	39.0-55.0	fl
Mean corpuscular haemoglobin	MCH	12.5-17.5	pg
Mean corpuscular haemoglobin concentration	MCHC	30.0-36.0	g/dl
White blood cell count	WBCC	5.5-15.5	x10 E9/l
Band Neutrophils		0.00-0.3	x10 E9/l
Neutrophils		2.5-12.5	x10 E9/l
Lymphocytes		1.5-7.0	x10 E9/l
Monocytes		0.00-0.85	x10 E9/l
Eosinophils		0.00-1.50	x10 E9/l
Basophils		0.00-0.1	x10 E9/l
Normoblasts			x10 E9/l

3.3 Results

3.3.1 Signalment

A total of 32 cats with FCGS and 16 healthy cats were included in the study. The mean age of the cats with FCGS was 7.6 years, standard deviation (SD) 4.5 years with a minimum age of 1 year and a maximum of 17 years. Most cats fell into the age category of 5-10 years old (61-119 months). The mean age of the healthy cats was 6.7 (SD 3.6) with a minimum age of 1.2 years and a maximum age of 13 years (Table 3.3 and 3.4, Figure 3.1). Nineteen cats from the FCGS group were male (17 neutered) and 12 cats were female (10 neutered). The sex of one cat was unknown. Six of the healthy cats were male (four neutered) and seven were female (five neutered). The sex of two healthy cats was unknown (Table 3.3, Figure 3.2). The majority of cats were domestic shorthair (DSH). In the FCGS group 77.4% were DSH and 19.4% were pedigree cats. In the healthy group 93.3% of the cats were DSH and one cat (6.7%) was pedigree (Table 3.3).

3.3.2 Vaccination status

The vaccination status was recorded for 22 of the cats from the FCGS group. Sixty-eight percent of these cats were vaccinated every 12-18 months. All vaccinated cats received vaccines for FCV, FHV-1 and feline panleukopenia virus (FPV). A total of 45.5% of the cats were vaccinated against FeLV and 9.1% of the cats were vaccinated against *Chlamydophila felis*. 27.3% of the cats were not regularly vaccinated and the vaccination status of one cat was unknown (Table 3.5).

3.3.3 Clinical signs

The clinical signs of 22 cats with FCGS were recorded. The signs that were most often recorded when the cats were presented to the veterinarian included halitosis in 54.6% of the cats, excessive salivation in 40.9%, dysphagia in 40.9%, weight-loss in 40.9% and a change in grooming behaviour in 40.9%. Thirty-two percent of the cats did not exhibit any of these features. The maximum number of clinical signs seen together was nine out of the eleven described signs (in cat F18) (Tables 3.6 and 3.7).

3.3.4 Previous medication

All cats in the current study received previous medication. Treatment of 20 cats with known history is shown in Table 3.8 and 3.9. Time since last treatment for antibiotics is shown on the date of the last tablet or two weeks after cefavecin injection, when action of the injection ends. Cats receiving treatment within two weeks prior to sampling were; 9 cats treated with antibiotics, 8 cats treated with NSAID's, one cat treated with corticosteroids and one cat treated with Interferon-ω.

Figure 3.1: Age distribution in 31 cats with FCGS and 14 healthy cats

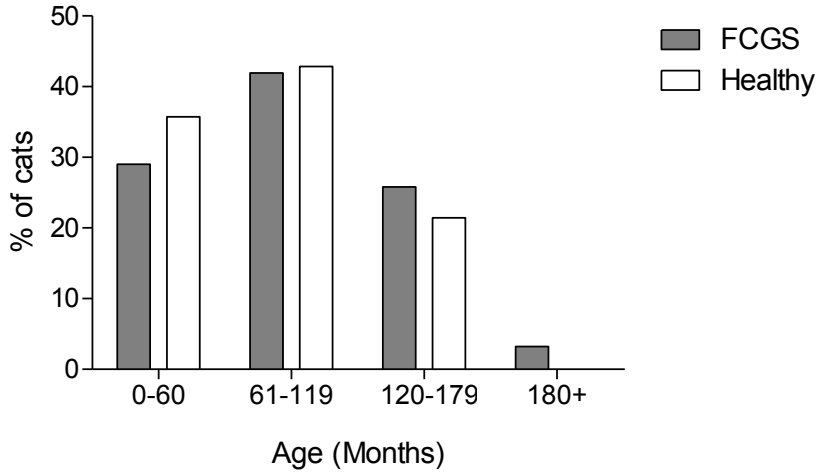


Table 3.3: Signalment of sampled cats

FCGS				Healthy			
Cat ID	**Age** (months)	**Breed**	**Sex**	**Cat ID**	**Age** (months)	**Breed**	**Sex**
F1	-	-	-	**H1**	-	-	-
F2	164	Siamese cross	FN	**H2**	96	Domestic shorthair	M
F3	104	Domestic shorthair	MN	**H3**	35	Domestic shorthair	MN
F4	164	Siamese	MN	**H4**	156	Domestic shorthair	FN
F5	90	Domestic shorthair	MN	**H5**	88	Domestic shorthair	FN
F6	47	Norwegian forest	MN	**H6**	32	Domestic shorthair	MN
F7	113	Domestic shorthair	MN	**H7**	51	Domestic shorthair	F
F8	14	Maine Coon	MN	**H8**	85	Domestic shorthair	FN
F9	66	Domestic shorthair	FN	**H9**	75	Domestic shorthair	F
F10	48	Domestic shorthair	F	**H10**	35	Domestic shorthair	FN
F11	80	Domestic shorthair	MN	**H11**	14	Maine Coon	F
F12	12	Domestic shorthair	MN	**H12**	88	Domestic shorthair	MN
F13	21	Domestic shorthair	FN	**H13**	115	Domestic shorthair	MN
F14	40	Domestic shorthair	MN	**H14**	-	Domestic shorthair	-
F15	87	British shorthair	FN	**H15**	131	Domestic shorthair	FN
F16	70	Domestic shorthair	FN	**H16**	131	Domestic shorthair	M
F17	114	Siamese	MN				
F18	65	Domestic shorthair	FN				
F19	90	Domestic shorthair	FN				
F20	155	Siamese	MN				
F21	72	Domestic shorthair	MN				
F22	15	Domestic shorthair	M				
F23	70	Domestic shorthair	MN				
F24	204	Domestic shorthair	F				
F25	126	Domestic shorthair	FN				
F26	17	Domestic shorthair	MN				
F27	43	Domestic shorthair	MN				
F28	156	Domestic shorthair	MN				
F29	120	Domestic shorthair	FN				
F30	111	Domestic shorthair	M				
F31	168	Domestic shorthair	FN				
F32	168	Domestic shorthair	MN				

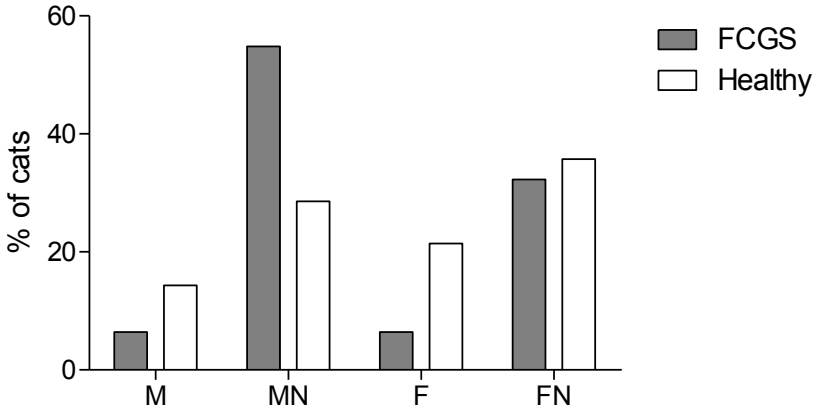
Signalment of 32 cats with FCGS and 16 control cats, F: female; FN: female neutered; M: male; MN: male neutered, -: no data available.

Table 3.4: Column statistics of the age in years

	FCGS	Healthy
Number of values	31	14
Minimum	1.000	1.167
25% Percentile	3.917	2.917
Median	7.250	7.208
75% Percentile	10.50	9.917
Maximum	17.00	13.00
Mean	7.565	6.738
Std. Deviation	4.486	3.594
Std. Error	0.8058	0.9605
Lower 95% CI of mean	5.919	4.663
Upper 95% CI of mean	9.210	8.813

Statistics of ages of 31 cats with FCGS and 14 healthy cats.

Figure 3.2: Sex distribution of 31 cats with FCGS and 14 healthy cats



M: male; MN: male neutered; F: female; FN: female neutered.

Table 3.5: Vaccination status

	Total vaccinated cats (%)	**FCV** (%)	**FHV-1** (%)	**FPV** (%)	**FeLV** (%)	**CF** (%)	**Unknown** (%)
Regular	68.2	68.2	68.2	68.2	45.5	9.1	
Non-regular	27.3	4.5	4.5	4.5	4.5		22.7
Unknown	4.5						4.5

Percentage of 22 cats with FCGS vaccinated against FCV, FHV-1, feline panleukopenia virus (FPV), FeLV and *Chlamydophila felis* (CF) every 12-18 months (regular) or over 18 months ago at the time of sampling (non-regular).

Table 3.6: Clinical signs shown by each individual cat

	Anorexia	Excessive salivation	Halitosis	Dysphagia	Ungroomed coat	Pawing at the mouth	Weight loss	Vomiting/retching	Change in grooming behaviour	Change in drinking behaviour	Facial swelling	None
F2			■						■			
F3		■		■		■	■	■			■	
F4				■			■					
F5	■	■	■	■			■	■	■		■	
F6			■				■		■		■	
F7		■	■	■					■			
F8		■	■	■		■	■		■			
F9	■	■	■	■		■	■	■				
F10				■			■					
F11		■	■	■	■	■		■	■			
F12												■
F13												■
F14												■
F15			■		■	■				■		
F16												■
F17			■		■	■			■			
F18		■	■	■	■	■	■	■	■	■		
F19												■
F22												■
F27												■
F29	■	■	■		■		■					
F32		■	■						■			

Clinical signs for individual cats (F#), for which data was available shown in black marked cells.

Table 3.7: Clinical signs

Clinical signs:	% of cats showing signs
Halitosis	54.6
Excessive salivation	40.9
Dysphagia	40.9
Weight loss	40.9
Change in grooming behaviour	40.9
Pawing at the mouth	31.8
Ungroomed coat	22.7
Vomiting/retching	22.7
Anorexia	13.6
Facial swelling	13.6
Change in drinking behaviour	9.1
Pyrexia	0
No signs	31.8

The percentage of the 22 cats with FCGS which show the described clinical signs.

Table 3.8: Days since medication before sample taking

Cat ID	Days since last antibiotic	Days since last NSAID	Days since last corticosteroid	Days since last Interferon-ω
F2	265	27	308	82
F3	52	5	N.A.	N.A.
F4	0	7	42	N.A.
F5	13	17	13	16
F6	70	21	99	N.A.
F7	18	10	18	N.A.
F8	4	48	21	N.A.
F9	4	4	380	N.A.
F10	2	12	63	N.A.
F11	175	9	242	367
F12	41	N.A.	N.A.	N.A.
F13	116	N.A.	N.A.	N.A.
F14	113	N.A.	N.A.	105
F15	3	3	N.A.	N.A.
F16	13	27	20	20
F17	169	155	62	N.A.
F18	8	11	238	3
F19	151	151	31	N.A.
F28	6	38	126	N.A.
F33	41	553	41	N.A.

Days since the last antibiotic tablet/injection, NSAID injection/dispense of oral suspension, corticosteroid injection/tablet and/or interferon injection/dispense of oral solution were given.

Table 3.9: Last supplied medication before sample taking

	Antibiotics	NSAIDs	Corticosteroid
F2	Clindamycin capsules	Meloxicam suspension	Methylprednisolone inj.
F3	Clindamycin capsules	Meloxicam suspension	N.A.
F4	Clindamycin capsules	Meloxicam suspension	Methylprednisolone inj.
F5	Clindamycin capsules	Meloxicam suspension	Prednisolone tablets
F6	Enrofloxacin tabl.	Meloxicam suspension	Methylprednisolone inj.
F7	Cefovecin inj.	Meloxicam inj.	Methylprednisolone inj.
F8	Amoxycillin/Clavulanate tabl.	Meloxicam suspension	Methylprednisolone inj.
F9	Cefovecin inj.	Meloxicam suspension	Dexamethasone inj.
F10	Clindamycin capsules	Meloxicam suspension	Methylprednisolone inj.
F11	Cefovecin inj.	Meloxicam suspension	Dexamethasone inj.
F12	Cefovecin inj.	N.A.	N.A.
F13	Clindamycin capsules	N.A.	N.A.
F14	Amoxycillin/Clavulanate tabl.	N.A.	N.A.
F15	Cefovecin inj.	Meloxicam suspension	N.A.
F16	Amoxycillin/Clavulanate inj.	Meloxicam suspension	Dexamethasone inj.
F17	Cefovecin inj.	Meloxicam suspension	Methylprednisolone inj.
F18	Cefovecin inj.	Meloxicam inj.	Methylprednisolone inj.
F19	Amoxicillin inj.	Meloxicam inj.	Dexamethasone inj.
F28	Amoxicillin inj.	Meloxicam suspension	Prednisolone tablets
F33	Cefovecin inj.	Meloxicam inj.	Depomedrone

Inj: injection, tabl.: tablet

3.3.5 Tooth health assessment

Plaque accumulation was recorded in 12 cats with FCGS. Sixty-six percent of the cats showed a moderate accumulation of plaque. No plaque accumulation was noted in 8.3% of cats suffering from FCGS (Table 3.10A).

Accumulation of dental calculus was recorded in 21 cats with FCGS. No calculus was seen in 23.8% of these cats. Mild accumulation was recorded in 28.6% of the cats, as was moderate accumulation, and 19.1% showed severe accumulation of calculus (Table 3.10A).

The presence and severity of tonsillitis was recorded in 12 cats with FCGS. Sixty-seven percent of the cats did not show signs of tonsillitis during physical examination of the oral cavity. Twenty-five percent had mild tonsillitis, 8.3% had moderate tonsillitis and none of the cats had severe tonsillitis at the time of examination (Table 3.10A).

All 21 cats that had an assessment of periodontal disease, showed at least grade 1 periodontal disease (gingivitis). Ten percent of the cats showed grade 2 and 33% showed grade 3 periodontal disease. Severe periodontal disease (grade 4) was not recorded in combination with FCGS (Table 3.10B).

The presence of TR lesions in combination with FCGS was assessed in 21 cats. Of these cats, 33% showed TR lesions (Table 3.10C).

3.3.6 Severity of oral inflammation

Recordings were made of the location and the severity of oral inflammation in 21 cats with FCGS (Table 3.11). In 19 cats at least one of the assessed locations was severely inflamed. Two of the eight assessed sites were most often severely inflamed; the maxillary attached gingiva and the mucosa lateral to the palatoglossal folds (Figure 3.3 and 3.4). At these two locations, grade 3 inflammation was recorded in 66.7% of the cats. Moreover, none of the cats was free of inflammation in these areas and these two locations showed at least mild inflammation in all 21 cats (Table 3.12).

Table 3.10: Clinical examination results

A					
	None (% of cats)	**Mild (% of cats)**	**Moderate (% of cats)**	**Severe (% of cats)**	
Plaque	8.3	16.7	66.7	8.3	
Calculus	23.8	28.6	28.6	19.1	
Tonsillitis	66.7	25	8.3	0	
B					
	Grade 0 (% of cats)	**Grade 1 (% of cats)**	**Grade 2 (% of cats)**	**Grade 3 (% of cats)**	**Grade 4 (% of cats)**
Periodontal disease	0	57.1	9.5	33.3	0
C					
	Present (% of cats)	**Not present (% of cats)**			
Tooth resorption lesions	33.3	66.7			

A: The severity of plaque (12 cats) and calculus (21 cats) accumulation and the presence of tonsillitis (12 cats) shown as a percentage of cats assessed. B: The grade of periodontal disease present (21 cats), as a percentage of cats assessed. C: Percentage of 21 cats assessed that were recorded to have tooth resorption lesions in combination with FCGS.

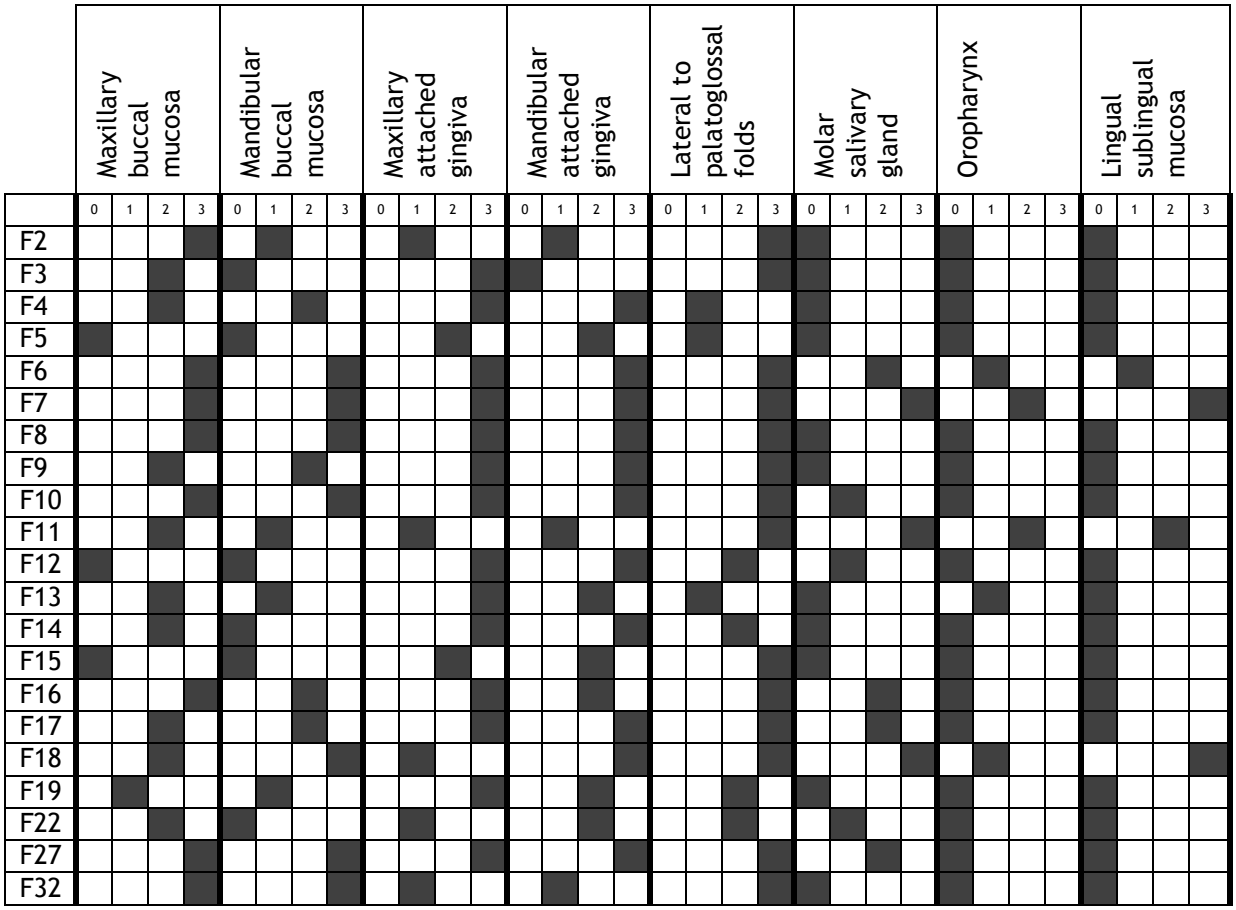
Figure 3.3: Intra-oral photograph of severe FCGS

Intra-oral photograph after anaesthetic premedication of case F7 showing severe involvement of the attached gingiva and mucosa lateral to the palatoglossal folds.

Figure 3.4: Side view of severe FCGS

Intra-oral photograph after anaesthetic premedication of case F7 showing severe involvement of attached gingiva and buccal mucosa.

Table 3.11: Inflammation severity for each anatomical site for each affected cat



Severity of inflammation for each individual cat (F#) for which data was available shown in black marked cells; 0: no inflammation; 1: mild inflammation; 2: moderate inflammation; 3: severe inflammation.

Table 3.12: Inflammation severity at each assessed site

Inflamed site	None (%)	Mild (%)	Moderate (%)	Severe (%)
Maxillary buccal mucosa	14.3	4.8	42.9	38.1
Mandibular buccal mucosa	28.6	19.1	19.1	33.3
Maxillary attached gingiva	0	23.8	9.5	66.7
Mandibular attached gingiva	4.8	14.3	28.6	52.4
Lateral to the palatoglossal folds	0	14.3	19.1	66.7
Molar salivary gland	52.4	14.3	19.1	14.3
Oropharynx	76.2	14.3	9.5	0
Lingual/sublingual mucosa	81.0	4.8	4.8	9.5

Percentages of inflammation severity per site in 21 cats with FCGS.

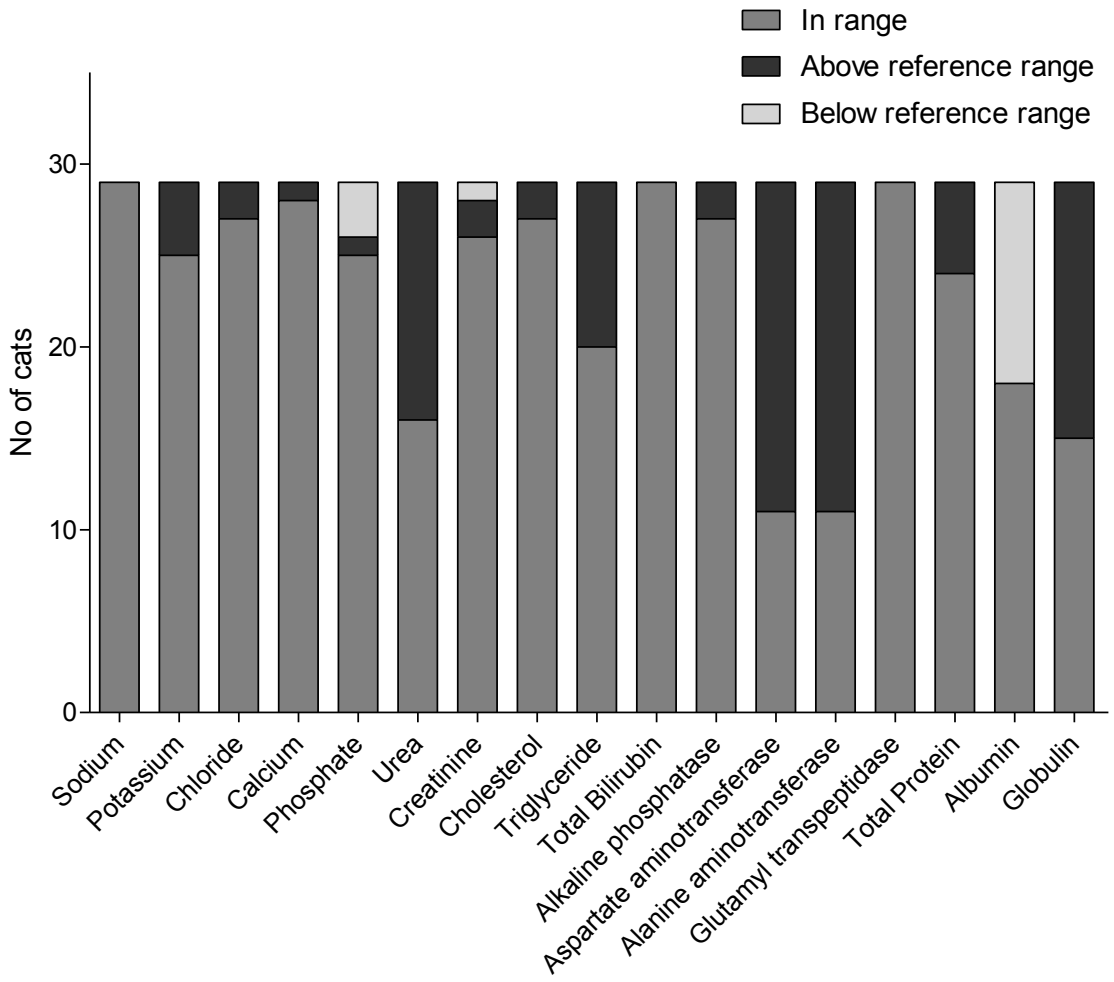
The two oral sites that were least affected were the oropharynx and lingual mucosa. These two locations were free of inflammation in 76.2% and 81.0% of the cats, respectively. In the oropharynx no severe inflammation was recorded and only two out of 21 cats showed severe inflammation at the lingual or sublingual mucosa (Tables 3.11 and 3.12).

The molar salivary gland area showed no signs of inflammation in 52.4% of cases. A variety of mild, moderate and severe inflammation was seen in the rest of the cases. The other assessed sites, the maxillary and mandibular buccal mucosa and the mandibular attached gingiva, showed signs of mild, moderate or severe inflammation in the majority of cases (Tables 3.11 and 3.12).

3.3.7 Blood biochemistry

The blood biochemistry results of 29 cats are shown in Figure 3.5 and Table 3.13. Sodium, potassium and sodium:potassium ratios were within the reference ranges in 100%, 86.2% and 93.1% respectively of the 29 cats tested. An increase in potassium was seen in four cats and a decrease of the ratio in two of these four cats. Chloride levels were increased in two cats (6.9%). One cat (3.4%) showed a slight increase in calcium levels. Phosphate levels were decreased in three cats (10.3%) and increased in one cat (3.4%). Urea and creatinine levels were increased in 13 cats (44.8%) and 2 cats (6.9%) respectively. Cholesterol levels were increased in two cats (6.9%) and triglyceride values increased in nine (31.0%) of the tested cats. Alkaline phosphatase (ALP) levels were increased in two cats (6.9%). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were both above the reference range in 18 cats (62.1%). The total protein concentration was above the reference range in five cats (17.2%). Albumin levels were decreased in 11 cats (37.9%) and globulin levels were increased in 14 cats (48.3%). The albumin-globulin ratio was decreased in 14 cats (48.3%). Total bilirubin and γ-glutamyl transpeptidase (GGT) levels were within the reference range in all cats.

Figure 3.5: Blood biochemistry results



The number of cats with blood biochemical values within, above or below the reference range.

Table 3.13: Biochemistry blood results of 29 cats with FCGS

Cat ID	Sodium (mmol/l)	Potassium (mmol/l)	Sod:Pot ratio	Chloride (mmol/l)	Calcium (mmol/l)	Phosphate (mmol/l)	Urea (mmol/l)	Creatinine (umol/l)	Cholesterol (mmol/l)	Triglyceride (mmol/l)	Total Bilirubin (umol/l)	ALP (U/l)	AST (U/l)	ALT (U/l)	GGT (U/l)	Total Protein (g/l)	Albumin (g/l)	Globulin (g/l)	Alb:Glob ratio
Ref Range	145-160	2.6-5.2	27-	94-120	1.6-2.65	1.29-2.84	2.7-9.2	91-180	1.8-5.2	-0.6	-10	-100	-30	-35	-15	60-85	26-36	27-45	0.6-1.5
F2	151.8	4.95	30.7	119.3	2.48	1.92	16.1	219	3.63	0.28	2.9	42	15	44	0	67	23	44	0.52
F3	155.7	2.85	54.6	119.9	2.44	0.74	9.5	148	2.35	1.53	4.4	61	23	33	0	60	30	30	1
F4	156.3	4.33	36.1	115.3	2.25	1.61	9.1	108	4.27	0.18	1.7	80	65	334	0	70	29	41	0.71
F5	150.2	4.22	35.6	116.8	2.35	1.55	7.9	117	4.94	0.71	0.7	52	20	28	0	67	30	37	0.81
F6	150.9	3.17	47.6	113	2.42	1.61	9	121	4.2	1.11	0	42	20	27	0	69	27	42	0.64
F7	149.1	3.79	39.3	110.8	2.3	1.26	6.8	133	2.56	0.27	0.4	33	29	26	2	95	22	73	0.3
F8	150.3	5.5	27.3	111.8	1.74	1.79	6.3	130	2.71	0.48	0	34	25	33	0	81	28	53	0.5
F9	151.9	4.8	31.6	109.9	2.12	2.36	6.4	91	4.1	0.14	3	9	43	71	2	86	27	59	0.5
F10	148.4	3.8	39.1	111.8	2.41	1.69	10.2	122	2.83	0.49	0	20	44	22	1	87	25	62	0.4
F11	146.8	5	29.4	112.8	2.14	2.47	9.1	129	4.29	0.09	1	13	42	29	0	82	22	60	0.4
F12	149.7	4.7	31.9	111.6	2.5	2.27	11.6	151	5.34	0.23	1	158	29	39	0	71	32	39	0.8
F13	156.9	4.6	34.1	114.6	2.41	2.01	8.9	126	1.93	0.12	2	46	48	45	0	80	34	46	0.7
F14	147.6	4	36.9	111.8	2.46	1.44	9.1	152	5.04	0.18	1	37	44	68	0	72	30	42	0.7
F15	149.3	4.4	33.9	113	2.19	1.68	10	143	3.64	0.15	2	42	47	63	0	76	24	52	0.5
F16	151.5	3.4	44.6	118.5	2.39	1.24	5.5	90	3.32	0.14	2	47	24	50	0	70	31	39	0.8
F17	152.9	3.6	42.5	114.5	2.38	1.35	12.4	117	3.16	0.33	0	46	42	83	2	78	29	49	0.6
F18	148.6	4	37.2	116.5	2.16	1.54	7	131	3.01	0.31	0	3	28	31	0	91	20	71	0.3
F19	146.8	4.7	31.2	112	2.3	1.9	7.2	135	2.19	0.61	4	67	20	35	0	60	27	33	0.8
F20	149.5	4.5	33.2	113.6	2.32	1.69	11.5	223	4.19	1.14	0	70	42	74		77	27	50	0.5

Table 3.13 continued

Cat ID	Sodium (mmol/l)	Potassium (mmol/l)	Sod:Pot ratio	Chloride (mmol/l)	Calcium (mmol/l)	Phosphate (mmol/l)	Urea (mmol/l)	Creatinine (umol/l)	Cholesterol (mmol/l)	Triglyceride (mmol/l)	Total Bilirubin (umol/l)	ALP (U/l)	AST (U/l)	ALT (U/l)	GGT (U/l)	Total Protein (g/l)	Albumin (g/l)	Globulin (g/l)	Alb:Glob ratio
Ref Range	145-160	2.6-5.2	27-	94-120	1.6-2.65	1.29-2.84	2.7-9.2	91-180	1.8-5.2	-0.6	-10	-100	-30	-35	-15	60-85	26-36	27-45	0.6-1.5
F21	147.2	6.9	21.3	113.4	2.18	3.14	10.2	128	3.36	0.44	1	38	81	51	2	77	24	53	0.5
F22	155.5	5.2	29.9	116	2.67	2.33	8.8	116	5.38	0.22	1	104	76	68	1	74	35	39	0.9
F23	149.8	4.9	30.6	114.9	2.29	1.46	9.1	103	3.86	1.47	3	47	56	72	3	70	27	43	0.6
F24	149.7	4.5	33.3	123.7	1.81	2.11	8.5	122		0.26	10	6	50	63	6	62	18	44	0.4
F25	145	5.2	27.9	119.1	2.11	2.13	11.7	173	3.69	0.52	2	47	43	79	0	69	19	50	0.4
F26	149.6	6	24.9	116.8	2.25	2.64	9.3	172	2.25	0.36	3	76	47	44	0	67	24	43	0.6
F27	149.4	4	37.4	114.6	2.21	1.71	9.9	91	2.34	1	0	35	30	29	0	82	27	55	0.5
F28	147.9	4.4	33.6	115.1	2.25	2.84	12.4	162	2.81	0.34	1	49	40	41	0	66	25	41	0.6
F29	154	5.4	28.5	120.1	2.23	1.86	7.3	101	3	1.01	0	54	47	30	0	60	28	32	0.9
F32	150.7	4.4	34.3	115.5	2.35	1.65	10	162	3.97	0.78	0	68	34	41	1	91	29	62	0.5

Values above reference range (purple), values below reference range (green). ALP: alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: glutamyl transpeptidase.

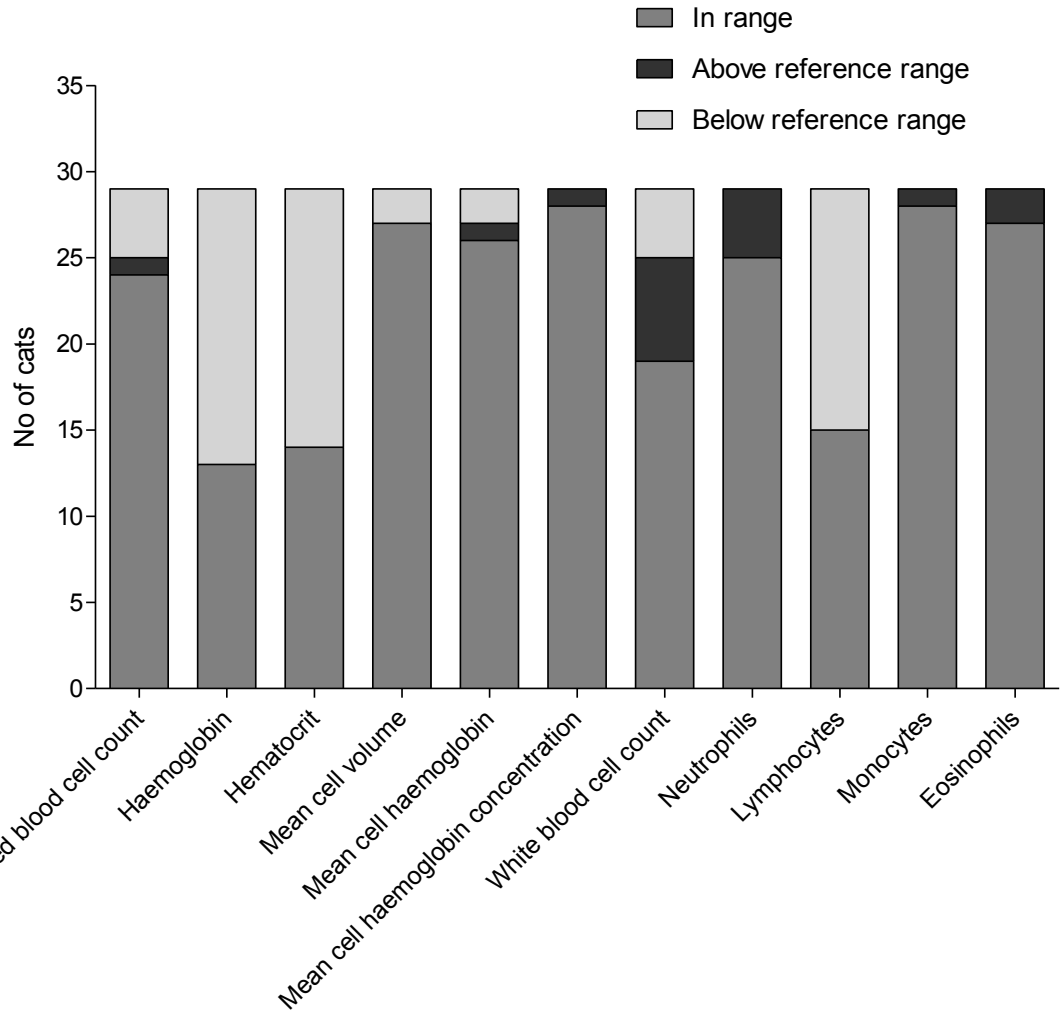
3.3.8 Haematology results

The haematology results of 28 cats are shown in Figure 3.6 and Table 3.14. The hematocrit (HCT) and haemoglobin (Hb) values were below the reference range in 15 (53.6%) and 16 (57.1%) of cats respectively. In addition, the red blood cell count (RBCC) was below the reference range in four cats (14.3%). The mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were within the reference ranges in 89.3-96.4% of the tested cats.

The WBCC was below the reference range in 3 cats (10.7%) and was above the reference range in six cats (21.4%). Lymphocyte numbers were below the reference range in 13 cats (46.4%). Neutrophils, eosinophils and monocytes were increased in number in 4 (14.3%), 2 (7.1%) and 1 (3.6%) of the cats with FCGS respectively. Band neutrophils were detected in six cats (21.4%) but were all within the reference range. Basophils were detected in six cats (21.4%), and were above the reference range in three cats (10.7%). Normoblasts were present in one cat (3.6%).

Blood smear analysis showed a poorly regenerative anaemia in 14 cats (50%). Increased rouleaux formation was seen in 15 cats (53.6%). Six cats (21.4%) showed a mild toxic change in the neutrophils. Spiculing was seen in 13 cats (46.4%) but was noted as a possible artefact in 10 cats (35.7%) (Table 3.15).

Figure 3.6: Haematology results



The number of cats with blood results within, above or below the reference ranges.

Table 3.14: Haematology results of 28 cats with FCGS

Cat ID	RBC (x10 E12/l)	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	WBC (x10 E9/l)	Band Neutrophils (x10 E9/l)	Neutrophils (x10 E9/l)	Lymphocytes (x10 E9/l)	Monocytes (x10 E9/l)	Eosinophils (x10 E9/l)	Basophils (x10 E9/l)	Normoblasts (x10 E9/l)
Ref range	5.0-10.0	10.0-15.0	30-45	39.0-55.0	12.5-17.5	30.0-36.0	5.5-15.5	0.00-0.3	2.5-12.5	1.5-7.0	0.00-0.85	0.00-1.50	0.00-0.1	
F2	5.29	6.5	21.5	40.6	12.3	30.3	8.34	-	7.423	0.5	0.197	0.25	0	-
F3	7.17	9.7	29.7	41.4	13.6	32.8	7.56	0.076	6.88	0.302	0.151	0.076	0.076	-
F4	6.4	10.5	33.4	52.2	16.4	31.5	6.51	-	5.989	0.26	0.13	0.13	0	-
F5	4.32	6.1	18.6	43.1	14.2	33	4.72	-	2.738	1.322	0.378	0.142	0	-
F6	5.86	8.3	25.2	43	14.2	33	10.25	-	7.38	1.948	0.41	0.512	0	-
F7	7.07	10.2	31.3	44.2	14.4	32.6	8.94	0.089	5.811	1.788	0.805	0.447	0	-
F8	8.12	12.2	37.1	45.7	15.1	33	10.9	-	8.611	0.545	0.645	0.981	0.109	-
F9	8.45	10.4	34.1	40.3	12.3	30.6	20.25	-	18.427	1.418	0.203	0.203	0	-
F10	5.31	7.7	24.8	46.7	14.5	31	13.52	-	10.005	2.298	0.27	0.946	0	-
F11	5.97	8.4	26.2	43.8	14.1	32.2	16.62	-	12.465	2.659	0.499	0.665	0.332	-
F12	10.09	13.1	37	36.7	12.9	35.2	7.64	0.153	2.598	4.584	0.229	0.076	0	0
F13	7.57	11.6	34.9	46.1	15.4	33.4	6	-	4.2	1.5	0.24	0.06	0	-
F14	8.03	12.1	36.9	46	15	32.7	5.54	-	3.601	1.662	0.111	0.166	0	-
F15	7.4	9.6	28.7	38.7	13	33.4	6.3	-	3.843	2.142	0.126	0.189	0	-
F16	5.89	9.8	28.9	49.1	16.6	33.8	6.06	-	4.484	1.091	0.182	0.303	0	-
F17	6.36	10.9	33.7	53.1	17.1	32.3	11.26	-	9.909	0.788	0.338	0.225	0	-
F18	4.86	8.1	25	51.4	16.7	32.5	13.57	-	10.042	1.9	0.95	0.543	0.136	-
F19	8.12	12.2	38.2	47.2	15	31.8	4.49	-	3.637	0.359	0.225	0.225	0.045	-

Table 3.14 continued

Cat ID	RBC (x10 E12/l)	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	WBC (x10 E9/l)	Band Neutrophils (x10 E9/l)	Neutrophils (x10 E9/l)	Lymphocytes (x10 E9/l)	Monocytes (x10 E9/l)	Eosinophils (x10 E9/l)	Basophils (x10 E9/l)	Normoblasts (x10 E9/l)
Ref range	**5.0-10.0**	**10.0-15.0**	**30-45**	**39.0-55.0**	**12.5-17.5**	**30.0-36.0**	**5.5-15.5**	**0.00-0.3**	**2.5-12.5**	**1.5-7.0**	**0.00-0.85**	**0.00-1.50**	**0.00-0.1**	
F20	7.52	11.6	35.1	46.6	15.4	33.1	**15.93**	-	11.948	1.912	0.159	**1.752**	0	0.159
F21	6.49	**9.5**	30.7	47.4	14.6	30.9	**17.24**	-	**14.309**	**0.69**	0.172	**2.069**	0	-
F22	7.38	11.2	33.8	45.8	15.2	33.2	8.93	0.268	7.055	**1.072**	0.179	0.357	0	0
F23	5.77	**9**	**27.6**	47.8	15.6	32.7	13.63	-	11.177	**1.363**	0.545	0.545	0	-
F24	**3.12**	**5.5**	**14.3**	45.7	**17.6**	**38.5**	**20.4**	-	**13.464**	4.896	0.816	1.224	0	-
F25	**3.32**	**5.5**	**14**	42.2	16.5	30.7	7.58	-	3.411	2.729	0.227	1.213	0	-
F26	6.48	10.3	31.6	48.7	15.9	32.6	6.47	0.194	4.205	1.876	0	0.129	0.065	-
F27	5.24	**8**	**23.2**	44.3	15.2	34.3	**20.68**	-	**14.269**	5.584	0.207	0.62	0	-
F28	5.79	**8.3**	**24.2**	41.8	14.3	34.3	**4.94**	-	4.501	**0.593**	0.099	0.198	0	-
F32	8.6	12.5	39.6	46.1	14.5	31.5	13.27	0.133	10.351	1.99	0.531	0.265	0	-

Values above reference range (purple), values below reference range (green). RBC: red blood cell count, Hb: haemoglobin, HCT: hematocrite, MCV: mean cell volume, MCH: mean cell haemoglobin, MCHC: mean cell haemoglobin concentration, WBC: white blood cell count. -: no value provided by the laboratory.

Table 3.15: Blood smear analysis

Findings	% of cats
Poorly regenerative anaemia	50
Increased rouleaux formation	53.6
Mild toxic change	21.4
Poikilocytes	14.3
Acanthocytes	10.7
Platelets above reference range	3.6
Heinz body formation	3.6
Keratocytes	3.6
Lymphoblasts	3.6
Plasmacytoid-lymphoid	3.6
Schistocytes	3.6
Spiculing	10.7
Spiculing, possible artefact	35.7

Description of findings in blood smears of 28 cats with FCGS.

3.4 **Discussion**

Investigations of the signalment of cats suffering from FCGS are sparse. It has been suggested that gingivitis/stomatitis/pharyngitis is mostly seen in young cats and is gradually progressive (Diehl and Rosychuk, 1993). In a UK study of 34 cats with FCGS, two age peaks were seen, 1 to 5 years and 10 to 13 years (Healey et al., 2007). A non-significant increase in age was seen in the group of cats with FCGS when compared to a random group of cats visiting the veterinary surgery. In the current study, the FCGS affected cats had a non-significant increase in age when compared to the healthy cats. The age distribution was similar in cats with FCGS and healthy cats. The age distribution differed from the results found by Healey et al. (2007) with most of the cats with FCGS in the current study being in the category of 5 to 10 years (61-119 months). The mean age in the present study of 7.6 years is comparable to other studies that show the age of cats with FCGS. Mean ages of 8.3 (Johnessee and Hurvitz, 1983), 7.1 (White et al., 1992) 8 (Hennet, 1997), 6.9 and 8.2 years (Hennet et al., 2011) have been described.

As described in Section 1.7.2, a variety of breeds including Siamese, Himalayan and Burmese have been suggested as being predisposed to FCGS (Frost and Williams, 1986; Diehl and Rosychuk, 1993). The current study showed a higher percentage of pedigree cats in the FCGS population when compared to a random group of 4858 cats visiting a veterinary surgery (Healey et al., 2007). In the current study, pedigree cats accounted for six (19.4%) of the 31 cats where the breed was recorded, compared to 482 (9.9%) of the 4858 cats studied by Healey et al. (2007). Siamese was the most prevalent breed. Of 31 cats with FCGS a total of four cats (12.9%) were Siamese or Siamese cross. Healey et al. (2007) reported that Siamese cats accounted for 93 (2%) of the total population of cats visiting a veterinary surgery. Although the present study shows a higher number of pedigree cats in the FCGS group, any breed predisposition remains questionable. The cases in this study were collected from a veterinary specialist and it is possible that a higher proportion of pedigree cats will be visiting a referral practice as compared to a first opinion practice as studied by Healey et al. (2007).

The sex distribution of cats with FCGS normally shows a proportion of female and male cats comparable to the normal population (Johnessee and Hurvitz, 1983; White et al., 1992; Healey et al., 2007). In this study a slightly higher proportion of male cats were seen, 19 (61.3%) of 31 cats for which the sex was recorded. This could be due to the small group of cats that was studied. An interesting finding was that a high proportion (83%) of the purebred cats with FCGS were male.

The source of the samples in a referral practice makes previous treatment from the first opinion practice inevitable. The cats shown in this study did not respond to initial treatment, which often includes antibiotics and steroid therapy. Any treatment given could have interfered with the results in the study. For the immunology and histopathology results corticosteroid treatment and interferon-ω are of importance. While all cats were showing the clinical signs of FCGS during time of sample taking, these immune modulators can interfere with the results. Five cats in the cohort received corticosteroids within one month of sample taking, and can therefore possibly have altered results, most likely, the reaction to an anti-inflammatory dose of corticosteroids is however a dampening of the immune reaction. For the bacteriology the antibacterial treatment is of importance. All cats received antibiotics and 9 cats within two weeks of sampling. Results could have been influenced by the use of antimicrobial therapy. The two most often used antibiotics were clindamycin and cefovecin. Clindamycin is known for its action against aerobic and anaerobic gram-positive cocci and anaerobic gram-negative bacteria. Cefovecin is a broad-spectrum antibiotic with bacteriocidal action against gram-positive and gram-negative bacteria.

Sixty-eight percent of 22 cats with FCGS in the study were vaccinated every 12-18 months for FCV, FHV-1 and FPV. This is comparable with a recent web-based questionnaire study on cat vaccination in the UK, in which 69% of cat owners reported vaccination in the last 12 months (Habacher et al., 2010). Another study that used a telephone-based questionnaire reported that 58% of cats had been vaccinated in the previous year (Murray et al., 2009). The vaccination status of the cats in the current study might have been higher than in a general population, due to the fact that the cats were referred to a specialist and owners of such cats might be more likely to ensure regular vaccination of their pets.

Halitosis was the most common reported clinical sign and was observed in 55% of cats. Halitosis is reported in other studies affecting 33% (White et al., 1992) and 75% (Bellei et al., 2008) of cats with gingivostomatitis. Other signs that were common in the current study were excessive salivation, dysphagia, weight loss and a change in grooming behaviour, which were all reported in 41% of cats. Dysphagia and anorexia together were reported in 44% of cases (White et al., 1992). Another study described 72% of the cases suffering from dysorexia and 16% of cases having dysphagia (Bellei et al., 2008). These signs are variable and difficult to standardise. These investigations require the use of questionnaire-based studies, and the owners are important in reporting the signs in their cats. The results will differ between households and studies. The questionnaire gives very useful information on the severity of the cases and provides the owners with key signs for monitoring any improvement during treatment.

Accumulation of calculus was recorded in 76% of the cats in this study, which was much higher than the 20% reported by White et al. (1992). It could be suggested that cats referred to a specialist have already been treated and have not responded to the initial treatment. Therefore it is possible that the cases seen in this study are more severe. All the FCGS cases seen in the study also had periodontal disease, with mild gingivitis being the minimal sign seen. This result is comparable to the gingivitis and periodontitis incidence of 93% reported by Hennet et al. (1997). FCGS has been described by Healey at al. (2007) in combination with 'visible dental diseases' in 70% of cases. Periodontopathy has been described in 69% of cases in a study of 32 cats with FCGS (Bellei et al., 2008). TR lesions as assessed radiographically were seen in 33% of the cats with FCGS. In other studies the proportion of cats with FCGS showing at least one TR lesion was 41% (Bellei et al., 2008) and 66% (Hennet, 1997).

In Section 1.2.1.2, the most important sites of inflammation in the oral cavity of cats with FCGS were described. The most severe form of FCGS is when there is inflammation at the palatoglossal folds and the attached gingiva. All the cases in the current study had at least mild inflammation at both these sites. Reports on the location of inflammation vary and for example caudal inflammation is present in 68%-100% of cats with FCGS (Johnessee and Hurvitz, 1983; Hennet, 1997; Healey et al., 2007; Bellei et al., 2008; Hennet et al., 2011). The lingual and sublingual mucosa (19.1%) and the oropharynx (23.8%) were the sites in the current study that demonstrated the lowest levels of inflammation. These findings are consistent with those from other studies that showed 9% to 18% of cases with involvement of the tongue (White et al., 1992; Hennet, 1997; Bellei et al., 2008). One study described inflammation of the pharynx and tongue as 'infrequent' (Healey et al., 2007) and another study showed no inflammation of the tongue in any of the nine cases investigated (Johnessee and Hurvitz, 1983).

In the introduction of this Chapter, FCGS is defined as a poorly described disease. With the current study, a contribution can be made that shows comparison to the more recent literature, describing the involvement of the caudal mucosa and attached gingiva and the rare involvement of lingual and sublingual inflammation. The cases shown in this study are referral cases and can therefore be considered to be the most severe and difficult to treat. FCGS should be described as an inflammation of the oral cavity with at least inflammation of the caudal oral cavity.

In the current study, most blood biochemistry changes that were observed were only mild. A mild increase in the enzymes AST and ALT was seen in 62.1% of the cases. Together with ALP and GGT, these enzymes are often used for the screening of hepatobiliary disease. However these enzymes may be elevated without any clinically

significant hepatobiliary disease (Webster, 2005). Corticosteroids are known to increase ALP, GGT, ALT and AST levels in dogs (Webster, 2005). Few studies have been done in cats, but high doses of glucocorticoids can increase ALT (Lowe et al., 2008). This reason can be excluded in the current study in cases that did not receive corticosteroids prior to the sample collection (Table 3.8 and 3.9). Hepatotoxicity due to long term use of NSAIDs has not been studied in cats (Sparkes et al., 2010), but NSAIDs are known for their potential to cause hepatotoxicity in dogs (MacPhail et al., 1998). Increase in liver enzymes can be induced by several other factors that include neoplasia, muscle injuries and systemic infections (Webster, 2005). The mild changes in AST and ALT are not considered clinically relevant in the current study and no other signs of liver disease were apparent in these cats.

A change in the albumin:globulin ratio was seen in 48.3% of the FCGS cases in the current study. The globulins were increased in 48.3% and albumin decreased in 37.9% of cases. Total protein was increased in 17.2% of cases. Serum globulin levels are known to increase in chronic infections and have been reported to be increased in cats with FCGS (Johnessee and Hurvitz, 1983; White et al., 1992; Mihaljevic, 2003). An increase in the total protein concentration was seen in 77% of cases by White et al (1992). No decrease of albumin has been reported in these studies. In the current study a compensatory hypoalbuminaemia was the most likely explanation of the lowered albumin levels (Couto, 2003b; duFort, 2005).

Urea levels were slightly increased in 44.8% of cats. It is likely that this had a prerenal cause (diBartola, 2005). Only a minority of the cases also had a slight increase in creatinine. The clinical relevance of the increased urea concentration needs to be assessed together with urine analysis and the clinical features of the patient.

Another biochemical change that was seen in a relatively high percentage of the cats was an increase in triglyceride levels (31.0% of the cats). Triglyceride increase can be a normal physiologic process after a meal, which normally resolves in 2-10 hours. Other causes for hyperlipidaemia include endocrine disorders and the use of glucocorticoids (Elliot, 2005).

Total blood counts were performed in 28 cats. The most commonly seen abnormality was a mild anaemia (53.6% of cats). The two most likely explanations of this are the anaemia of chronic disease (ACD) and the anaemia of inflammatory disease (AID). ACD is characterised by a mild, normocytic, normochromic, non-regenerative anaemia and with shortened erythrocyte life span (Gaschen, 2005). Similar findings are seen in AID which also shows a mild normocytic, normochromic, non-regenerative anaemia. AID is

mediated by inflammatory cytokines and is seen as the most common cause of anaemia in veterinary medicine. It is caused by a relative deficiency of iron, a decline in red cell survival and a decrease in erythropoietic response. Iron reduction is a metabolic response to deprive infectious organisms of the iron needed for their metabolism, and the decline in red cell survival is likely to be the most important factor in the onset of anaemia in cats (Feldman, 2005).

In the current study the WBCC was above the reference range in 21.4% of the cats and below the reference range in 10.7% of the cats. Johnessee and Hurvitz (1983) reported a mild elevation in WBCC in 33% of cats with FCGS and a leukocytosis in 20% of cats was observed by White et al. (1993). White blood cells increase in inflammation and infection through the acute phase response in which cytokines activate the leucocyte production (Paltrinieri, 2008). Other causes of elevated white blood cell levels include stress and glucocorticoid administration. A decrease in the WBCC can be caused by an increased white blood cell consumption (duFort, 2005; Paltrinieri, 2008). Lymphocytes were reduced in 46.4% of the cases in the current study, a similar finding to that observed previously in a study of nine cats with FCGS of which five showed an absolute lymphopenia and five showed a neutrophilia with a mild left shift (Johnessee and Hurvitz, 1983). Lymphopenia in cats can be caused by stress or by administration of corticosteroids and other often seen causes are acute viral infections e.g. FeLV, FIV and FPV. A ‘stress-induced’ change may be seen in cats, the typical ‘stress leukogram’ shows a decrease in lymphocyte numbers in combination with an increase in neutrophil numbers under the influence of an endogenous release (or exogenous administration) of corticosteroids (Couto, 2003a). In the current study 14.3% of the cases had an increase in neutrophil numbers and a mild toxic change was seen in 21.4% of cases. A toxic change is the morphological change of the cytoplasma in neutrophils and can be seen during bacterial infections or severe inflammation. Increases in the numbers of eosinophils and monocytes were also observed, and this can be caused by chronic infections and tissue damage. A toxic change in the neutrophils is regarded as an important indicator of the severity of disease in cats (Segev et al., 2006; Paltrinieri, 2008).

In the current study a group of cats in the age range 1 to 17 years was investigated. A higher proportion of purebred cats were seen during the study when compared to a population attending first opinion veterinary practice. The clinical signs were mostly related to the oral inflammation and the pain it causes. The inflammation was most severe in the caudal parts of the oral cavity and the maxillary attached gingiva and all cats were affected by periodontal disease. The mild changes in blood biochemistry and

haematology that were reported in this study are most likely related to the chronic inflammatory disease and the ‘stress’ it causes.

Chapter 4 Virological investigations

4.1 Introduction

A number of different viruses have been implicated in the aetiopathogenesis of FCGS particularly FeLV, FIV, FHV-1 and FCV.

In cats infected with FeLV, oral inflammation is a common clinical sign (Cotter et al., 1975; Frost and Williams, 1986). No definite correlation with FCGS has been shown and the percentage of cats positive for FeLV in FCGS populations is not greater than 17% (Johnessee and Hurvitz, 1983; Knowles et al., 1989; White et al., 1992; Hennet, 1997; Quimby et al., 2008).

The most commonly described clinical signs of cats infected with FIV are oral lesions. Studies have investigated the possible relationship between FIV and FCGS. Up to 80% of cats with FCGS are positive for FIV (Knowles et al., 1989) and an increase in the severity of oral lesions in cats with FCGS was seen when FIV infection was present (Dawson et al., 1991; Tenorio et al., 1991; Waters et al., 1993).

Few studies were able to show a correlation between FCGS and FHV-1 (Quimby et al., 2008; Dowers et al., 2010). Clinical signs of FHV-1 infection can include oral ulceration but this is not common (Gaskell et al., 2007). One study showed 88% of cats with FCGS were infected with a combination of FCV and FHV-1, compared to 21% of the cats with only periodontal disease (Lommer and Verstraete, 2003).

The role of FCV in FCGS has been investigated in many studies, with most showing that a high percentage of cats with FCGS are positive for the virus (Knowles et al., 1989; Lommer and Verstraete, 2003; Bellei et al., 2008). When cats with caudal stomatitis were selected from a group of cats with oral inflammation, 100% tested positive for the presence of FCV (Reubel et al., 1992).

In this chapter, the presence of FeLV, FIV, FHV-1 and FCV in healthy cats and those with FCGS was assessed.

4.2 Materials and methods

All virus diagnostics were performed by the Veterinary Diagnostics Service, School of Veterinary Medicine, University of Glasgow

4.2.1 FeLV ELISA

Plasma samples were tested for FeLV gag protein p27 by an ELISA to detect FeLV infection (Lutz et al., 1983). The test was performed using two anti-p27 monoclonal antibodies coated onto an ELISA plate. Serum samples were added in addition to a biotinlyated anti-p27 antibody; after the reaction time it was developed with a streptavidin-akaline-phosphatase system. Samples with a positive optical density (OD) reading were considered reactive for p27 antigen. Positive tests were confirmed using virus isolation (Jarrett et al., 1964).

4.2.2 FIV IFA

Plasma samples were analysed using an Immunofluorescence antibody (IFA) test. The test was carried out using infected and uninfected cells in wells of a 96-well teflon-coated microscope slide. Serial dilutions of test sample were placed on the wells and allowed to react; an anti-feline IgG conjugated with fluorescein isothiocyanate (FITC) was added before final examination under an ultraviolet (UV) microscope. The endpoint was taken as the last dilution showing fluorescence.

4.2.3 FCV and FHV-1 virus isolation

Virus isolation was performed on the VTM samples. The samples were centrifuged to pellet any debris and 0.5 ml was inoculated onto a feline embryonic fibroblast (FEA) cell line. Cells were checked daily for the appearance of the typical cytopathic effect (CPE) before being declared negative if no CPE was seen after the fourth day.

4.2.4 FHV-1 PCR

The VTM samples were tested for FHV-1 using a real-time PCR assay based on the feline herpesvirus thymidine kinase gene (Accession No. M26660). For crude DNA preparation, pelleted samples were resuspended in 2% 0.1M dithiothreitol (DTT) and boiled. All reactions were carried out in 96-well optical reaction plates in 25 μl volumes. DNA

amplification was performed using a 7500 real-time PCR System (Applied Biosystems, Paisley, UK) and the results were analysed using the 7500 System Sequence Detection Software (Applied Biosystems).

4.2.5 Statistical analysis

Graphical representations and Fisher's exact tests of virological investigations were prepared in GraphPad Prism for Windows, version 5.

4.3 Results

The data for the presence of FeLV, FIV, FHV-1 and FCV in cats with and without FCGS is shown in Table 4.1 and Table 4.2 respectively and in Figure 4.1.

4.3.1 Feline leukaemia virus

Of the 29 cats with FCGS tested for the FeLV antigen, none were positive. Of six healthy cats tested, two (33.3%) were positive for FeLV antigen. A Fisher's exact test showed that a significantly higher proportion of the healthy cats were positive for FeLV antigen when compared to the cats with FCGS (p=0.03).

4.3.2 Feline immunodeficiency virus

Of the 29 cats with FCGS tested for the presence of FIV antibodies, one (3.4%) cat was positive. Of six healthy cats tested, two (33.3%) were positive for FIV antibodies.

4.3.3 Feline herpes virus - 1

Twenty-nine cats with FCGS were tested for FHV-1 by qPCR, two (6.9%) cats were positive. Thirty-one cats were tested for FHV-1 by virus isolation. No virus was isolated in any of these cats. In the 15 healthy cats tested, no virus was isolated and none of the cats tested positive by the qPCR test.

4.3.4 FCV

Thirty-one cats with FCGS were tested for FCV and the virus was isolated from 22 (71%) cats. Fifteen healthy cats were tested and FCV was isolated from two (13.3%) cats. A Fisher's exact test showed that the presence of FCV was significantly higher in cats with FCGS when compared to the healthy cats (p=0.0004).

Table 4.1: Presence of FeLV, FIV, FCV and FHV-1 in cats with FCGS

Cat ID	FeLV antigen	FIV antibodies	Virus isolation FCV/FHV-1	FHV-1 PCR
F1	-	-	-	-
F2	Negative	Negative	FCV isolated	Negative
F3	Negative	Negative	No virus isolated	Negative
F4	Negative	Negative	FCV isolated	Negative
F5	Negative	Negative	No virus isolated	Negative
F6	Negative	Negative	FCV isolated	Negative
F7	Negative	Negative	FCV isolated	Negative
F8	Negative	Negative	FCV isolated	Negative
F9	Negative	Negative	FCV isolated	Negative
F10	Negative	Negative	FCV isolated	Negative
F11	Negative	Negative	FCV isolated	Negative
F12	Negative	Negative	FCV isolated	Negative
F13	Negative	Negative	No virus isolated	Negative
F14	Negative	Negative	No virus isolated	Negative
F15	Negative	Negative	No virus isolated	Negative
F16	Negative	Negative	FCV isolated	Negative
F17	Negative	Negative	FCV isolated	Negative
F18	Negative	Negative	FCV isolated	Negative
F19	Negative	Negative	No virus isolated	Negative
F20	Negative	Negative	FCV isolated	Negative
F21	Negative	Positive	FCV isolated	Negative
F22	Negative	Negative	FCV isolated	Negative
F23	Negative	Negative	FCV isolated	Negative
F24	Negative	Negative	FCV isolated	Negative
F25	Negative	Negative	No virus isolated	Negative
F26	Negative	Negative	FCV isolated	Negative
F27	Negative	Negative	FCV isolated	Negative
F28	Negative	Negative	FCV isolated	Positive
F29	Negative	Negative	No virus isolated	Negative
F30	-	-	FCV isolated	Negative
F31	-	-	No virus isolated	Positive
F32	Negative	Negative	FCV isolated	-

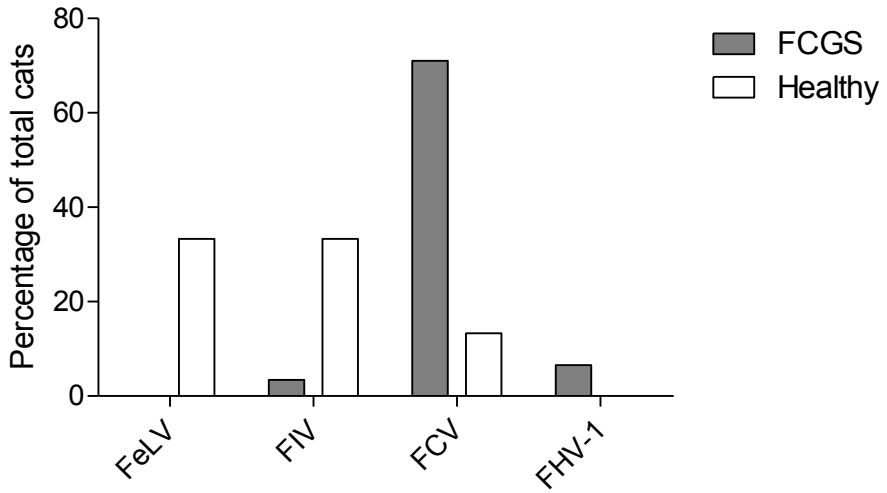
-: Not tested.

Table 4.2: Presence of FeLV, FIV, FCV and FHV-1 in healthy cats

Cat ID	FeLV Antigen	FIV Antibodies	Virus isolation FCV/FHV-1	FHV-1 PCR
H1	-	-	-	-
H2	Negative	Positive	FCV isolated	Negative
H3	Negative	Positive	No virus isolated	Negative
H4	Positive	Negative	No virus isolated	Negative
H5	Negative	Negative	No virus isolated	Negative
H6	Negative	Negative	No virus isolated	Negative
H7	-	-	No virus isolated	Negative
H8	-	-	No virus isolated	Negative
H9	-	-	No virus isolated	Negative
H10	-	-	No virus isolated	Negative
H11	-	-	No virus isolated	Negative
H12	-	-	No virus isolated	Negative
H13	-	-	No virus isolated	Negative
H14	-	-	FCV isolated	Negative
H15	-	-	No virus isolated	Negative
H16	Positive	Negative	No virus isolated	Negative

-: Not tested

Figure 4.1: Percentage of cats positive for each virus in the healthy and FCGS groups



4.4 Discussion

Feline viruses have been implicated in the aetiopathogenesis of FCGS. The presence of the viruses FeLV, FIV, FHV-1 and FCV in cats with and without FCGS was assessed.

In the current study, none of the 29 cats with FCGS that were tested were positive for the FeLV antigen. Studies on FeLV in cats with FCGS show varying results but the numbers of cats positive for FeLV are relatively low. In two studies, the prevalence of FeLV was between 4% and 17% (Knowles et al., 1989; White et al., 1992). Other studies have shown no tested cats with FCGS to be positive for the FeLV antigen (Johnessee and Hurvitz, 1983; Thompson et al., 1984; Hennet, 1997; Quimby et al., 2008). Of six healthy cats that were tested in the current study, two (33.3%) cats were positive for FeLV, a higher number than that previously reported where 1-5% of cats were asymptomatically infected with FeLV (Hosie et al., 1989; Levy, 2005; de Lange, 2008; Gleich et al., 2009). However only a small number of healthy cat samples were tested in the present study. Knowles et al. (1989) showed that 4% of cats with FCGS and 11% of cats in a time-matched control group were positive for FeLV. The evidence available suggests that FeLV is unlikely to play a role in the aetiopathogenesis of FCGS.

Of the 29 cats with FCGS tested in the current study, one (3.4%) tested positive for FIV antibodies. Other studies have shown higher levels of positivity but results are highly variable, ranging from 13% (Hennet, 1997) to 81% (Knowles et al., 1989) of cats with FCGS being positive for FIV. In the healthy cohort in the current study, two (33.3%) cats tested positive. This relatively high prevalence may be due to the low number of healthy cats tested and the fact that two cats were from a cat shelter and both tested positive. High numbers of FIV-positive cats in the control groups have been observed in other studies, with FIV prevalences ranging from 8.3-50% (Knowles et al., 1989; Quimby et al., 2008). The role of FIV in FCGS is uncertain, but the severity of clinical signs may increase in FIV-positive cats (Dawson et al., 1991; Tenorio et al., 1991; Waters et al., 1993).

In the current study two methods were used to determine the presence of FHV-1, namely virus isolation and PCR. Since FCV grows more rapidly than FHV-1, this can mask the growth of FHV-1 in cats also infected with FCV when virus isolation is used (Radford et al., 2009). Reduced survival of FHV-1 during sampling and transport has also been described (Reubel et al., 1993). Therefore qPCR is considered to be a more reliable and sensitive test for FHV-1 (Sykes et al., 1997), and was performed in tandem with virus isolation. Of the cats tested, two (6.9%) cats were positive for FHV-1 by qPCR in the

FCGS group and none were positive by virus isolation. Of the two cats that were positive for FHV-1 by qPCR, one was also positive for FCV by virus isolation. Few studies have tested cats with FCGS for the presence of FHV-1. One study determined the presence of FHV-1 and FCV in 25 cats with FCGS and 24 cats with periodontal disease by virus isolation (Lommer and Verstraete, 2003). Of the cats with FCGS, 88% shed both viruses as did 22% of the cats with periodontal disease. One cat (4%) with FCGS was positive for only FHV-1. Another study showed that 100% of cats with FCGS were positive for FHV-1 antibodies by ELISA, which compared with 97% of the cats in the control group (Quimby et al., 2008). When PCR was performed none of the cats with FCGS and 8.3% of the control group were positive. High prevalence rates of FHV-1 are often observed by serological testing. This is due to the widespread use of vaccines for FHV-1 and FCV, which increases the antibody titres in the population of healthy cats (Radford et al., 1997; Quimby et al., 2008). The vaccines against FHV-1 protect against disease but not against infection or the development of a carrier-state (Gaskell et al., 2007) Viral shedding seems to be reduced in vaccinated cats compared to unvaccinated controls. Of the 15 healthy cats studied in the current study, none tested positive for FHV-1. The prevalence of FHV-1 is thought to be approximately 1% in clinically healthy cats (Binns et al., 2000). The current study could not confirm a correlation between FHV-1 and FCGS.

Of 31 tested cats in the current study, 22 (71%) were positive for FCV by viral isolation. As with FHV-1, vaccination against FCV protects against classical oral/respiratory disease reasonably well but does not protect against infection or development of a carrier-state (Radford et al., 2007). This explains the high percentage of FCV positive cats regardless of vaccination. FCV has been described in cats with FCGS with a prevalence ranging from 40.5% (Dowers et al., 2010) to 92% (Knowles et al., 1989) of cases (Thompson et al., 1984; Lommer and Verstraete, 2003; Bellei et al., 2008). One study showed that none of nine cats with FCGS were positive for FCV by PCR (Quimby et al., 2008). FCV is most likely to play a role in the aetiopathogenesis of FCGS but studies have failed to induce FCGS by infecting cats with FCV isolates collected from cats with FCGS (Knowles et al., 1991). Over the ten months duration of the study, chronic oral inflammation did not develop and only acute signs were observed. However, most studies have shown that high numbers of cats with FCGS are infected with FCV. Reubel et al. (1992) showed that cats with caudal stomatitis were all positive for FCV. Most studies, however, showed that less than 100% of the cats with FCGS are positive for FCV. When virus isolation is used for detection, false negative results are possible due to inactivation of the virus during transport, the presence of small numbers of virions and the presence of antibodies in the sample (Radford et al., 2009). The current study showed that 13.3% of the healthy cats tested were positive for FCV. Prevalence rates of

between 10% and 40% have been reported in normal cats (Wardley et al., 1974; Gaskell, 2005; Radford et al., 2007).

In conclusion, the prevalence of both, FCV and FHV-1 was higher in the FCGS group compared to the healthy group. Conversely, the prevalence of FeLV and FIV was higher in the healthy cats.

Chapter 5 Identification of bacteria associated with FCGS

5.1 Introduction

The aetiopathogenesis of FCGS is unclear and multiple factors have been suggested including, viruses and an immune-mediated component (Tenorio et al., 1991; Dowers et al., 2010; Southerden, 2010). Bacteria have also been implicated to play a role in the aetiopathogenesis of FCGS (Love et al., 1989; Sims et al., 1990; Harvey, 1991).

Several studies have isolated bacteria from the feline oral cavity (Mallonee et al., 1988; Love et al., 1989; Love et al., 1990; Mihaljevic and Klein, 1998). Bacteria that have been isolated from the healthy oral cavity include *Bacteroides* species, *Actinomyces* species and *Pasteurella* species (Love et al., 1990). An overview of known bacterial species in the feline oral cavity is given in Tables 1.2 and 1.3.

Studies on the bacterial flora in oral-associated diseases of cats have primarily concentrated on periodontal disease (Mallonee et al., 1988; Norris and Love, 1999a). In FCGS the grade of periodontal disease ranges from none to severe but gingivitis is often part of the clinical signs. Periodontal disease is considered to be of a different pathogenic background and, as in human periodontal disease, specific bacteria can be described as an indicator for the severity of the disease (Norris and Love, 1999a; Booij-Vrieling et al., 2010).

To investigate the possible importance of bacteria in FCGS, a comparison of the bacterial flora in the healthy oral cavity with that in cats with FCGS has been made. This is the first study to use 16S rRNA gene cloning and sequencing to identify the bacterial flora in cats. The use of 16S rRNA gene sequencing makes it possible to identify a broader range of bacteria than would be expected using conventional methods only. Culture-independent techniques give the opportunity to identify non-cultivable species or slow-growing bacteria that would not be identified using culture-dependent methods. The technique has proven successful in detecting bacteria in the complex flora of the gut and human oral cavity (Hutter et al., 2003; Woo et al., 2008; Suchodolski et al., 2010). Using this culture-independent method together with conventional culture methods allows an even wider range of bacteria to be detected and makes it possible to demonstrate the complex microbiological diversity that is found in the oral cavity.

5.2 Materials and methods

5.2.1 Bacteriological culture methods

5.2.1.1 Culture of the samples

From the material eluted from each swab, six 10-fold serial dilutions were prepared. From each dilution, 50 µl was plated by use of a spiral plater (SpiralSystems inc., Cincinatti, USA) on both Colombia agar (Sigma-Aldrich) containing 7.5% v/v defibrinated horse blood for aerobic culture and fastidious anaerobe agar (FAA) (BioConnections, Leeds, UK) containing 7.5% v/v defibrinated horse blood for anaerobic culture. Aerobic incubation of the Colombia blood agar (CBA) was performed at 37ºC. FAA plates were incubated at 37ºC in an anaerobic chamber with an atmosphere of 85% N_2, 10% CO_2 and 5% H_2. After incubation for a maximum of seven days, up to eight morphologically distinct colonies were sub-cultured and incubated for three days under the same conditions to obtain pure colonies.

Culture of the samples by the Veterinary Diagnostic Service was performed on Colombia agar containing 5% v/v sheep blood (E&O laboratories Ltd., Bonnybridge, UK) and MacConkey agar (E&O Laboratories Ltd.) aerobically for 48h. FAA containing 5% horse blood (E&O Laboratories) was incubated anaerobically for 48h and chocolate agar (E&O Laboratories Ltd.) was incubated at an atmosphere of 5%CO_2 for 48h. All incubation was performed at 37°C. Isolated bacteria were identified by API system (bioMérieux UK Ltd., Hampshire, UK).

5.2.1.2 Cryopreservation of the isolates

A scraping of each pure culture was taken with a sterile loop and immersed in a mixture of de-ionised water, beef extract, peptone, sodium chloride, 20% glycerol and 20-25 ceramic beads in cryopreservation vials (Technical Service Consultants Ltd, Heywood, UK). The vials were inverted six times and set for 30 s to let the suspension be absorbed by the ceramic beads. The broth was removed and the beads stored at -80ºC. When required, the isolates were cultured on CBA and FAA by rubbing the bead over the agar. Plates were incubated as described before. From each isolate a scraping was taken and immersed in 95 µl nuclease-free water. DNA was extracted and the microorganisms were identified by 16S rRNA gene sequencing as described below.

5.2.2 16S rRNA gene sequencing

5.2.2.1 DNA extraction

DNA extraction of the FAB swab eluate was performed by proteinase-K digestion. Proteinase-K (Invitrogen, Paisley, UK), a non-specific serine protease, was added to a final concentration of 1000 µg/ml and incubated at 55°C for a minimum of 60 min. Each sample was then incubated for 10 min at 100°C to ensure denaturation of the proteinase-K. The samples were diluted 1:10, 1:50 and 1:100 and stored at -20°C until required. To extract DNA from the bacterial isolates the same method was used with a scraping of the isolated bacteria from the plate eluted in nuclease-free water containing 1000 µg/ml proteinase-K.

5.2.2.2 Bacterial 16S rRNA gene PCR amplification

Each dilution (1:10, 1:50 and 1:100), of the DNA extract and the neat sample were amplified by PCR. To amplify the 16S rRNA gene, universal primers 63F and 1387R were used (numbering is based on the *Escherichia coli* 16S rRNA gene sequence) (Marchesi et al., 1998). The primer sequences were 63F 5'CAG GCC TAA CAC ATG CAA GTC 3' and 1387R 5' GGG CGG WGT GTA CAA GGC 3' (W=A+T). Primers were synthesised commercially (Sigma Genosys, Cambridge, UK). The PCR reactions were carried out in a total volume of 50 µl containing 5 µl of the extracted DNA dilution and 45 µl reaction mixture. The reaction mixture was prepared using 1 x *GoTaq*® PCR buffer (Promega, Southampton, UK), 1.25 units of *GoTaq*® polymerase (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM deoxynucleotide triphosphates (dNTPs) (New England Biolabs, Hitchin, UK) and each primer at a concentration of 0.2 µM. The PCR cycling protocol comprised an initial denaturation phase of 2 min at 95ºC followed by 35 cycles of 95ºC, denaturation for 1 min, 60ºC annealing for 1 min, 72ºC extension for 1.5 min and a final extension cycle of 10 min at 72ºC.

5.2.2.3 PCR quality control

Negative and positive controls were included with each batch of samples being analysed. The positive control comprised a standard PCR reaction mixture containing 10 ng bacterial DNA instead of sample; the negative control contained sterile water instead of sample. Each PCR product was subjected to gel electrophoresis as described in Section 5.2.2.4.

5.2.2.4 Gel electrophoresis

A 2% agarose gel was prepared by dissolving 2 g of agarose (Roche, Burgess Hill, UK) in 100 ml 0.5% TBE buffer by heating and swirling. The solution was left to cool to 50°C before adding ethidium bromide at a concentration of 0.5 µg/ml. The gel was poured into a gel box and after solidifying, 5 µl of each PCR product was mixed with 2 µl 6 x loading buffer (New England Biolabs) and loaded into the wells, and the last well was loaded with 1 µl 100 bp ladder (New England Biolabs, Hitchin, UK) and 2 µl 6x loading buffer. The gel was run at 70 V for 1 hr. Examination of the gel was performed using a Gel Doc™ XR+ System with Image lab™ software (Bio-Rad Laboratories Inc., Hemel Hempstead,UK).

5.2.2.5 Bacterial 16S rRNA gene cloning

The 16S rRNA genes were cloned into the cloning vector pSC-A-amp/kan using the StrataClone™ PCR Cloning Kit (Agilent Technologies, Cheshire, UK). For incubation, L-agar plates containing 100 µg/ml of ampicillin (Sigma-Aldrich), plated with 40 µl of 5-bromo-3-indolyl-ß-D-galactopyranoside (Bluo-Gal) (40 µg/µl) were used. A 5-min ligation reaction allowed Adenine-Uracil (A-U) base-pairing between the U-overhangs of the vector and the A overhangs of the PCR products. Strand ligation was mediated by topoisomerase-I. The linear molecule was transformed into the competent cell line that expressed Cre-recombinase. Cre-recombinase mediated the formation of a circular DNA molecule able to replicate in cells growing on ampicillin-containing agar (Figure 5.1).

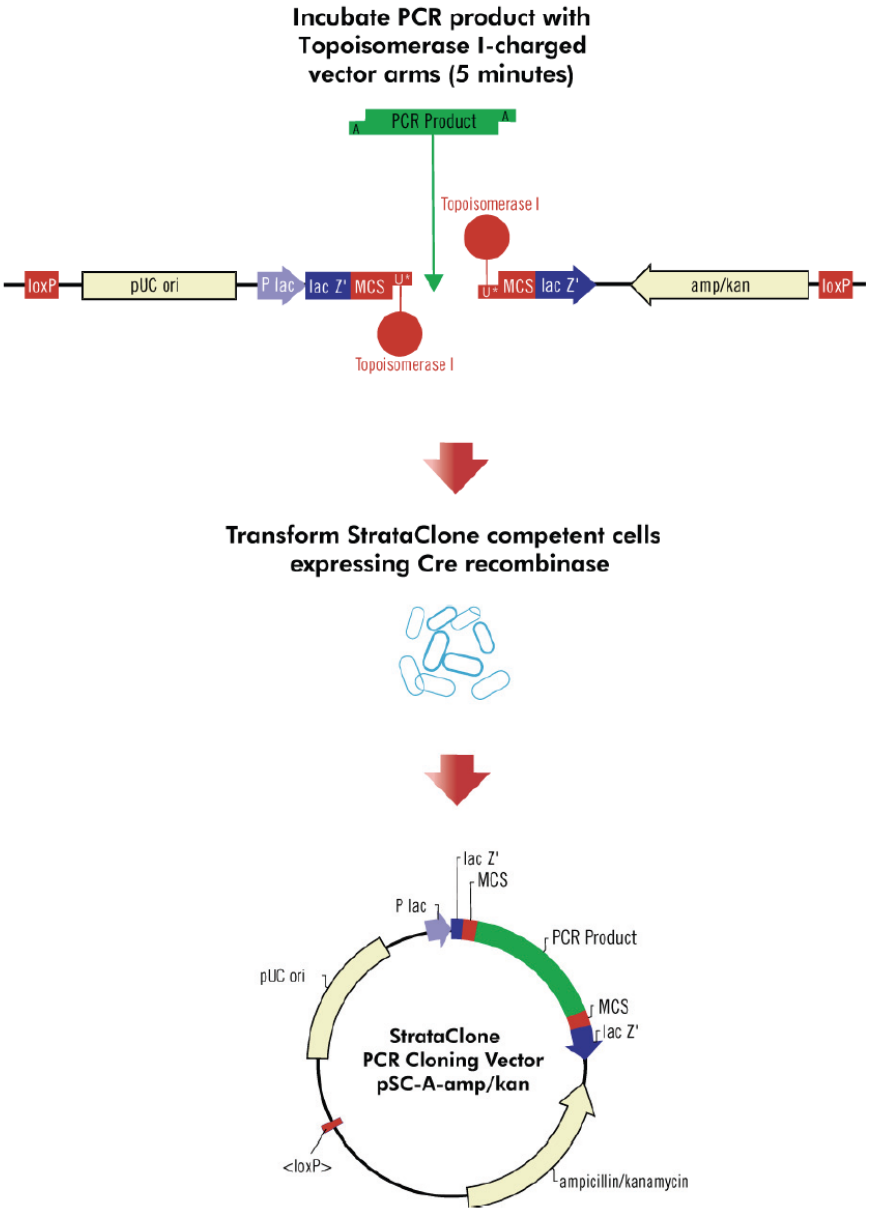
5.2.2.6 Bacterial 16S rRNA gene insert PCR re-amplification

Following cloning, the 16S rRNA gene inserts were re-amplified by PCR. Approximately fifty white clones were randomly selected from each generated library and amplified using the primer pair M13 SIF (5' to 3') CCC TCG AGG TCG ACG GTA TC and M13 SIR (5' to 3') CTC TAG AAC TAG TGG ATC CC. The M13SIF binding site is located 61 base pairs downstream of the M13 reverse primer binding-site, and the M13SIR binding site is located 56 base pairs upstream of the M13 forward primer binding-site in the pSC-A-amp/kan cloning vector (Figure 5.2). The PCR reactions were carried out in a total volume of 30 µl. The mastermix was prepared using 1.1 x ReddyMix PCR mastermix (containing 1.5 mM $MgCl_2$) (Thermo Scientific, Epsom, UK), 0.2 µM of each primer and nuclease-free water to a total volume of 30 µl. One clone was added to the reaction mixture and the PCR cycling protocol used was as previously described (Section 5.2.2.2). Each single clone used was grown on a fresh ampicillin/agar plate and incubated overnight.

5.2.2.7 Clone cryopreservation

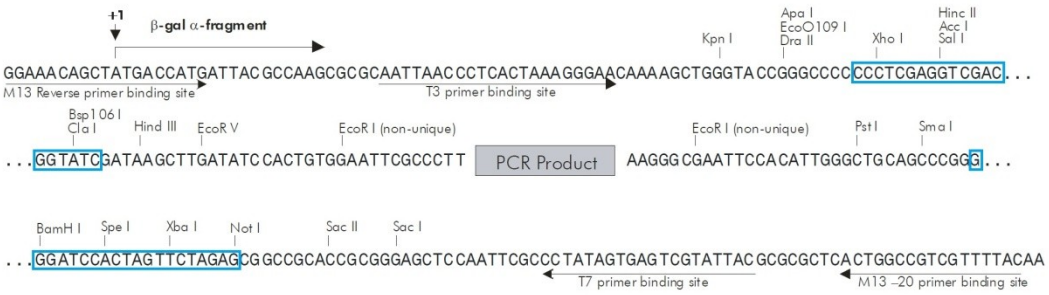
After incubation, each clone was taken from the plate and transferred into bijoux flasks containing 2 ml LB-broth. The clones were incubated under agitation at 37°C overnight and 0.5 ml of the medium containing the clones was then transferred into 1.5 µl tubes and mixed with 0.5 ml of 0.5% glycerol. The clones were stored at -80ºC until required.

Figure 5.1: Overview of the Strataclone PCR cloning method



Reproduced from StrataClone PCR Cloning Kit Instruction Manual (#240205). The cloning vector in the kit contained two DNA arms, charged with topoisomerase I on one end, containing modified U overhangs and a *loxP* recognition sequence on the other end. The vector also contained a *lacZ'* α-complementation cassette, when an insert was present the *lacZ'* gene was disrupted, the Bluo-Gal present on the agar plates was cleaved in the presence of an intact *lacZ'* gene which presented as a blue colour, therefore colonies containing no insert presented as blue, while colonies with an insert presented as white on the agar plates after incubation.

Figure 5.2: PSC-A-amp/kan PCR cloning vector PCR Product insertion Site region



Adapted from StrataClone PCR Cloning Kit Instruction Manual (#240205). Sequence shown: 4261-4270, 1-250. Blue boxes show primer location for SIF/SIR.

5.2.2.8 Restriction enzyme analysis

Each re-amplified 16S rRNA gene insert was analysed using restriction enzyme analysis. Approximately 0.5 µg of each PCR product was digested in a total volume of 15 µl with 2.0 U of each of the restriction enzymes *Rsa*I and *Mnl*I (Fermentas Life Sciences, York, UK) at 37ºC for 1 hr. The restriction fragments were visualised by agarose gel electrophoresis as described previously. For each library, clones were first sorted in groups according to the *Rsa*I restriction digestion profiles. Further discrimination was achieved by digestion with *Mnl*I. Clones with identical restriction profiles for both enzymes were grouped together in distinct restriction fragment length polymorphism (RFLP) groups.

5.2.2.9 PCR product purification

The PCR product of one representative clone from each RFLP group was purified using the 'QiaQuick PCR Purification Kit' (Qiagen, Crawley, UK). Five volumes of Buffer PB (Qiagen) were added to one volume of PCR reaction and mixed. The mix was transferred into the QiaQuick spin column and centrifuged for 60 s to allow the DNA to absorb onto the silica membrane. The DNA was washed with 750 µl of buffer PE (Qiagen) and centrifuged for 60 s. The flow-through was discarded and the spin column was centrifuged for another 60 s to ensure the ethanol containing buffer PE was removed completely. The column was placed into a clean 1.5 ml centrifuge tube and the DNA eluted in 30 µl of nuclease-free water by centrifuging for 60 s at 13000 rpm. The DNA yield was assessed using the ND-1000 NanoDrop® (Thermo Scientific) spectrophotometer.

5.2.2.10 Sanger DNA Sequencing

Each purified 16S rRNA gene insert was sequenced. To perform sequence reactions the SequiTherm EXCEL™ II DNA Sequencing Kit (Cambio Ltd., Cambridge, UK) with IRD800-labelled 357f primer was used in a LI-COR Gene ReadIR 4200S automated DNA sequencing system (MWG Biotech, Milton Keynes, UK). The loading gel was prepared as follows: 21 g of urea (Sigma-Aldrich), 7.5 ml of rapid gel (Affymetrix/USB, High Wycombe, UK), 28.0 ml double distilled water, 5.0 ml 10 x TBE Buffer and 500 µl DMSO (Fisher Scientific, Loughborough, UK) were added to a one-armed flask, mixed and degassed using a vacuum pump. The gel solution was poured into a beaker and 50 µl TEMED (Sigma-Aldrich) and 350 µl (0.1 g/ml) ammonium persulphate (Sigma-Aldrich) were added. Prior to pouring, the gel plates were cleaned with different chemicals in the following order; 0.5M HCl, 0.5M NaOH, double distilled water and ethanol. The gel

plates were put together, separated by plastic spacers. The gel was poured from the top and the loading comb was inserted. The gel was left to polymerise for 1.5 h before placing into the LI-COR automated DNA sequencing system with 1 x TBE Buffer, following the manufacturer's instructions. The SequiTherm reactions were set up in 4 tubes per sample to which 2 µl of one of the four termination mixtures (A, C, T, G) was added. Sequence mixtures were prepared for each reaction containing 2.5 µl sequencing buffer, 1 µl *Taq* DNA polymerase, 1.5 µl 357F primer, template DNA (Cambio Ltd.) and deionised water to a total volume of 18 µl. 4 µl of the mixture was added to each of the termination mixes. A drop of mineral oil was placed on top of each reaction. The sequencing cycling protocol had the following parameters: (i) initial denaturation at 95ºC for 30 s, (ii) 10 s at 95ºC, 30 s at 57ºC and 30 s at 70ºC for 20 cycles and (iii) 10 s at 95ºC and 30 s at 70ºC for 15 cycles. After thermocycling, 3 µl of Stop/Loading Buffer was added and approximately 1.5 µl of each reaction mixture was loaded onto the gel and run on the LI-COR DNA sequencing system overnight.

DNA sequencing was also performed by GATC Biotech, London and DNA Sequencing & Services, University of Dundee using Applied Biosystems Big-Dye Version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

5.2.2.11 Analysis of sequencing data

Sequence data generated from the LI-COR system were compiled using LI-COR Base ImagIR 4.0 software and converted to FASTA format. Sequence data obtained from external sources were provided in a variety of formats (FASTA format from GATC Biotech and raw sequences from DNA Sequencing & Services) that did not need conversion before searching the databases. All sequence data were compared against the EMBL/GenBank sequence databases using the nucleotide MEGABLAST algorithm. The program was run through the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov /BLAST). Sequences that had at least 98% identity with a known sequence were considered to be of the same species as the matching sequence with the highest score. Sequences with less than 98% identity were classified as potentially novel phylotypes. On several occasions the matching sequences will be described as 'uncultured bacteria' in the result section. These are sequences in the database that have not been previously cultured but have been identified by sequencing techniques and are uploaded to the database under the common name of 'uncultured bacteria'.

5.2.3 Phylogenetic analysis

5.2.3.1 Sequence analysis of clones representing potentially novel phylotypes

Sequences with less than 97% identity with any known sequence from the databases were tentatively classified as potentially novel phylotypes at the start of the project (Section 5.3.2.5). From FCGS samples F1-F6 and healthy samples H1-H3 the potentially novel clones were revived from storage on ampicillin/agar plates and incubated overnight at 37°C. The clones were subjected to PCR with primers M13SIF and M13SIR as described previously. Each clone was sequenced with primers M13SIF and M13SIR to obtain the nearly complete 16S rRNA gene sequence (1202 to 1325 bp).

5.2.3.2 Phylogenetic analysis

Near full-length 16S rRNA gene sequences were aligned using the ClustalW2 multiple sequencing alignment tool through the phylogenetic analysis program *MEGA* version 5 (Tamura et al., 2011). The maximum-likelihood method was used to perform phylogenetic analysis based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood was used and initial trees were obtained automatically; when the number of common sites was < 100 or less than one quarter of the total number of sites, the maximum parsimony method was used. The BIONJ method with MCL distance matrix was used in all other cases. The phylogenetic tree was drawn to scale and the branch lengths were measured in the number of substitutions per site. Positions containing gaps and missing data were eliminated.

5.3 Results

5.3.1 Culture-dependent methods

5.3.1.1 Bacterial culture

Using standard culture methods, 22 FCGS and 13 healthy samples showed bacterial growth following both aerobic and anaerobic incubation. Blood plates were incubated for three to six days with spiral plated ten-fold serial dilutions of FAB in which the swab samples were emerged (Table 5.1). The growth density varied greatly per individual sample and growth was sparse in samples F7, F11, F18, F19 and F21. Up to eight morphologically different isolates were collected from each plate and isolated (Table 5.1).

5.3.1.2 DNA extraction and PCR

A standard proteinase-K DNA extraction method was used on all isolates and the 16S gene was successfully amplified from 131 healthy and 161 FCGS isolates. All amplified 16S rRNA genes were sequenced.

5.3.1.3 BLAST database comparison

All 16S rRNA sequences were compared against the BLAST database and the isolates that were identified from 22 FCGS and 13 healthy samples are shown in Table 5.2. The most frequently isolated bacteria in the FCGS samples were *P. multocida* subsp. *multocida*, (9.9%), *P. multocida* subsp. *septica* (9.9%) and uncultured bacterium (21.7%). The most frequently isolated bacteria from healthy samples were *P. multocida* subsp. *septica* (9.9%) and uncultured bacterium (30.5%). A large number of isolated bacteria were matched in the BLAST database to uncultured bacteria for both the healthy and FCGS samples. A subdivision of these results is made in Table 5.3 according to the accession number. For F12-F22 and H6-13 bacterial counts were performed (Table 5.4). In the FCGS group, an increase in the average CFU/ml can be seen in all *Pasteurella* sp. (except *P. stomatitis*) when compared to the healthy group.

Table 5.1: Number of different isolates from all samples

Sample	Number of isolates		Plated dilution		Incubation time (days)
	CA	FAA	CA	FAA	
F1	N.R.	N.R.	N.R.	N.R.	N.R.
F2	5	6	N.R.	N.R.	3
F3	7	4	N.R.	N.R.	4
F4	6	8	N.R.	N.R.	4
F5	7	7	N.R.	N.R.	3
F6	8	7	N.R.	N.R.	3
F7	8	8	neat	neat	5
F8	7	8	N.R.	N.R.	5
F9	8	5	N.R.	N.R.	5
F10	7	6	N.R.	N.R.	6
F11	5	6	N.R.	N.R.	5
F12	6	3	10^{-3}	10^{-2}	6
F13	5	5	10^{-6}	10^{-5}	5
F14	8	6	10^{-2}	10^{-3}	5
F15	5	4	10^{-3}	10^{-2}	6
F16	6	6	10^{-2}	10^{-3}	5
F17	3	3	10^{-5}	10^{-2}	4
F18	1	2	neat	10^{-1}	5
F19	4	2	10^{-2}	10^{-2}	4
F20	4	4	10^{-3}	10^{-3}	5
F21	3	3	neat	neat	5
F22	4	4	10^{-2}	10^{-2}	6
H1	N.R.	N.R.	N.R.	N.R.	N.R.
H2	6	6	N.R.	N.R.	4
H3	6	7	N.R.	N.R.	4
H4	8	6	N.R.	N.R.	5
H5	6	4	10^{-2}	10^{-2}	5
H6	6	5	10^{-2}	10^{-2}	4
H7	8	8	10^{-2}	10^{-2}	5
H8	8	8	10^{-2}	10^{-2}	6
H9	8	6	10^{-3}	10^{-3}	6
H10	8	8	10^{-1}	10^{-1}	6
H11	8	8	10^{-3}	10^{-1}	5
H12	8	8	10^{-2}	10^{-2}	6
H13	8	8	10^{-2}	10^{-2}	5

Number of morphologically different bacteria isolated from 22 FCGS samples and 13 healthy samples and the number of different bacteria identified by 16S rRNA gene sequencing.

CA: Columbia agar, FAA: Fastidious anaerobe agar, N.R.: Not recorded.

Table 5.2: Identification of bacterial isolates

Species	Healthy	FCGS
	No. of isolates (% of total) n=131	No. of isolates (% of total) n=161
Actinomyces canis	4 (3.1)	1 (0.6)
Actinomyces hordeovulneris		8 (5.0)
Anaerococcus sp./*Peptostreptococcus* sp.*	1 (0.8)	
Bacillus simplex		1 (0.6)
Bacillus sp.	1 (0.8)	
Bacteroides heparinolyticus		1 (0.6)
Bacteroides pyogenes		1 (0.6)
Bacteroides tectus	2 (1.5)	4 (2.5)
Bergeyella sp.	3 (2.3)	1 (0.6)
Bergeyella zoohelcum	2 (1.5)	
Capnocytophaga cynodegmi	1 (0.8)	
Carnobacterium divergens	1 (0.8)	
Catonella sp.	1 (0.8)	
Chryseobacterium sp.		2 (1.2)
Citrobacter braakii		1 (0.6)
Clostridium perfringens	1 (0.8)	
Comamonadaceae bacterium		1 (0.6)
Corynebacterium felinum	1 (0.8)	
Corynebacterium sp.	1 (0.8)	
Cupriavidus basilensis		1 (0.6)
*Curculio sikkimens/Serratia grimesii/Pectobacterium carotovorum**	2 (1.5)	
Cythophaga sp.	1 (0.8)	
Enterococcus casseliflavus	1 (0.8)	
Enterococcus faecalis		1 (0.6)
Enterococcus sp.	1 (0.8)	1 (0.6)
*Escherichia coli/Shigella flexneri**		5 (3.1)
Eubacteriaceae bacterium	1 (0.8)	
Filifactor villosus	3 (2.3)	1 (0.6)
Fusobacterium equinum	1 (0.8)	
Fusobacterium rusii		1 (0.6)
Gordonia otidis		1 (0.6)
Hafnia sp.		2 (1.2)
Lactobacillales bacterium	2 (1.5)	2 (1.2)
Microbacterium sp.		2 (1.2)
Moraxella ovis	1 (0.8)	1 (0.6)
Moraxella sp.	1 (0.8)	
Mycoplasma arginini	1 (0.8)	
Neisseria canis		2 (1.2)
Neisseria shayeganii		2 (1.2)
Neisseria zoodegmatis	3 (2.3)	
Neissseria sp.	7 (5.3)	
Pantoea agglomerans	1 (0.8)	
Pasteurella canis		4 (2.5)
Pasteurella multocida strain	3 (2.3)	1 (0.6)
Pasteurella multocida subsp. *multocida*	1 (0.8)	16 (9.9)
Pasteurella multocida subsp. *septica*	13 (9.9)	16 (9.9)
Pasteurella multocida subsp. *septica/multocida**		7 (4.3)
Pasteurella pneumotropica	3 (2.3)	5 (3.1)
Pasteurella sp.	3 (2.3)	5 (3.1)
Pasteurella stomatis	2 (1.5)	
Peptostreptococcus sp.	2 (1.5)	

Table 5.2 continued

Species	Healthy	FCGS
	No. of isolates (% of total) n=131	No. of isolates (% of total) n=161
Porphyromonas canoris		1 (0.6)
Porphyromonas circumdentaria	5 (3.8)	1 (0.6)
Porphyromonas gulae g	2 (1.5)	1 (0.6)
Porphyromonas sp.	1 (0.8)	1 (0.6)
Propionibacterium acnes	1 (0.8)	
Pseudomonas reactans		1 (0.6)
Pseudomonas sp.		3 (1.9)
Rhodococcus		1 (0.6)
Shigella sp		2 (1.2)
Staphylococcus aureus		1 (0.6)
Staphylococcus epidermidis	2 (1.5)	
Staphylococcus pasteuri		1 (0.6)
Staphylococcus sp.		1 (0.6)
Streptococcus minor	1 (0.8)	2 (1.2)
Streptococcus sobrinus		1 (0.6)
Tannerella forsythia	1 (0.8)	
Uncultured bacterium	40 (30.5)	35 (21.7)
Uncultured beta proteobacterium		1 (0.6)
Uncultured *Capnocytophaga*		1 (0.6)
Uncultured *Citrobacter*		3 (1.9)
Uncultured *Haemophilus sp.*	1 (0.8)	
Uncultured *Micrococcus*		1 (0.6)
Uncultured *Porphyromonas*		1 (0.6)
Virgillobacillus halophilus		4 (2.5)
Xanthomonadaceae bacterium	5 (3.8)	1 (0.6)

Bacterial species identified by 16S rRNA gene sequencing of isolates obtained following microbiological culture from 13 healthy samples and 22 FCGS samples.
* Unable to distinguish between two or more species, therefore grouped generically.

Table 5.3: Isolates identified as previously uncultured bacteria

Accession number	Healthy	FCGS
	No of isolates (% of total) n=131	No of isolates (% of total) n=161
AB368999	1 (0.8)	1 (0.6)
AY850486		1 (0.6)
DQ447792	1 (0.8)	
DQ860033		1 (0.6)
EF511977		1 (0.6)
EF516089		1 (0.6)
EU472784		1 (0.6)
EU535692		1 (0.6)
EU681991		4 (2.5)
EU762526		1 (0.6)
FM872599		1 (0.6)
GQ010287	1 (0.8)	
GQ010438		1 (0.6)
GQ110814	1 (0.8)	3 (1.9)
GQ110821		1 (0.6)
GQ111117	1 (0.8)	
GQ111197	1 (0.8)	
GQ111280	2 (1.5)	1 (0.6)
GQ114820	1 (0.8)	
GQ158316	7 (5.3)	
HM278514	1 (0.8)	
HM326498	4 (3.1)	
JF032321	1 (0.8)	
JF041223	1 (0.8)	1 (0.6)
JF092143		1 (0.6)
JF104340	1 (0.8)	1 (0.6)
JF108085		2 (1.2)
JF108316	2 (1.5)	
JF108357	2 (1.5)	
JF147582	1 (0.8)	
JF154679	2 (1.5)	5 (3.1)
JF174825	2 (1.5)	1 (0.6)
JF175431	3 (2.3)	1 (0.6)
JF176465		1 (0.6)
JF180219		1 (0.6)
JF223924	1 (0.8)	
JF224016		1 (0.6)
JF240330		1 (0.6)
JF240812	1 (0.8)	
JF241047	1 (0.8)	
JF241102	1 (0.8)	

Previously uncultured bacteria (ordered by accession number) isolated from 13 healthy and 22 FCGS samples.

Table 5.4: Bacterial counts of the isolates

Species	Healthy		FCGS	
	Average CFU/ml per sample	(no of samples)	Average CFU/ml per sample	(no of samples)
Actinomyces canis	$4.1\ 10^{3}$	(2)		
Bacteroides heparinolyticus			$8.0\ 10^{4}$	(1)
Bacteroides pyogenes			$6.0\ 10^{4}$	(1)
Bacteroides tectus	$2.3\ 10^{4}$	(1)		
Bergeyella sp.	$2.3\ 10^{4}$	(2)	$8.0\ 10^{3}$	(1)
Bergeyella zoohelcum	$1.9\ 10^{4}$	(2)		
Capnocytophaga cynodegmi	$1.0\ 10^{5}$	(1)		
Citrobacter braakii			$8.0\ 10^{3}$	(1)
Corynebacterium sp.	$4.0\ 10^{3}$	(1)		
Cythophaga sp.	$1.0\ 10^{3}$	(1)		
Enterococcus sp.			$1.1\ 10^{3}$	(1)
Filifactor villosus	$1.8\ 10^{4}$	(1)	$8.0\ 10^{4}$	(1)
Fusobacterium equinum	$2.0\ 10^{3}$	(1)		
Hafnia sp.			$1.6\ 10^{8}$	(1)
Lactobacillales bacterium	$1.7\ 10^{4}$	(1)	$1.5\ 10^{2}$	(1)
Moraxella sp.	$2.2\ 10^{5}$	(2)		
Neisseria canis			$1.5\ 10^{4}$	(1)
Neisseria shayeganii			$9.0\ 10^{6}$	(2)
Neisseria zoodegmatis	$1.4\ 10^{5}$	(3)		
Neissseria sp.	$1.9\ 10^{4}$	(2)	$8.0\ 10^{3}$	(1)
Pasteurella multocida strain	$1.3\ 10^{4}$	(2)	$2.6\ 10^{4}$	(1)
Pasteurella multocida subsp. *multocida*			$8.2\ 10^{4}$	(2)
Pasteurella multocida subsp. *septica*	$1.4\ 10^{4}$	(5)	$9.1\ 10^{7}$	(2)
Pasteurella sp.	$4.2\ 10^{4}$	(2)	$5.2\ 10^{7}$	(1)
Pasteurella stomatis	$6.0\ 10^{3}$	(2)		
Peptostreptococcus sp.	$2.0\ 10^{3}$	(1)		
Porphyromonas circumdentaria	$6.2\ 10^{4}$	(4)	$6.0\ 10^{5}$	(1)
Porphyromonas gulae g	$5.3\ 10^{4}$	(1)		
Porphyromonas sp.	$8.0\ 10^{5}$	(1)		
Propionibacterium acnes	$6.0\ 10^{3}$	(1)		
Pseudomonas sp.			$3.3\ 10^{6}$	(1)
Staphylococcus epidermidis	$2.0\ 10^{2}$	(1)		
Staphylococcus pasteuri			$2.0\ 10^{6}$	(1)
Streptococcus minor			$1.8\ 10^{5}$	(1)
Tannerella forsythia	$1.2\ 10^{3}$	(1)		
Uncultured bacterium	$3.5\ 10^{4}$	(8)	$3.3\ 10^{4}$	(5)
Uncultured beta proteobacterium			$4.0\ 10^{4}$	(1)
Uncultured *Capnocytophaga*			$1.0\ 10^{4}$	(1)
Uncultured *Citrobacter*			$1.8\ 10^{7}$	(1)
Uncultured *Porphyromonas*			$8.0\ 10^{4}$	(1)
Xanthomonadaceae bacterium	$3.6\ 10^{4}$	(3)		

Average bacterial counts in colony forming units (CFU) per millilitre per sample, of the isolated bacteria from nine FCGS (F12-F21) and eight healthy samples (H6-H13).

5.3.1.4 Veterinary Diagnostics Service – microbiological culture

Samples F2 to F19 were cultured as part of the Veterinary Diagnostic Service at the University of Glasgow companion animal diagnostics laboratory. The results are shown in Table 5.5 next to the different species per sample detected by 16S rRNA gene sequencing. From the diagnostic laboratory no results were given for samples F11 and F18.

5.3.2 Culture-independent methods

5.3.2.1 Bacterial DNA amplification

All 22 FCGS samples analysed were positive for the presence of bacteria as determined by 16S rRNA PCR. A small amount of bacterial DNA was identified in samples F7, F11 and F21. All 13 healthy samples were positive for the presence of bacteria.

5.3.2.2 Cloning

All 35 samples were subjected to cloning. During cloning, the use of bluo-gal ensured blue coloured colonies were formed when no insert was present. Blue, light blue and white colonies formed on the plates. Light blue colonies became darker and easier to distinguish from the white colonies when kept at 4°C for at least 4 days. Of the 22 FCGS samples, F7 had a low cloning efficiency, showing a high percentage of blue and light blue colonies in comparison to the white colonies. Low colony numbers were also observed in samples F11 and F21 and re-amplification failed to isolate clones with 16S rRNA gene inserts. Purification of the PCR product before cloning did not change this outcome. All thirteen healthy samples were cloned successfully. From each individually cloned sample, a library of approximately 50 clones was re-amplified with the exception of sample F7 where, due to low cloning efficiency, only 27 clones were successfully re-amplified by PCR (Table 5.6).

5.3.2.3 Enzyme digestion

All re-amplified clones were digested with the enzymes *Rsa*I and *Mnl*I and depending on the different product sizes the clones were grouped according to their restriction fragments length polymorphism (RFLP) profiles. One clone was sequenced from each RFLP group. Between four and 34 clones were sequenced per individual sample (Table 5.6).

Table 5.5: FCGS isolates

Sample	16S sequencing	Veterinary diagnostics (culture)
F2	*Pasteurella multocida multocida/septica* *Pasteurella pneumotropica* *Staphylococcus aureus* *Pseudomonas* *Chryseobacterium*	*Pasteurella multocida* (moderate pure)
F3	*Pasteurella multocida multocida/septica* *Pasteurella pneumotropica*	*Pasteurella multocida* (profuse) *Staphylococcus* (sparse)
F4	*Pasteurella multocida multocida* *Bacteroides tectus* *Moraxella ovis* *Staphylococcus* sp. *Streptococcus sobrinus*	*Pasteurella multocida* (profuse pure)
F5	*Pasteurella multocida multocida/septica* *Pasteurella pneumotropica* *Pasteurella* sp. *Chryseobacterium* sp. *Cupriavidus basilensis* *Enterococcus faecalis* *Pseudomonas reactans* *Pseudomonas* sp.	*Pasteurella multocida* (moderate) *Streptococcus* (sparse) *Gram negative rods* (sparse)
F6	*Pasteurella canis* *Pasteurella multocida septica* *Bacteroides tectus* *Fusobacterium rusii* *Porphyromonas canoris*	*Pasteurella multocida* (moderate pure)
F7	*Actinomyces hordeovulneris* *Rhodococcus*	*Cellulomonas/microbacterium* sp. *Porphyromonas asaccharolytica* *Bacteroides ureolyticus* (moderate mixed culture)
F8	*Pasteurella* sp. *Bacteroides tectus* *Actinomyces hordeovulneris* *Shigella* sp.	*Coliform* (sparse) *Pasteurella multocida* (moderate) *Bacteroides* (profuse)
F9	*Actinomyces canis* *Actinomyces hordeovulneris* *Microbacterium* sp. *Pasteurella multocida multocida*	*Corynebacterium proinquum* *Streptococcus* (sparse mixed culture)
F10	*Bacillus simplex* *Xanthomonadaceae* bacterium *Comamoadeceae* bacterium *Actinomyces hordeovulneris*	*Pasteurella multocida* (profuse pure)
F11	*Pasteurella* sp. *Porphyromonas gulae*	No results
F12	*Pasteurella multocida septica* *Porphyromonas* sp.	*Pasteurella multocida* (profuse pure)

Table 5.5 continued

Sample	16S sequencing	Veterinary diagnostics (culture)
F13	*Neisseria shayeganii* *Pasteurella* sp. *Hafnia* sp. *Staphylococcus pasteuri* *Pasteurella multocida septica*	*Pasteurella* sp. *Streptococcus* (profuse mixed culture)
F14	*Pasteurella multocida multocida* *Bacteroides pyogenes* *Filifactor villosus* *Neisseria shayeganii*	*Pasteurella multocida* *Pasteurella* sp. (moderate profuse mixed culture)
F15	*Pseudomonas* sp. Uncultured beta *proteobacterium* Uncultured *Capnocytophaga*	Gram negative large rods (sparse)
F16	*Bacteroides heparinolticus* *Bergeyella* sp. *Pasteurella multocida* *Porphyromonas circumdentaria*	*Pasteurella multocida* (moderate) Beta-*haemolitic Escherichia coli* (sparse)
F17	*Escherichia coli/Shigella flexneri* *Pasteurella multocida septica*	Beta haemolytic *Escherichia coli* (profuse pure)
F18	*Enterococcus* sp. *Neisseria canis*	No results
F19	*Pasteurella multocida multocida* *Neisseria* sp.	*Pasteurella multocida* (sparse) Gram negative *coccobacillus* (sparse)

Morphologically different bacteria identified by 16S rRNA gene sequencing, and most represented bacteria isolated from Columbia agar and fastidious anaerobe agar by the laboratory at the Veterinary Diagnostics Service.

Table 5.6: Analysed clones per sample library

FCGS			Healthy		
Sample	Total clones analysed	Number of RFLP groups	Sample	Total clones analysed	Number of RFLP groups
F1	51	27	H1	50	34
F2	50	19	H2	56	22
F3	50	12	H3	51	29
F4	52	9	H4	54	20
F5	50	24	H5	53	22
F6	51	28	H6	46	17
F7	27	10	H7	51	15
F8	48	15	H8	51	18
F9	50	13	H9	51	23
F10	48	15	H10	49	14
F11	0	0	H11	51	10
F12	51	15	H12	51	14
F13	49	17	H13	49	15
F14	47	22			
F15	51	5			
F16	51	19			
F17	51	4			
F18	46	10			
F19	52	10			
F20	51	8			
F21	0	0			
F22	50	7			

Total number of analysed clones and the number of RFLP groups per individual sample.

5.3.2.4 BLAST database comparison

From the clone libraries generated from the 20 FCGS samples, 976 clones were analysed and 289 clones were sequenced. Bacteria identified with a similarity of 98% or more with known species in the used databases (Genbank, EMBL, DDBJ, PDB) are shown in Table 5.7. The most frequently identified bacteria in the cats with FCGS were *P. multocida* subsp. *multocida* (14.1% of clones analysed) *P. multocida* subsp. *septica* (11.5%), *Pseudomonas* sp. (7.3%), *Tannerella forsythia* (6.6%) and *Porphyromonas circumdentaria* (5.6%). A variety of uncultured bacteria represented 7.7% of all analysed FCGS clones. No individual uncultured species was over-represented in the FCGS group (Table 5.8)

From the 13 healthy samples a total of 663 clones were analysed and 253 clones were sequenced. The bacteria identified are shown in Table 5.7. The predominant species in the healthy group were *Xanthomonadaceae* bacterium (6.2% of clones analysed), *Capnocytophaga canimorsus* (5.4%), *Capnocytophaga cynodegmi* (4.8%), *Bergeyella* species (4.5%) and *P. multocida* subsp. *septica* (4.4%). A large proportion of uncultured bacteria (29%) were found in the healthy group. Two of these uncultured bacteria were particularly well represented in these cats: GQ111280 (4.2%) which is the partial sequence of an uncultured bacterium clone nbw623c11c1 identified from a human skin swab of the antecubital fossa (Grice et al., 2009); and HM326498 (3.6%), a partial sequence of an uncultured bacterium clone ncd461e11c1 (direct submission) which was also identified from a human arm skin swab sample (Table 5.8). The five most prevalent bacterial species found in the healthy and FCGS samples are shown in Figure 5.1.

The different phyla that were identified in the FCGS and healthy samples were compared (Figure 5.2). Healthy samples contained predominantly uncultured bacteria, Bacteroidetes and Proteobacteria. In the FCGS samples, Proteobacteria was predominant and a high proportion of Bacteroidetes was also seen. When the distribution of the genera within healthy and FCGS samples was compared, the predominant groups in the healthy samples were uncultured bacteria and *Capnocytophaga* species, whereas the predominant species in the FCGS group were *Pasteurella* species (Figure 5.3).

Table 5.7: Bacterial species identified by culture-independent (16S rRNA gene cloning) methods

Species	Healthy				FCGS			
	No. of clones analysed (% of total) n=663		No. of clones sequenced (% of total) n=253		No. of clones analysed (% of total) n=976		No. of clones sequenced (% of total) n=289	
Abiotrophia defectiva	1	(0.2)	1	(0.4)				
Acinetobacter					2	(0.2)	2	(0.7)
Actinobaccilus capsulatus							1	(0.3)
Actinomyces					2	(0.2)	1	(0.3)
Actinomyces canis					2	(0.2)	2	(0.7)
Advenella sp./*Pelistega europaea*/*tetrathibacter kashmirensis**	1	(0.2)	1	(0.4)				
Afipia genosp.					4	(0.4)	2	(0.7)
Bacterium cp04.13	4	(0.6)	4	(1.6)	2	(0.2)	1	(0.3)
Bacterium enrichment culture	10	(1.5)	5	(2.0)				
Bacteroides pyogenes					1	(0.1)	1	(0.3)
Bacteroides tectus	2	(0.3)	1	(0.4)	3	(0.3)	3	(1.0)
Bergeyella sp.	30	(4.5)	11	(4.3)	3	(0.3)	2	(0.7)
Capnocytophaga canimorsus	36	(5.4)	6	(2.4)	6	(0.6)	3	(1.0)
Capnocytophaga cynodegmi	32	(4.8)	5	(2.0)	1	(0.1)	1	(0.3)
Citrobacter amalonaticus/ *Citrobacter* sp.*	1	(0.2)	1	(0.4)				
Clostridium botulinum/ *Clostridium sporogenes**	1	(0.2)	1	(0.4)				
Cythophaga sp.	1	(0.2)	1	(0.4)	9	(0.9)	3	(1.0)
Desulfomicrobium orale	14	(2.1)	5	(2.0)	16	(1.6)	4	(1.4)
Eubacterium sp.					2	(0.2)	2	(0.7)
Flexistipes-like sp. Oral					2	(0.2)	1	(0.3)
Fusobacterium canifelinum					4	(0.4)	4	(1.4)
Haemophilus felis	2	(0.3)	2	(0.8)	1	(0.1)	1	(0.3)
Kebsiella sp.	4	(0.6)	1	(0.4)				
Lysobacter sp.	1	(0.2)	1	(0.4)				
Moraxella canis	23	(3.5)	4	(1.6)				
Moraxella lacunata					1	(0.1)	1	(0.3)
Moraxella ovis	3	(0.5)	2	(0.8)				
Moraxella sp.	12	(1.8)	5	(2.0)	1	(0.1)	1	(0.3)
Neisseria canis					19	(1.9)	2	(0.7)
Odoribacter denticanis					9	(0.9)	3	(1.0)
Pasteurella canis					2	(0.2)	1	(0.3)
Pasteurella multocida strain	13	(2.0)	7	(2.8)	2	(0.2)	1	(0.3)
*Pasteurella multocida multocida/septica**					41	(4.2)	4	(1.4)
Pasteurella multocida multocida	2	(0.3)	2	(0.8)	138	(14.1)	25	(8.7)
Pasteurella multocida. septica	29	(4.4)	8	(3.2)	112	(11.5)	18	(6.2)
Pasteurella pneumotropica	20	(3.0)	7	(2.8)	11	(1.1)	4	(1.4)

Table 5.7 continued

Species	Healthy		FCGS	
	No. of clones analysed (% of total) n=663	No. of clones sequenced (% of total) n=253	No. of clones analysed (% of total) n=976	No. of clones sequenced (% of total) n=289
Pasteurella sp.	6 (0.9)	3 (1.2)	26 (2.7)	6 (2.1)
Pasteurella stomatis	1 (0.2)	1 (0.4)		
Peptococcus sp. (oral)			24 (2.5)	6 (2.1)
Peptostreptococcus sp.	1 (0.2)	1 (0.4)		
Porphyromonas cangigivalis			2 (0.2)	1 (0.3)
Porphyromonas canoris	3 (0.5)	2 (0.8)	14 (1.4)	4 (1.4)
Porphyromonas circumdentaria	7 (1.1)	2 (0.8)	55 (5.6)	15 (5.2)
Porphyromonas gulae			4 (0.4)	2 (0.7)
Pseudomonas reactans			13 (1.3)	6 (2.1)
Pseudomonas sp.	1 (0.2)	1 (0.4)	71 (7.3)	15 (5.2)
Pseudomonas synxantha			1 (0.1)	1 (0.3)
Shigella flexneri			26 (2.7)	2 (0.7)
Simonsiella sp.	2 (0.3)	2 (0.8)		
Stenotrophomonas maltophilia			1 (0.1)	1 (0.3)
Stenotrophomonas sp.			1 (0.1)	1 (0.3)
Tannerella forsythia	6 (0.9)	4 (1.6)	64 (6.6)	13 (4.5)
Treponema sp.			7 (0.8)	5 (1.7)
Treponema vincentii			1 (0.1)	1 (0.3)
Uncultured bacterium	192 (29.0)	70 (27.7)	75 (7.7)	46 (15.9)
Uncultured *Capnocytophaga*	21 (3.2)	4 (1.6)	44 (4.5)	7 (2.4)
Uncultured *citrobacter*			42 (4.3)	4 (1.4)
Uncultured delta *proteobacterium*			4 (0.4)	1 (0.3)
Uncultured *endomicrobia*			2 (0.2)	1 (0.3)
Uncultured *lachnospiraceae*			1 (0.1)	1 (0.3)
Uncultured *propionivibrio* sp.	3 (0.5)	1 (0.4)		
Uncultured *Pseudomonas* sp.			8 (0.8)	5 (1.7)
Uncultured Termite group			1 (0.1)	1 (0.3)
Uncultured *Treponema*			1 (0.1)	1 (0.3)
Xanthomonadaceae bacterium	41 (6.2)	10 (4.0)	2 (0.2)	2 (0.7)

Bacterial species (at least 98% identity) identified by 16S rRNA gene sequencing of clones from 13 healthy and 20 FCGS samples. n= total amount of samples analysed or sequenced, in columns total amount of samples with at least 98% identity.
* Unable to distinguish between two or more species, therefore grouped generically.

Table 5.8: Uncultured bacteria identified by culture-independent (16S rRNA gene cloning) methods

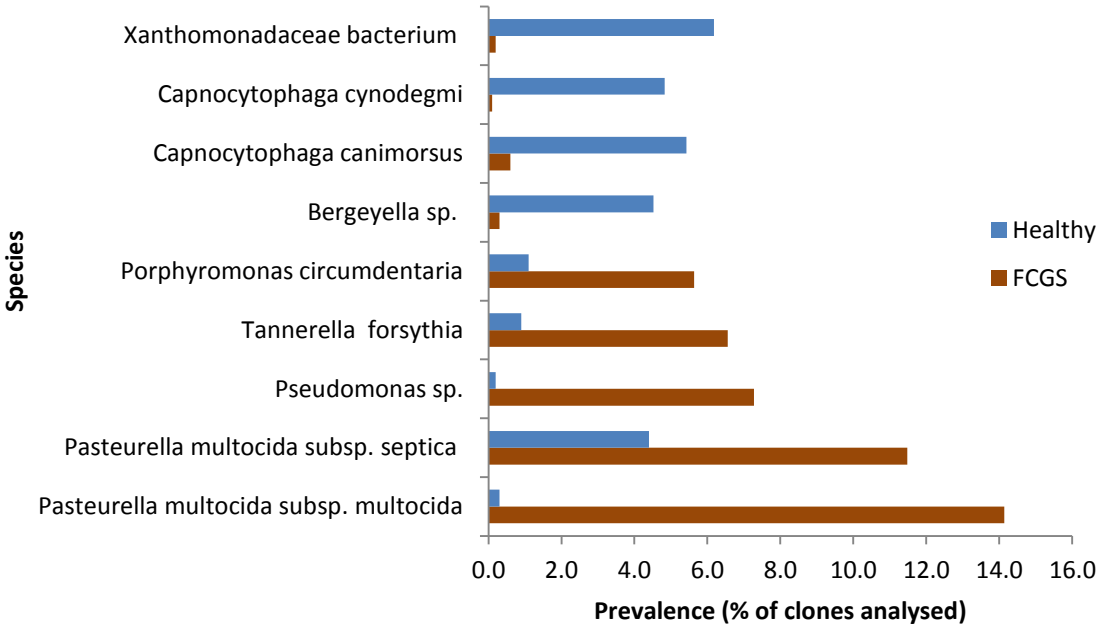
Species	Healthy		FCGS	
	No. of clones analysed (% of total) n=663	No. of clones sequenced (% of total) n=253	No. of clones analysed (% of total) n=976	No. of clones sequenced (% of total) n=289
DQ113703.1	10 (1.5)	2 (0.8)		
DQ113720.1	3 (0.5)	1 (0.4)		
DQ113762.1	5 (0.8)	2 (0.8)		
DQ925900.1			5 (0.5)	5 (1.7)
EU540333.1			1 (0.1)	1 (0.3)
EU681966.1	1 (0.2)	1 (0.4)		
EU681991.1			1 (0.1)	1 (0.3)
EU681995.1	1 (0.2)	1 (0.4)		
EU828420.1			1 (0.1)	1 (0.3)
FJ892840.1			1 (0.1)	1 (0.3)
FJ893172.1			1 (0.1)	1 (0.3)
FJ893207.1			2 (0.2)	1 (0.3)
FJ894994.1			6 (0.6)	1 (0.3)
GQ009341.1			1 (0.1)	1 (0.3)
GQ010438.1	3 (0.5)	1 (0.4)	1 (0.1)	1 (0.3)
GQ010546.1			4 (0.4)	2 (0.7)
GQ016546.1	6 (0.9)	3 (1.2)	6 (0.6)	4 (1.4)
GQ111117.1	8 (1.2)	5 (2.0)	3 (0.3)	2 (0.7)
GQ111197.1	1 (0.2)	1 (0.4)		
GQ111229.1	1 (0.2)	1 (0.4)	2 (0.2)	1 (0.3)
GQ111278.1	2 (0.3)	1 (0.4)	5 (0.5)	4 (1.4)
GQ111280.1	28 (4.2)	3 (1.2)	1 (0.1)	1 (0.3)
GQ111288.1	1 (0.2)	1 (0.4)		
GQ111657.1	4 (0.6)	2 (0.8)		
GQ113620.1	5 (0.8)	2 (0.8)		
GQ114820.1	3 (0.5)	3 (1.2)		
GQ158348.1	1 (0.2)	1 (0.4)		
GQ358873.1	1 (0.2)	1 (0.4)		
HM272460.1	1 (0.2)	1 (0.4)		
HM276442.1			2 (0.2)	1 (0.3)
HM278396.1	1 (0.2)	1 (0.4)		
HM278403.1			1 (0.1)	1 (0.3)
HM313082.1			2 (0.2)	1 (0.3)
HM326498.1	24 (3.6)	8 (3.2)	4 (0.4)	1 (0.3)
HM328219.1	2 (0.3)	1 (0.4)		
HM330153.1	5 (0.8)	2 (0.8)	4 (0.4)	3 (1.0)
HM330648.1	4 (0.6)	1 (0.4)		
HM335226.1			2 (0.2)	2 (0.7)
HM336336.1	2 (0.3)	1 (0.4)		
HM336349.1	10 (1.5)	1 (0.4)		

Table 5.8 continued

Species	Healthy		FCGS	
	No. of clones analysed (% of total) n=663	No. of clones sequenced (% of total) n=253	No. of clones analysed (% of total) n=976	No. of clones sequenced (% of total) n=289
HM336383.1	1 (0.2)	1 (0.4)		
HM340972.1	8 (1.2)	4 (1.6)	3 (0.3)	2 (0.7)
JF005737.1	1 (0.2)	1 (0.4)		
JF041223.1			2 (0.2)	1 (0.3)
JF042996.1	2 (0.3)	2 (0.8)		
JF045026.1	1 (0.2)	1 (0.4)		
JF092109.1	4 (0.6)	3 (1.2)	5 (0.5)	3 (1.0)
JF104340.1	1 (0.2)	1 (0.4)		
JF108085.1	2 (0.3)	2 (0.8)		
JF125670.1	15 (2.3)	2 (0.8)		
JF223781.1	3 (0.5)	1 (0.4)	6 (0.6)	1 (0.3)
JF232582.1	3 (0.5)	1 (0.4)		
JF240378.1			1 (0.1)	1 (0.3)
JF240413.1	1 (0.2)	1 (0.4)		
JF240735.1	16 (2.4)	2 (0.8)		
JF240930.1			1 (0.1)	1 (0.3)
JF240949.1			1 (0.1)	1 (0.3)
JF241102.1	1 (0.2)	1 (0.4)		

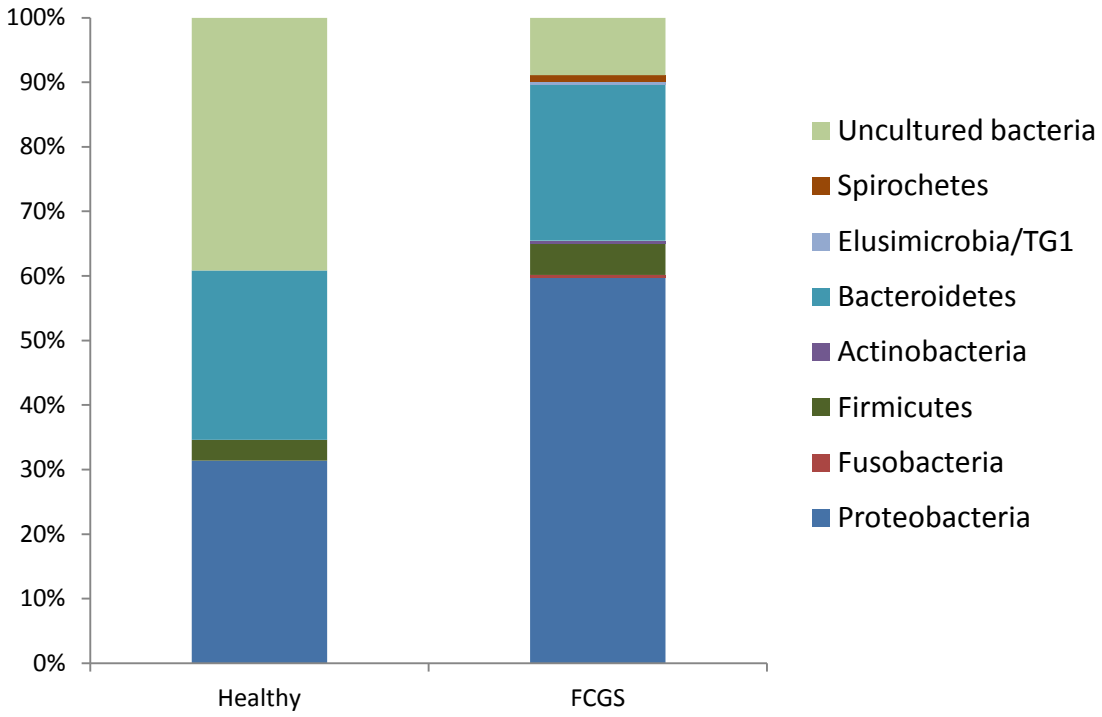
Uncultured bacteria cloned from thirteen healthy samples and twenty-two FCGS samples.

Figure 5.3: Most frequently identified bacterial species in the FCGS and healthy samples



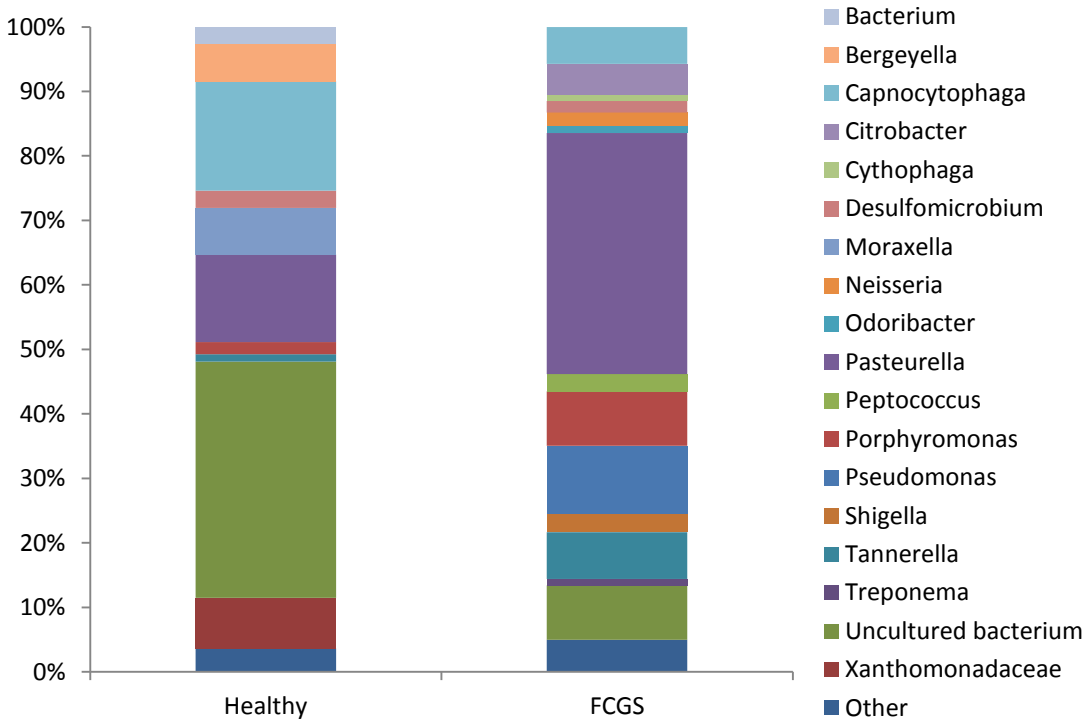
Five most prevalent bacterial species shown as a percentage of the total of all clones from 20 FCGS and 13 healthy samples.

Figure 5.4: Distribution of phyla in FCGS and healthy samples



Distribution of the different phyla identified by 16S rRNA gene sequencing of the clone libraries from healthy and FCGS samples.

Figure 5.5: Distribution of genera in FCGS and healthy samples



Distribution of the different genera identified by 16S rRNA gene sequencing of the clone libraries from healthy and FCGS samples.

5.3.2.5 Potentially novel phylotypes

Species with less than 98% identity to a known sequence from the BLAST database were classified as potentially novel phylotypes. In the 20 FCGS samples 93 clones were potentially novel represented in 48 potentially novel RFLP groups. One hundred and thirty-seven clones in the 13 healthy samples were potentially novel, represented in 70 potentially novel RFLP groups. In order to determine if some of the phylotypes were truly novel, near full-length 16S rRNA gene sequences were obtained for the clones representing potentially novel phylotypes from six FCGS (F1-F6) and three healthy (H1-H3) samples. The potentially novel phylotypes whose 16S rRNA was not sequenced fully from the remaining 14 FCGS and 10 healthy samples are shown in Table 5.9.

From samples F1 to F6, 25 clones from 17 RFLP groups were classified as potentially novel phylotypes. From three healthy samples (H1-H3) 69 clones from 37 different RFLP groups were classified as potentially novel phylotypes. The near full-length 16S rRNA gene was sequenced for 54 clones from the different RFLP groups. From 17 FCGS clones, four were found to be novel phylotypes and from the healthy samples a total of 18 clones were confirmed as being novel phylotypes. The species most closely related to the identified novel phylotypes are shown in Table 5.10. Phylogenetic analysis for all species that were identified in these nine samples and for the 22 novel phylotypes is shown in Figure 5.4

In the nine samples, six phyla were identified; Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Spirochaetes and Proteobacteria. Two novel clones branched into the Bacteroidetes phylum, three novel clones (including one FCGS clone) into the Firmicutes phylum and one clone branched into the Actinobacteria phylum. Fifteen novel clones (two from FCGS and 13 from healthy samples) branched away from the known species in a separate cluster, which also contained uncultured *Capnocytophaga* AF426105.

Table 5.9: Bacteria most closely related to potentially novel phylotypes

Species	Healthy				FCGS			
	No. of clones analysed (% of total) n=663		No. of clones sequenced (% of total) n=253		No. of clones analysed (% of total) n=976		No. of clones sequenced (% of total) n=290	
Actinomyces sp.	2	(0.3)	2	(0.8)				
Bacterium	2	(0.3)	1	(0.4)				
Bacteroidales oral clone	1	(0.2)	1	(0.4)	1	(0.1)	1	(0.3)
Bacteroides sp.	4	(0.6)	2	(0.8)	2	(0.2)	1	(0.3)
Bacteroides tectus					2	(0.2)	1	(0.3)
Bergeyella sp.	1	(0.2)	1	(0.4)				
Capnocytophaga canimorsus	10	(1.5)	4	(1.6)	2	(0.2)	1	(0.3)
Catonella sp.					1	(0.1)	1	(0.3)
Chryseobacterium sp.	1	(0.2)	1	(0.4)				
Comamonas sp.	2	(0.3)	1	(0.4)				
Desulfovibrio sp.					1	(0.1)	1	(0.3)
Eubacterium brachii					2	(0.2)	1	(0.3)
Mannheimia sp.	1	(0.2)	1	(0.4)				
Micromonas micros					2	(0.2)	1	(0.3)
Moraxella ovis					2	(0.2)	2	(0.7)
Neisseria sp.	1	(0.2)	1	(0.4)				
Parvimonas sp.					1	(0.1)	1	(0.3)
Pasteurella multocida m*ultocida*					1	(0.1)	1	(0.3)
Pasteurella pneumotropica	1	(0.2)	1	(0.4)	2	(0.2)	2	(0.7)
Pasteurella pneumotropica/ stomatitis					1	(0.1)	1	(0.3)
Pasteurella sp.	1	(0.2)	1	(0.4)				
Pasteurella trehalosi	1	(0.2)	1	(0.4)				
Peptococcus sp. (oral)					3	(0.3)	3	(1.0)
Porphyromonas sp.	10	(1.5)	5	(2.0)	1	(0.1)	1	(0.3)
Prevotella sp.	4	(0.6)	1	(0.4)				
Sufflavibacter maritimus	1	(0.2)	1	(0.4)				
Treponema sp.					1	(0.1)	1	(0.3)
Uncultured *actinobacterium*					7	(0.7)	2	(0.7)
Uncultured *anaerovorax*	1	(0.2)	1	(0.4)				
Uncultured bacterium	46	(6.9)	23	(9.1)	34	(3.5)	17	(5.9)
Uncultured *Bacteroidetes*	7	(1.1)	2	(0.8)	3	(0.3)	1	(0.3)
Uncultured *Capnocytophaga*	19	(2.9)	10	(4.0)				
Uncultured *Catonella*	2	(0.3)	1	(0.4)				
Uncultured *Endomicrobium*	7	(1.1)	1	(0.4)	12	(1.2)	2	(0.7)
Uncultured *Firmicutes*	2	(0.3)	2	(0.8)	6	(0.6)	2	(0.7)
Uncultured *Fusibacter*	2	(0.3)	1	(0.4)				
Uncultured *Lachnospiraceae*					5	(0.5)	3	(1.0)
Uncultured *Peptococcus*	1	(0.2)	1	(0.4)				
Uncultured *Treponema*					1	(0.1)	1	(0.3)

Table 5.9 continued

Species	Healthy		FCGS	
	No. of clones analysed (% of total) n=663	No. of clones sequenced (% of total) n=253	No. of clones analysed (% of total) n=976	No. of clones sequenced (% of total) n=290
Virgibacillus marismortui	1 (0.2)	1 (0.4)		
Virgibacillus marismortui/ *Bacillus permians**	5 (0.8)	1 (0.4)		
Virgibacillus sp./*Salibacillus* sp.*	1 (0.2)	1 (0.4)		

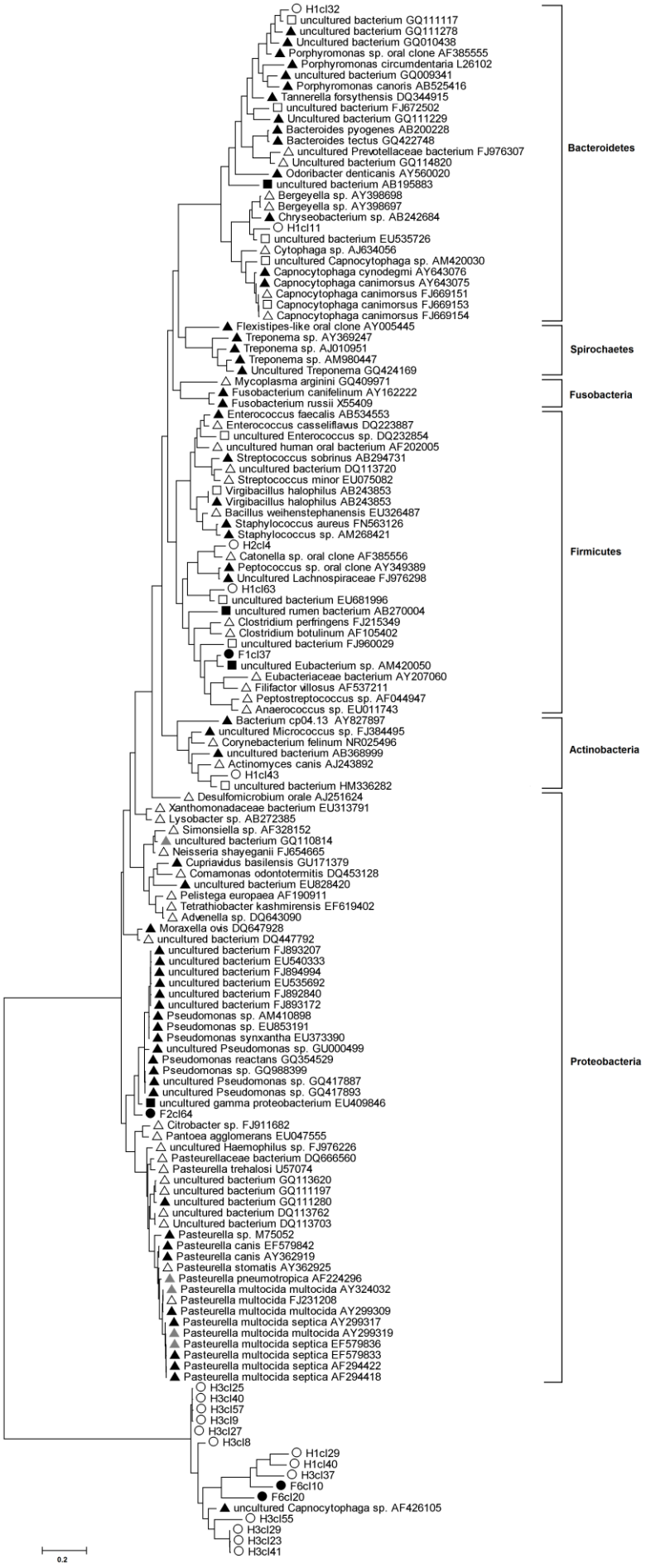
Potentially novel phylotypes identified by 16S rRNA sequencing of clones from 20 FCGS and 13 healthy samples.
* Unable to distinguish between two or more species, therefore grouped generically.

Table 5.10: Phylotypes confirmed as novel

Clone	Accession No.	Most closely related bacterium	Identity (%)
H1cl11	EU535726.1	Uncultured bacterium clone nbt10b01	95.1
H1cl29	AB243853.1	*Virgibacillus halophilus*	96.8
H1cl32	GQ111117.1	Uncultured bacterium clone nbw621a08c1	96.4
H1cl40	DQ232854.1	Uncultured *Enterococcus* sp. clone F28	93.9
H1cl43	HM336282.1	Uncultured bacterium clone ncd1073b06c1	93.9
H1cl63	EU681996.1	Uncultured bacterium clone DH5-10	94.2
H2cl4	FJ672502.1	Uncultured bacterium clone Ll142-5G3	96.1
H3cl8	AM420030.1	Uncultured *Capnocytophaga* sp.	94.6
H3cl9	AM420030.1	Uncultured *Capnocytophaga* sp. partial	93.8
H3cl23	FJ669153.1	*Capnocytophaga canimorsus* strain BD05-00029	92.6
H3cl25	AM420030.1	Uncultured *Capnocytophaga* sp.	94.2
H3cl27	AM420030.1	Uncultured *Capnocytophaga* sp.	94.2
H3cl29	FJ669153.1	*Capnocytophaga canimorsus* strain BD05-00029	92.7
H3cl37	FJ960029.1	Uncultured bacterium clone D940_095	95.2
H3cl40	AM420030.1	Uncultured *Capnocytophaga* sp.	93.8
H3cl41	FJ669153.1	*Capnocytophaga canimorsus* strain BD05-00029	92.5
H3cl55	EU535726.1	Uncultured bacterium clone nbt10b01	96.9
H3cl57	AM420030.1	Uncultured *Capnocytophaga* sp.	93.7
F1cl64	EU409846.1	Uncultured gamma proteobacterium clone	95.8
F2cl10	AB270004.1	Uncultured rumen bacterium	95.4
F2cl20	AB195883.1	Uncultured bacterium gene	90.6
F3cl37	AM420050.1	Uncultured *Eubacterium* sp.	96.3

Novel bacterial phylotypes identified by 16S rRNA sequencing of clones from three healthy and six FCGS samples.

Figure 5.6: Phylogenetic analysis



Legend to Figure 5.6

All bacterial species identified in six FCGS and three healthy samples (including confirmed novel phylotypes)

Clones representing novel phylotypes: ●, FCGS; ○, healthy

Clones representing known phylotypes (Dolieslager et al., 2011); ▲, FCGS only, △, healthy only; ▲, FCGS and healthy

Known phylotypes to which novel phylotypes are most closely related as determined by BLAST analysis: ■, FCGS; □, healthy

5.4 Discussion

Although it has been suggested that bacteria are an important part of the aetiopathogenesis of FCGS, their specific roles are unclear. Over the years, several studies have been conducted to identify bacterial species in the oral cavity of the cat. The most important bacteria that are implicated to play a role in FCGS are gram-negative anaerobes and *Bartonella* species (Sims et al., 1990; Glaus et al., 1997).

Research on *Bartonella* species shows contradictory results on the possible relationship between *Bartonella henselae* and feline oral diseases. In one study, a possible link between gingivitis and seropositivity for both FIV and *Bartonella* species was shown (Ueno et al., 1996). An increase in the frequency of diagnosing stomatitis in cats that are seropositive for *B. henselae* has also been reported (Glaus et al., 1997). In a study with 190 cats, no correlation was described between *Bartonella* species and oral lesions when PCR was performed on oral swab samples; however the same study showed cats were three to four times more likely to suffer from oral lesions when seropositive for *B. henselae* and *B. clarridgelae* (Namekata et al., 2010). A recent large-scale study used isolation of *Bartonella* species from blood samples in combination with serology tests. A correlation between FCGS and isolation of *Bartonella* species was found but no correlation between seropositivity and FCGS could be discovered (Sykes et al., 2010). Other studies failed to show any correlation between FCGS and the presence of *Bartonella* species (Quimby et al., 2008; Belgard et al., 2010; Dowers et al., 2010).

In the current study no *Bartonella* species were detected in oral swabs from cats with FCGS or orally healthy cats. It is perhaps unsurprising that *Bartonella* species were not cultured. The organism needs a prolonged incubation period with an average of 21 days to form colonies on blood agar plates (Maggi et al., 2005). The standard culture media and incubation periods used to ensure growth of a broad range of bacteria would not be suitable for culture of *Bartonella* species. Also, there are no reports of *Bartonella* species having been cultured directly from oral swabs; detection of *Bartonella* species from the oral cavity has been achieved solely by the use of molecular detection methods. It is possible that *Bartonella* DNA may have originated from other sources (Quimby et al., 2008; Namekata et al., 2010). One way for bacterial DNA to enter the oral cavity is by grooming. In the case of *Bartonella* species, the ingestion of flea dirt in which *Bartonella henselae* can persist for at least three days might be significant (Finkelstein et al., 2002; Quimby et al., 2008).

No bacterial DNA from *Bartonella* species was detected by 16S PCR in any of the samples analysed in the current study. The universal primer combination of 63f and 1387r that was utilised has been used previously to successfully detect strains of *Bartonella* species (Bai et al., 2008). A BLASTn test confirmed that the primers would be able to amplify *Bartonella henselae* DNA. In 16S rRNA gene sequencing the phenomenon of primer bias should always been taken into account. In the present study several organisms were isolated by culture that were not identified by culture-independent methods. Primer bias leads to unequal amplification of PCR products and consequent inaccuracies in the true number of species present within a sample (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998).

Culture-dependent methods were initially used to analyse the bacterial flora at the gingival margin of cats with healthy gingiva and cats with inflamed gingiva (Love et al., 1989). The bacteria that were isolated consisted of a combination of *Bacteroides* species, currently renamed and including *Bacteroides*, *Prevotella* and *Porphyromonas* species. All of these isolated species were found in higher numbers at the healthy gingival margin compared to the inflamed gingival margin. The current study focused on a combination of culture-dependent and culture-independent methods in an attempt to identify the microbial flora in cats with FCGS and in healthy cats. This is the first study to use molecular cloning and sequencing of bacterial 16S rRNA genes to identify bacteria in the oral cavity of the cat.

One of the key findings in the current study is the proportion of *P. multocida* species in the cats with FCGS compared to the healthy oral cavity. *P. multocida* was identified in high numbers by all identification methods used (culture, direct 16S rRNA gene sequencing and in the culture results from the Veterinary Diagnostic Service).

P. multocida, a gram negative proteobacterium, is a commonly found bacterium of the feline oral cavity (Baldrias et al., 1988; Love et al., 1990; Hariharan et al., 2011) and is also known for its important role in cat-bite infections (Love et al., 2000). *P. multocida* has also been found in feline periodontal disease, where its numbers were found to decrease with an increase of disease severity (Mallonee et al., 1988). Another study showed that the organism was significantly more prevalent in cats with inflamed gingiva compared to healthy gingiva, although cats with mild inflammation showed the biggest increase in *P. multocida* (Mihaljevic and Klein, 1998). Two single case studies on FCGS samples showed large numbers of *P. multocida* (Reindel et al., 1987; Addie et al., 2003). *P. multocida* is a commensal in the oral cavity of the cat but strains of *P. multocida* are the named causative agent in several diseases, including atrophic rhinitis in swine, bovine haemorrhagic septicaemia and avian fowl cholera (Shivachandra et al.;

Subaaharan et al.; Davies et al., 2003). Swabs used in this study were taken directly from the oral lesions and showed a high concentration of bacteria at these sites, which implicates a possible role of *P. multocida* in this disease. It should be noted that *P. multocida* was not detected in all cats with FCGS, and colonisation of several other species was also seen. The species of *P. multocida* that were identified in cats with FCGS were *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. In the current study, *P. multocida* subsp. *septica* was found in high numbers in both healthy and FCGS samples but *P. multocida* subsp. *multocida* was found in very low numbers in the healthy cats and is the most represented species in the cats with FCGS.

Another striking difference between the healthy and FCGS groups in the present study was the presence of *Porphyromonas* species, especially *P. circumdentaria*. *Porphyromonas* species were highly represented in the cats with FCGS but were identified in much lower numbers in the healthy samples. *P. gingivalis* has been accepted as one of the key aetiological agents in human periodontal disease and is often referred to in combination with *T. forsythia* and *Aggregatibacter actinomycetemcomitans* (Slots et al., 1986; Bragd et al., 1987; Socransky et al., 1998). *P. gingivalis* was not found in the current study but three other *Porphyromonas* species were identified. Finding *Porphyromonas* species in the feline oral cavity is not new, since in the first studies on feline oral microbiology black pigmented *Bacteroides* species, that may have been *Porphyromonas* species, were isolated (Mallonee et al., 1988). *Porphyromonas* species have been isolated from cats with oral diseases and also from the healthy gingival margin (Love et al., 1989). In relation to periodontal disease in cats, *Porphyromonas* appears to be a good indicator of disease risk, as is the case in human periodontal disease. A positive correlation has been seen between the grade of periodontal disease and the quantity of *Porphyromonas* species at the canine and premolar sites where three species (*P. gingivalis*, *P. circumdentaria*, and *P. salivosa)* were studied (Norris and Love, 1999a). Periodontal pathogens found in cats and their owners were compared using culture-independent methods (Booij-Vrieling et al., 2010). The *Porphyromonas* species found in cats were *P. gulae* and in humans *P. gingivalis*. *T. forsythia* was found in both humans and cats. Human strains of *P. gingivalis* are being studied and a broad variety of virulence factors have been described. The variety of proteases described in *P. gingivalis* is not described in *P. circumdentaria* and therefore the mechanism behind the tissue destruction caused by hydrolysation of serum and tissue proteins cannot be transferred to *P. circumdentaria* (Grenier and Vu Dang, 2011). Further research into the host interaction of different *Porphyromonas* species, alone or in synergy with other possible pathogenic bacteria can give us insight into the role they might play in FCGS.

T. forsythia (formerly known as *Bacteroides forsythus* or *Tannerella forsynthensis*) is another well represented bacterium in the FCGS samples in the present study. As described previously, *T. forsythia* is a well-known periodontal pathogen in humans and virulence mechanisms have been studied. Activity of proteases and several surface factors are shown to play a role in the pathogenity of *T. forsythia* as are the synergetic abilities with other human pathogens like *P. gingivalis* and *F. nucleatum* (Sharma, 2010). It has been described in the feline oral cavity in relation to periodontal disease but not in relation to FCGS. Unspecified *Bacteroides* species have previously been described in oral diseases in cats (Mallonee et al., 1988; Love et al., 1989). *T. forsythia* has been identified in plaque samples from cats with and without periodontal disease, where the proportion of *T. forsythia* was higher in cats with periodontitis (Booij-Vrieling et al., 2010). *Tannerella* species have been identified regularly by culture-independent methods but were only isolated on only one occasion in the current study. The organism requires a specific growth medium and the standard culture methods that were performed would not allow this species to be successfully cultured in high numbers (Wyss, 1989).

In two of the FCGS samples, a high proportion of the bacteria identified were *Pseudomonas* species. *Pseudomonas* species are known to be present in the oral cavity of the cat during health and disease (Mihaljevic and Klein, 1998) but also cause a variety of problems in pets, such as otitis externa and otitis media, urinary tract infections and pyoderma (Kowalski, 1988; Gatoria et al., 2006; Hillier et al., 2006). *Pseudomonas* species are not known to be of importance in oral diseases.

Potentially novel phylotypes were found in healthy and FCGS samples in the current study. A near full-length 16S rRNA gene sequence was obtained for the clones representing potentially novel phylotypes from six FCGS and three healthy samples. Eighteen clones from the three healthy samples analysed were confirmed as being novel phylotypes, compared to only four clones from the six FCGS samples. In the FCGS samples, 2.3% of all 304 analysed clones were novel and in the healthy cats 19.6% of the 158 analysed clones were novel. In these samples a high proportion of *P. multocida* species was found in the FCGS samples and there was less microbial diversity compared to the healthy samples (Dolieslager et al., 2011). Therefore the lower proportion of novel phylotypes found in the FCGS samples is unsurprising. Phylogenetic analysis of these novel phylotypes showed a cluster of 15 clones branching away from known phylotypes. This cluster included one uncultured *Capnocytophaga* AF426105. Six clones were found branched into three known phyla. The cluster of 15 clones implicates a group of previously unidentified bacteria that might be found frequently in the oral

cavity of the cat. These identified novel bacterial phylotypes need further characterisation to elucidate their role in oral health and disease.

Of the samples analysed, two (F11 and F18) demonstrated very sparse growth. No bacterial identification was performed on these samples in the Veterinary Diagnostic Services laboratory. A further two samples (F11 and F21) had a very low cloning efficiency and not enough inserts could be re-amplified from the clones to generate a library for analysis. The 16S rRNA PCR showed small amounts of DNA on the gel from these samples and the quantity was not enough to obtain a sufficiently efficient cloning reaction, as demonstrated by the low colony numbers observed after cloning. 16S rRNA gene sequencing was performed on the isolates for identification purposes. DNA could not be extracted from all isolates using the standard DNA extraction methods. During this study a standard proteinase-K extraction protocol was used to enable a time efficient and reliable DNA extraction from most bacteria. DNA from some bacteria, which are more challenging to lyse would not be able to be extracted by these standard methods. It should be taken into account that although a wide range of bacteria were identified in this study, it is likely that some species will not have been successfully identified. By using three different methods (standard diagnostic culture, culture with 16S rRNA gene sequencing and direct 16S rRNA gene sequencing) in two different laboratories, we tried to maximise identification of the different bacterial species present.

The antibacterial treatment given to cats suffering from FCGS could have influenced the bacterial species found. Antibiotic treatment had been given at different times and had been terminated up to one year prior to the study (Table 3.8 and 3.9). At the time of swab collection all cats had clinical signs of FCGS (as described in Section 1.2.1.2) despite any treatment given. Clindamycin is the antibacterial therapy most frequently described for treatment in FCGS. Clindamycin inhibits bacterial protein synthesis by binding to the 50S subunit of the ribosome (Scott, 2005). Clindamycin is often used in oral inflammation as it has a good action against anaerobes including *Fusobacterium*, *Peptostreptococcus* and *Actinomyces* species (Noli and Boothe, 1999). A study on orbital abcesses in dogs and cats showed however that none of the isolates in cats was susceptible to clindamycin (Wang et al., 2009). Orbital abcesses can be caused by tooth root inflammation or foreign body penetration through the oral cavity. Bacteria isolated in these abcesses are often of oral origin. As seen in this study *Pasteurella* sp. and *Bacteroides* sp. were most often isolated. Susceptibility *in vitro* can of course differ from the *in vivo* susceptibility and factors as tissue penetration and the presence of pus in abcesses can play a role, however if these two species, which were regularly isolated from the cats in the present study are not susceptible to clindamycin detecting these

bacteria could have been a result of the antibacterial treatment. Other antibacterial therapies that had been used prior to sampling included cefovecin. Cefovecin, an extended-spectrum cephalosporin antibiotic, is registered for the use in periodontal disease and has a known action against oral bacteria including *P. multocida* (Stegemann et al., 2006). In the current study, cefovecin has been prescribed in several cases. Numbers of individual bacterial species in the current study could have been disturbed by the use of antibacterial treatment. Which is why further studies into pathogen host interaction will be beneficial. A broad variety of bacteria have been discovered and, all cats were suffering from lesions during the time of sampling. In an ideal world, sampling lesions from cats during the same stage of disease without any treatment will give us the best results. In the current study, a variety of antimicrobials were used and a relative high number of samples were taken which showed a wide range of bacterial species. The study gives a good view on which species may be involved in the aetiopathogenesis of FCGS but given the fact that antibacterial treatment was used, species that are present in smaller numbers should not be overseen as they could be playing an important role.

As outlined in the Section 1.4.2, not many studies have specifically focussed on the bacteriology of FCGS. Clinical signs of different feline oral diseases are often categorised under the title of FCGS (as described in Section 1.2.1.1). In this study, a group of cats with similar clinical signs and all having at least mild inflammation of the area lateral to the palatoglossal folds were included. All swabs were taken from the palatoglossal fold area and the results are therefore not comparable with previously published plaque-focused studies. In the current study we focussed on bacteria found locally on the lesions to find possible causative agents of FCGS. Studies on periodontal disease which focus on the site of the disease use plaque samples for analysis. In the current study, bacteria at the site of inflammation tended to show little diversity with a high abundance of only one or two species per sample being seen. Repeatedly high bacterial counts of *P. multocida* subspecies, *T. forsythia*, *Pseudomonas* and *Porphyromonas* species were found in different combinations. When the identified phyla in healthy and FCGS samples were compared, phyla that were not found in the healthy oral cavity but which were seen in the cats with FCGS included Fusobacterium and spirochaetes. A shift was seen from a mix of Bacteroidetes, uncultured bacteria and Proteobacteria in the healthy samples to predominantly Proteobacteria in the cats with FCGS. These results support the likelihood of an opportunistic infection by bacteria present in the oral cavity. This seems to correlate with the clinical experience of a temporary response to antimicrobial therapy in these cats. Confirmation that these maby be the most important bacteria in this disease could be obtained by the use of

high throughput, next generation sequencing followed by further research into the interaction of these specific bacteria with different aspects of the immune system.

Chapter 6 Histopathological changes in tissue biopsies of cats with FCGS

6.1 Introduction

As described in section 1.2.1.1 and Table 1.1, many different terms are used in the literature to identify FCGS. Many refer to both the histopathological features of the lesions and the location of these lesions. Feline plasma cell gingivitis/pharyngitis, plasma cell stomatitis/pharyngitis and lymphoplasmacytic gingivitis are examples of the different names that are used (Johnessee and Hurvitz, 1983; White et al., 1992; Lyon, 1994).

The oral mucosa consists of two components; the epithelium and the supporting connective tissue called the lamina propria (Nanci, 2008). The boundary between mucosa and submucosa is difficult to identify. The submucosa contains fat, small vessels and minor salivary glands in variable amounts throughout. The epithelium is a stratified squamous epithelium. Cells seen in the normal epithelium are melanocytes, Langerhans cells, Merkel cells and low numbers of inflammatory cells. The lamina propria is the connective tissue supporting the oral epithelium and consists of cells, blood vessels, neural elements and connective tissue fibres. Fibroblasts, macrophages, mast cells and other inflammatory cells are normally seen in the lamina propria. Fibroblast numbers increase during wound healing. Small numbers of plasma cells and lymphocytes can be observed in normal tissue, larger numbers represent a pathological change. In acute reactions, neutrophils are the dominant cell type and in chronic conditions lymphocytes, plasma cells and macrophages are often seen. In the current study the majority of the changes described affected the epithelium and lamina propria.

Cats with clinical evidence of FCGS (Section 1.2.1.2) have been divided into two distinct pathological syndromes (Barker et al., 1992). Feline ulcerative stomatitis and glossitis or lymphocytic plasmacytic stomatitis is described as a chronic inflammation of the ‘fauces’, and can involve inflammatory changes at the palate, gingiva and tongue. A chronic active inflammation of the oral mucosa and submucosal connective tissues is seen and the dominant cells are lymphocytes and plasma cells. These changes are more prevalent in older cats and can include periodontitis. The second group is feline plasma cell gingivitis/pharyngitis or feline chronic gingivostomatitis, which is characterised by lesions in the palatoglossal folds often extending to the

palatopharyngeal arches and the gingiva. The mucosa is hyperplastic, ulcerated and the cells found are mainly plasma cells. Mott cells, plasma cells containing Russell bodies, as well as binucleated plasma cells can often be identified. Other cells described are neutrophils, lymphocytes and histiocytes (Barker et al., 1992). It is not clear if these two groups, which are distinguishable on the basis of cell type but can be associated with a similar clinical appearance, are the same disease. To date no studies have been conducted that compare the pathological findings in detail with other features of FCGS. In the current study the pathological findings of the palatoglossal fold tissue of these cats has been scored on severity of inflammation according to the scoring system previously described (Harley et al., 2011). The two pathological groups as described by Barker et al. (1992) are also used as a guideline to separate the samples.

6.2 Material and methods

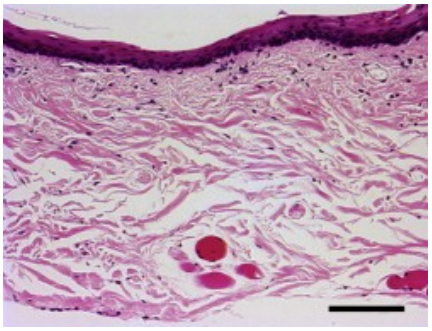
6.2.1 Sample collection and processing

Tissue biopsies were obtained from the palatoglossal fold area of cats under general anaesthesia. The tissue was fixed in 4% neutral buffered paraformaldehyde and sent to the veterinary diagnostic laboratory at the University of Glasgow. The tissue biopsies were embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin (H&E staining). Routine histological evaluation was performed.

6.2.2 Scoring of inflammatory changes

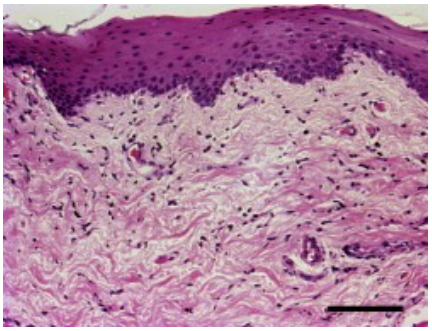
The severity of the inflammation in each biopsy was scored according to changes in the epithelium and the lamina propria. The epithelial changes included inflammatory cell infiltration and hyperplasia, degeneration and ulceration. The lamina propria changes included inflammatory cell infiltration, presence of granulation tissue and the presence of fibronecrotic material. The inflammatory cell infiltration within the lamina propria was scored. The scoring system used was that described by Harley et al. (2011) (Table 6.1).

Table 6.1: Grading system for histopathological changes



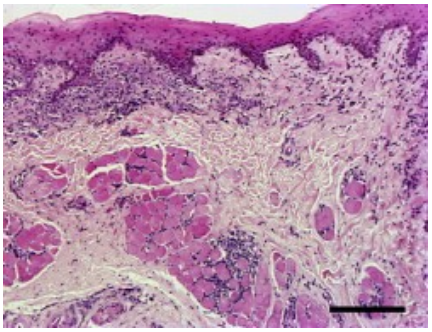
Grade 0: Normal

Stratified squamous epithelium with sparse intraepithelial lymphocytes. The lamina propria/submucosa contains sparse scattered mast cells and lymphocytes. The lymphocytes may form small subepithelial aggregates.



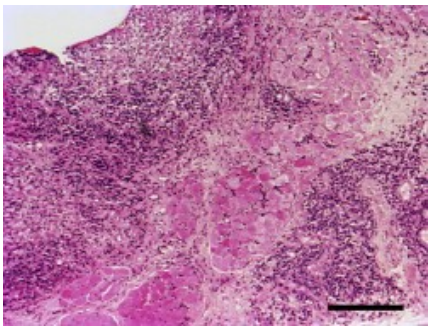
Grade 1: Minimal to mild inflammation

Variable, mild epithelial hyperplasia and parakeratosis. May have slightly increased numbers of intraepithelial lymphocytes and sparse exocytosing neutrophils.
The lamina propria/submucosa contains a sparse to light, perivascular to interstitial population of plasma cells, lymphocytes, mast cells and rare macrophages.



Grade 2: Moderate inflammation

Epithelial hyperplasia variably with regions of degeneration or ulceration. Mild to moderate numbers of intraepithelial lymphocytes variably mixed with macrophages and neutrophils. The lamina propria/submucosa contains a moderate inflammatory cell infiltration of lymphocytes and plasma cells mixed with variable numbers of macrophages and neutrophils. The inflammatory cells may form a distinct 'lichenoid' band within the superficial lamina propria. In the submucosa the infiltrating cells often extend around skeletal muscle fibres.



Grade 3: Severe inflammation

Often extensive regions of epithelial degeneration, spongiosis and ulceration and superficial exudation with many macrophages, neutrophils and lymphocytes.
The lamina propria/submucosa contains a dense inflammatory infiltrate with variable proportions of lymphocytes, plasma cells, macrophages and neutrophils. In some sections the lamina propria is expanded or replaced by immature granulation tissue and fibrinonecrotic debris.

Reproduced from Harley et al. (2011)

6.2.3 Grouping according to cell type

All biopsies were grouped according to the different types of cell infiltration previously described in FCGS. Two groups according to cell type were standardised (Barker et al., 1992):

- Group 1: Combined infiltration of plasma cells and lymphocytes

- Group 2: Majority of plasma cells, including Mott cells, binucleate plasma cells and fewer neutrophils, lymphocytes and macrophages

6.2.4 Statistical analysis of the histopathological changes

The independent samples t-test was chosen to compare the mean age of the cats and the Mann-Whitney test to compare the duration of the disease since onset for each of the two groups defined in Section 6.2.3. The null hypothesis (H0) was that no significant difference occurred between the two groups. A Spearman's correlation was performed to test the correlation between the severity of the inflammation (Section 6.2.2) and the cell type group (Section 6.2.3). Differences were considered statistically significant at $p<0.05$.

6.3 Results

6.3.1 Cell types and histopathological features

Of the 31 biopsies that were analysed, two were non-diagnostic: F19 due to crush artefacts and F29 due to insufficient sample size. In the other 29 biopsies, the epithelium and lamina propria were assessed. Glandular tissue was present in six biopsies; F21 showed no changes in the glandular tissue but inflammation adjacent to the ducts was observed in the remaining five samples (Table 6.2). The epithelium showed hyperplasia in 26 cats and exocytosis of inflammatory cells into the epithelium was seen in 25 cats. Of these cats, neutrophils were present in the epithelium in 14, plasma cells in three, lymphocytes in two and unidentified leucocytes in 12. Inflammatory cell types identified in the lamina propria were predominantly plasma cells in 14 cats, a combination of plasma cells and lymphocytes in 11, a combination of neutrophils and plasma cells as the predominant cells in two and mainly lymphocytes in two. Neutrophils marginating within vessels were seen in 25 of 31 cats.

Eight healthy tissue samples were analysed (Table 6.3). One sample was non-diagnostic due to only salivary gland tissue being present. Hyperplasia of the epithelium was minimal in two cats, mild in two cats and moderate in one cat. Five cats showed few inflammatory cells and two cats showed a mild inflammatory infiltrate in the lamina propria. A minimal to mild infiltration of neutrophils was seen marginating in vessels in four cats and minimal sialoadenitis was seen in two cats. One cat was diagnosed as having a mild gingivitis; six cats were thought to be normal.

6.3.2 Severity of inflammation scores

For the 29 tissue biopsies that were assessed, the inflammation score was graded (Harley et al., 2011). Pathological changes that were classified between two categories were scored as mild to moderate (1-2) or moderate to severe (2-3). Severity was graded mild in two cats, mild to moderate in five cats, moderate in ten cats, moderate to severe in five cats and severe in seven cats (Table 6.4, Figure 6.1).

Table 6.2: Cell types and histopathological features in epithelium and lamina propria in 29 FCGS samples

		F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17
Epithelium	Dysplasia*										+		+				
	Erosion*			+					+	+					+		+
	Hyperpigmentation*				+												
	Hyperplasia	+	+	+	++	+		++	+	++	+	++	+++	+		+	++
	Rete pegging*							+		+		+	+				
	Spongiosis*	+	+	+	+			+		+	+		+	+	+	+	+
	Thinning*	+									+					+	
	Ulceration*	+		+		+	+			+		+		+			+
	Bacteria					++		+				+					
	Leucocytes	+		+	+					+++		+	+				++
	Lymphocytes							+									
	Neutrophils			+		+	+				++					+	
	Plasma cells							+			+++						
Lamina propria	Lymphocytes	++	+	+	++	+++	+	+++	+	++	++	++		+	++	+	+
	Macrophages	+												+			+
	Mitotic figures																
	Neutrophils	+	+	++		++	+	+	+	+	++	+	+	+	++	+	
	Plasma cells	++	+	+++	+	+++	++++	+++	+	++	++++	++	+	+++	++++	+++	+++
	Binucleate plasma cells		+														
	Mott cells		+	++			+				++			+	+		+
	Bacteria					++						+					
	Fibrosis*	+	+	+		+						+					
	Granulation tissue*						+	+	+			+		+	+		
	Necrosis*					+	+	+		+		+		+			
	Oedema*		+		+			+				+	+		+		+
Marginating neutrophils		++	++	+	+	+	++			+	++	+	+	+		++	+
Sialoadenitis				+													

Table 6.2 continued

		F18	F19	F20	F21	F22	F23	F24	F25	F26	F27	F28	F29	F30	F31	F32
Epithelium	Dysplasia*					+		+								
	Erosion*	+			+		+				+				+	+
	Hyperpigmentation*														+	
	Hyperplasia		+	+	+	+	+		+	+	+	++		+	+	+
	Rete pegging*		+			+	+		+					+		
	Spongiosis*	+		+	+	+		+	+	+		+			+	
	Thinning*															
	Ulceration					+		+	+		+	+				
	Bacteria									+		+				
	Leucocytes	+			+				+			+++		+		
	Lymphocytes			++												
	Neutrophils			++	+	+	++	+		++	++				+	+
	Plasma cells			++												
Lamina propria	Lymphocytes			+++	+	+	+	+	+	++	++		+	++	++++	++
	Macrophages						+	+				+				
	Mitotic figures						+								+	
	Neutrophils	++		++	+	+++	+++	++	++	+	++	+++			++	++
	Plasma cells	++++		+++	+++	+++	++++	++++	++++	++	++	++++	+	++	+++	+++
	Binucleate plasma cells			+				+				+				
	Mott cells	+		+	++		+	++	++	+		++				++
	Bacteria											+				
	Fibrosis*			+		+		+		+	+					
	Granulation tissue*					+						+				
	Necrosis*					+				+		+				
	Oedema*			+	+			+	+	+	+					+
Marginating neutrophils		++		+	+++	++	+++	+	+	+	++		+		++	+
Sialoadenitis							+		++	++						+

Grading: +: Mild, ++: Moderate, +++: Moderate/severe, ++++: Severe. In subjects with a *, + means present.

Table 6.3 Cell types and histopathological features in epithelium and lamina propria of eight healthy samples

		H2	H3	H4	H5	H6	H14	H15	H16
Epithelium	Dysplasia*								
	Erosion*								
	Hyperpigmentation*								
	Hyperplasia	+/-		++	+	+/-			+
	Rete pegging*				+				
	Spongiosis*								
	Thinning*								
	Ulceration*								
	Bacteria								
	Leucocytes								
	Lymphocytes								+/-
	Neutrophils								
	Plasma cells								
Lamina propria	Lymphocytes	+		+/-	+	+/-	+/-	+/-	+/-
	Macrophages	+							
	Mitotic figures								
	Neutrophils								
	Plasma cells	+		+/-	+	+/-	+/-	+/-	+/-
	Binucleate plasma cells								
	Mott cells								
	Bacteria								
	Fibrosis*								
	Granulation tissue*								
	Necrosis*								
	Oedema*								
Marginating neutrophils		+					+/-	+/-	+
Sialoadenitis							+/-	+/-	

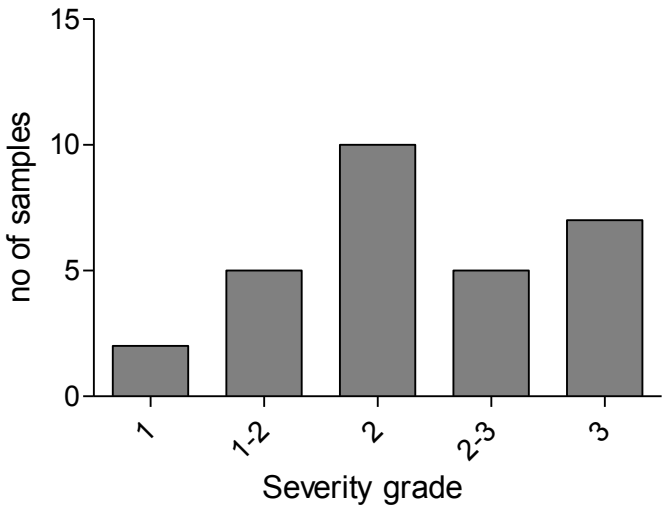
Grading: +/-: Minimal, +: Mild, ++: Moderate, +++: Moderate/severe, ++++: Severe. In subjects with a *, + means present.

Table 6.4: Cell groups, severity grades, age and time since onset of disease in 31 cats with FCGS

Cat ID	Cell group	Severity grade	Severity clinical inspection	Age (months)	Time since onset (months)
F2	1	1-2	3	164	14
F3	1	1	3	104	14
F4	2	2	1	164	2
F5	1	1-2	1	90	3
F6	1	2-3	3	47	6
F7	2	3	3	113	21
F8	1	2	3	14	2
F9	1	1	3	66	20
F10	1	2	3	48	4
F11	2	3	3	80	81
F12	1	2	2	12	7
F13	2	1-2	1	21	6
F14	2	2	2	40	11
F15	2	2-3	3	87	10
F16	2	2	3	70	7
F17	2	2	3	114	7
F18	2	2-3	3	65	25
F19	-	-	2	90	21
F20	1	2	-	155	-
F21	2	2	3	72	-
F22	2	2-3	2	15	-
F23	2	3	3	70	-
F24	2	3	3	204	-
F25	2	3	-	126	-
F26	1	1-2	-	17	-
F27	1	2	3	43	22
F28	2	3	-	156	-
F29	-	-	-	120	9
F30	1	1-2	-	111	-
F31	1	3	-	168	-
F32	2	2-3	3	168	28

All samples were grouped according to cell type, cell group 1: lymphoplasmacytic infiltrate, cell group 2: plasmacytic infiltrate. All samples were graded according to severity. Shown in combination with the clinical appearance of the caudal oral cavity, the age in months and the time since disease onset in months.

Figure 6.1: Number of FCGS samples showing different severity of inflammation grades



1: mild, 1-2: mild-moderate, 2: moderate, 2-3: moderate-severe, 3: severe

Variable results were seen when histopathological severity grading was compared to the clinical inspection of the caudal area of the oral cavity. Of the cats with clinical scores recorded, seven showed higher clinical severity scores, two showed higher histopathological scores and 13 cats showed a similar score in clinical and histopathology severity (Table 6.4).

6.3.3 Cell groups

From the 29 biopsy samples analysed, thirteen were categorised as cell group 1, a chronic inflammation in which plasma cells and lymphocytes are the dominant cells. The remaining sixteen were categorised as cell group 2, involving high numbers of plasma cells (Table 6.4, Figure 6.2).

When cats from each severity group were classified according to the cell group, a significant correlation ($p=0.004$) was seen by a Spearman's correlation test, showing that the majority of samples in the higher inflammation grades showed plasma cell infiltration (group 2) and the majority of samples in the lower inflammation grades were infiltrated with lymphocytes and plasma cells (group 1) (Figure 6.3).

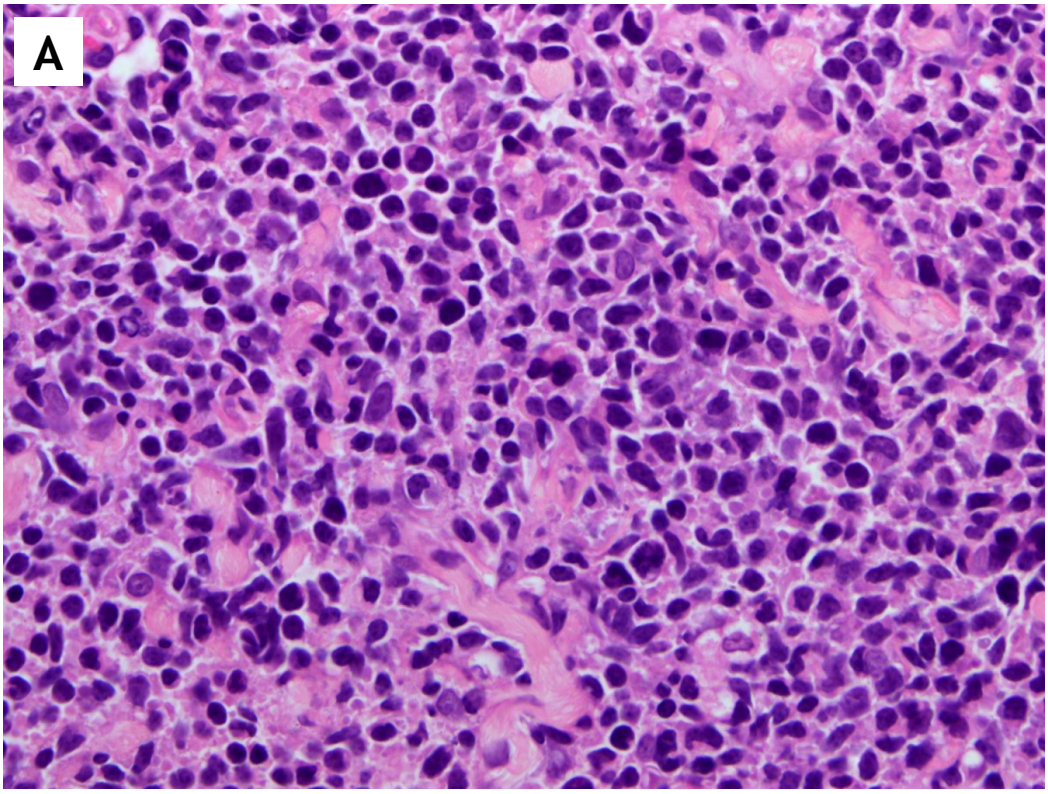
6.3.4 Comparison of age and the cell groups

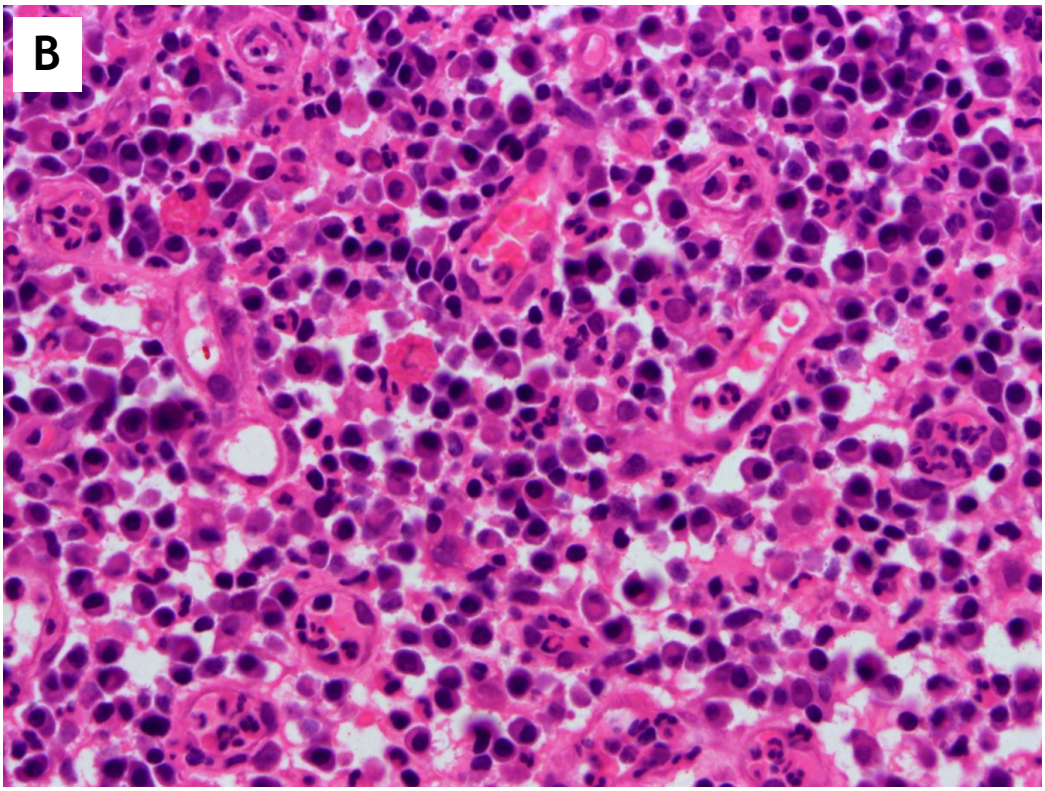
When the mean age of the cats classified as cell type group 1 was compared to the mean age of the cats in group 2 by a t-test, no significant difference was seen ($p=0.88$). The mean age in months ± SD for group 1 ($n=13$) was 79.92 ± 56.78 and for group 2 was ($n=16$) 97.81 ± 54.82 (Table 6.4, Figure 6.4).

6.3.5 Comparison of time since onset of disease and cell groups

Comparison of the time since onset of the disease in the cats placed in cell group 1 with the time since onset in the cell group 2 cats by a Mann-Whitney test showed no significant difference ($p=0.12$). The mean time since onset in months ± SD for group 1 ($n=10$) was 9.800 ± 7.193 and for group 2 ($n=11$) was 20.18 ± 21.85 (Table 6.4, Figure 6.5)

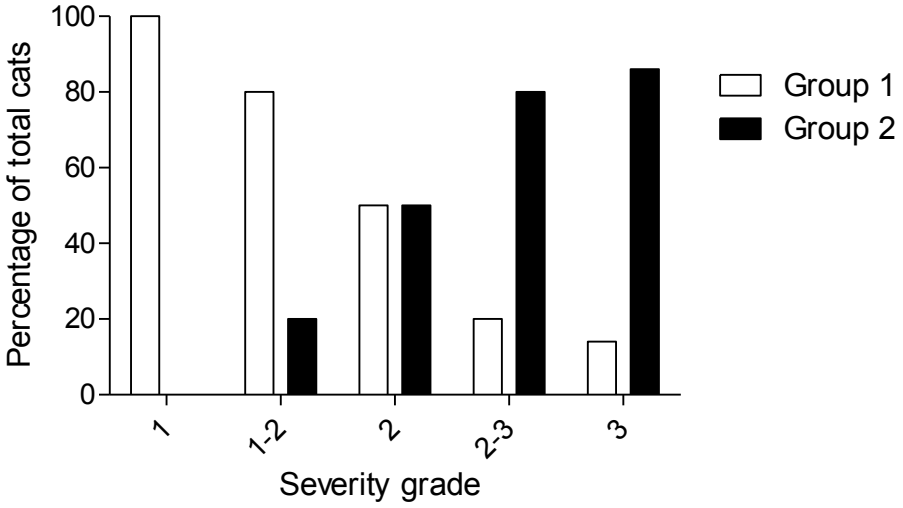
Figure 6.2: Photomicrographs of biopsies of the palatoglossal folds





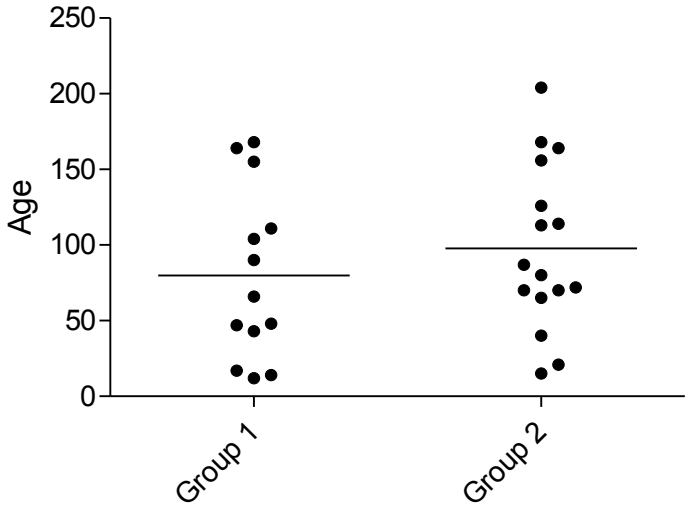
A: Severe lymphoplasmacytic infiltrate of the lamina propria B: Severe plasmacytic infiltrate of the lamina propria with Mott cells and neutrophils marginating the vessels.

Figure 6.3: Percentage of cats in the different severity of inflammation groups according to predominant cell group



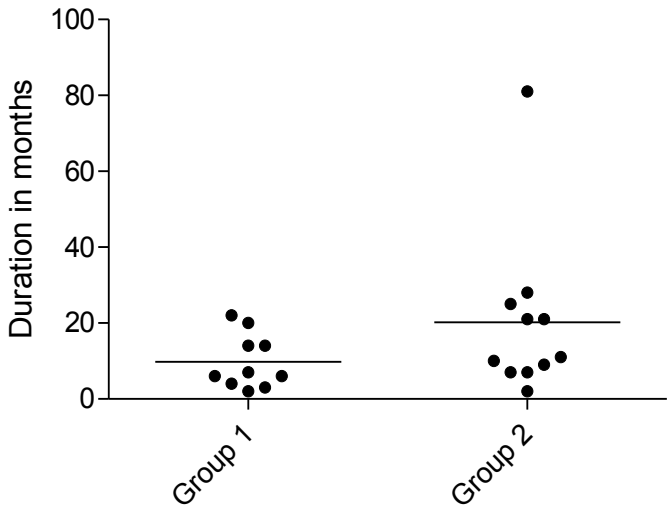
Group 1: lymphoplasmacytic infiltrate, group 2: plasmacytic infiltrate. Inflammation severity: 1: mild, 1-2: mild-moderate, 2: moderate, 2-3: moderate-severe, 3: severe.

Figure 6.4: Age distribution of the two cell groups



Each ● represents one biopsy, the line represents the mean.

Figure 6.5: Duration since onset of disease in the two cell groups



Each ● represents one biopsy, line represents the mean.

6.4 Discussion

Several studies have investigated the histopathology of FCGS. Standard findings within the epithelium of FCGS lesions include hyperplasia, ulceration, dysplasia and suppuration (Johnessee and Hurvitz, 1983; Hennet, 1997; Baird, 2005). In the current study, many of the FCGS biopsies showed hyperplasia of the palatoglossal folds. This was also evident in the healthy biopsies where it was considered 'normal'. Ulceration, dysplasia and suppuration are pathological. A large proportion of the FCGS biopsies showed ulceration and erosion with 69% showing at least one of the two lesions. Dysplasia was much less common (13.7%).

Pathological findings in the mucosa and submucosa are described as being lymphoplasmacytic (Hennet, 1997), lymphoplasmacytic and eosinophilic (Baird, 2005), sheets of plasma cells (Frost and Williams, 1986), predominantly plasma cells with binucleated cells and cells containing Russell bodies (Mott cells) and variable numbers of neutrophils, lymphocytes and histiocytes (Johnessee and Hurvitz, 1983). Other studies have described two different patterns, one being diffuse primarily plasmacytic and lymphocytic and the other having primarily plasma cells (White et al., 1992; Lyon, 2005; Wiggs, 2007). An acute reaction in the oral mucosa is normally associated with neutrophil infiltration while chronic reactions show lymphocytes, plasma cells, monocytes and macrophages. Suppurative inflammation is characterised by neutrophils as the predominant cell type (Pedersen, 1992; Nanci, 2008).

A number of samples in the current study had an infiltrate with a majority of plasma cells and low numbers of lymphocytes. This infiltrate, often seen in combination with Mott cells and binucleate plasma cells, can normally be seen in diseases with a suggested immune-mediated background such as rheumatoid arthritis and feline plasma cell pododermatitis (Perry et al., 1997; Dias Pereira and Faustino, 2003). Binucleate plasma cells were commonly found in multiple samples in the current study. Plasma cells are considered to be a fully differentiated state of the B-lymphocyte and therefore do not normally divide further. Binucleate and multinucleate plasma cells have been located in human rheumatoid arthritis (Perry et al., 1997) and several human oral diseases (Jinnfei and Ellabban, 1986), and have also been described in feline plasma cell pododermatitis (Dias Pereira and Faustino, 2003). The origin of binucleate plasma cells is controversial. One study suggested that they form through cell fusion (Jinnfei and Ellabban, 1986) but a later study reported that the morphology was more consistent with plasma cells that had undergone division (Perry et al., 1997). A rare disease in humans with a similar histopathological appearance is plasma cell mucositis

(Puvanendran et al., 2012). Histopathology shows an acanthotic epidermis and rete ridges as seen in histopathology of FCGS. A dense infiltrate of plasma cells is seen as well as Russell-bodies. The aetiopathogenesis of plasma-cell mucositis is not known but often a history of immunological dysfunction is described in the patients. Treatment is unreliable but most often corticosteroids are used in patients suffering from the disease.

The cats in this study were all considered to have a similar clinical appearance, and to be suffering FCGS. The biopsies could, however, be divided into two groups according to the histopathological appearance as described by Barker et al. (1992). Group 1: lymphocytic plasmacytic stomatitis with a chronic inflammation, characterised predominantly by plasma cells and lymphocytes (present in 45% of cases) and group 2: feline plasma cell gingivitis, with predominantly plasma cells in the lamina propria and showing fewer lymphocytes, neutrophils and macrophages (present in 55% of cases). The two types of cell infiltrate were compared to the severity of the infiltrate. A significant correlation could be seen, with the most severely inflamed samples being part of the plasmacytic group (group 2) and the less severely inflamed samples being categorised into the lymphoplasmacytic group (group 1). White et al. (1992) showed that 70% of cats with FCGS had a predominantly plasma-lymphocytic infiltrate and 30% had a predominantly plasmacytic infiltrate. Russell bodies, neutrophils and eosinophils could be found in some sections. Another study reported that lymphocytes or plasma cells were predominant in 25% of cases and neutrophils were predominant in 50% of cases (Harvey, 1991). The study also showed that there was no significant correlation between gingival index and the type of cells seen; however, the severity of inflammation and gingival index were significantly correlated. A much higher proportion of cats in our study represented the plasmacytic group when compared to previous studies. Taking into account that the more severe cases were predominantly plasmacytic in nature, it is possible that a higher number of severe cases were seen in this study due to the origin of the samples. Samples were collected at a referral veterinary dentist where one could expect a higher number of severe cases being presented.

Barker et al. (1992) reported that the lymphoplasmacytic type of infiltrate is more common in older cats. This was not verified by the current study. No difference in age was seen between the two cell-type groups. For 21 cats, an estimated start date of the disease was recorded but no difference could be seen in the time since disease onset between the two cell-type groups. The lymphoplasmacytic infiltrate has previously been described to be more common in cats having lesions of the tongue, palate and gingiva (Barker et al., 1992). In the current study, four cats possessed lesions of the tongue and

five had lesions of the oropharynx. Neither of the two cell-type groups was overrepresented in these cats. The clinical appearance was not always a good predictor of the histopathological features.

The current study showed that there are two different cell-type groups seen within the FCGS biopsies: group 1, showing a predominant lymphoplasmacytic infiltrate and group 2 showing a predominant plasmacytic infiltrate. There is a possible correlation between severity of inflammation and the cell-type groups seen.

Chapter 7 Aspects of the innate immune response in cats with FCGS

7.1 Introduction

In addition to other factors, alterations in the innate immune response have been suggested to play a role in the aetiopathogenesis of FCGS (Harley et al., 1999). Changes that indicate this are observed in cytokine expression, histopathology and immunoglobulin concentration in serum and saliva (White et al., 1992; Harley et al., 2003b; Arzi et al., 2010b; Harley et al., 2011).

Histopathological investigation of FCGS shows a chronic inflammation typically characterised by plasma cells in combination with variable numbers of lymphocytes, neutrophils and macrophages (White et al., 1992; Lyon, 2005). Quantification of the cell types has shown an increase in the number of mast cells in cats affected by FCGS when compared to a group of healthy cats (Arzi et al., 2010b; Harley et al., 2011). Mast cells are an important cell group in the innate immune system and can influence the immune response by releasing mediators such as: cytokines, vasoactive amines and enzymes following stimulation (Walsh, 2003). Mast cells are commonly detected in the oral mucosal lamina propria and submucosa of healthy cats (Harley et al., 2003a). Immunohistochemistry on glossopalatine mucosal biopsies from cats with FCGS showed a significant increase in the number of mast cells and in the numbers of cells expressing cluster of differentiation (CD) antigens (CD3, CD4, CD8, CD79a) immunoglobulins (Ig) (IgG, IgM, IgA) and, the Langerhans cell antigen (L1) in the lamina propria and submucosa when compared to healthy cats (Harley et al., 2011). The most prevalent cells were those expressing CD79a, IgG and L1.

Serum and salivary immunoglobulin levels have been shown to be elevated in cats with FCGS (White et al., 1992; Harley et al., 2003b). Analysis of cytokine gene expression in cats with FCGS has shown that they possess a mixed Th1-Th2 response as indicated by higher levels of expression of IL-2, IL-4, IL-6, IL-10, IL-12 and IFN-γ when compared to a predominantly Th1 type response in the healthy oral cavity (Harley et al., 1999). Cats with FCGS were subsequently treated but no significant change in the cytokine expression levels was detected before and after treatment.

So far, studies on the immune response in cats with FCGS have not investigated the roll of Toll-like receptors (TLRs). TLRs are important in the early host defence against

pathogens (Akira and Takeda, 2004). Stimulation of TLRs leads to variable gene expression, which then leads to activation of innate and acquired immunity. A change in the expression of TLRs can give information about the possible pathogenic agents involved in the aetiopathogenesis of a disease.

In this chapter, the expression of several cytokine and TLR genes was investigated in orally healthy cats and those with FCGS. Statistical analysis was performed to investigate the relationship between the five most commonly identified bacterial species, virological data and histopathological features, with changes in immune mediators (cytokines) and receptors (TLRs).

7.2 Materials and methods

7.2.1 Tissue samples

Tissue biopsies were collected from the mucosa lateral to the palatoglossal folds from a total of 39 cats. Tissue was collected from 31 cats with FCGS and 8 healthy cats. One biopsy was directly submerged in RNAlater (Sigma-Aldrich) and another was placed in neutral buffered 4% paraformaldehyde. The samples were then posted to the laboratory. The tissue samples in RNAlater were stored at 4°C until required.

7.2.2 Tissue homogenisation

Three different methods were tested to homogenise the tissue prior to RNA extraction:

I. Bead beater (Biospec Products, Bartlesville, USA)

The sample was sliced into approximately 1.0 mm pieces and added with lysis buffer to a 2.0 ml screw cap microtube with 1.0 mm beads. The sample was then placed into the bead beater for 3 min.

II. Tissue Ruptor (Qiagen)

The biopsy was placed into lysis buffer and disrupted for 1 min at full speed with the tissue ruptor using disposable autoclavable tips.

III. Motorised pellet pestle (Sigma-Aldrich)

The sample was sliced into approximately 1.0 mm pieces and placed into lysis buffer. The sample was homogenised for 1 min with a disposable autoclavable pestle.

7.2.3 RNA extraction

RNA extraction was performed on samples homogenised by method III, which proved to be the easiest method with best tissue disruption and homogenisation achieved. The samples were homogenised for 1 min by a motorised pellet pestle when submerged in buffer RLT (Qiagen). RNA was extracted using the RNA extraction kit RNeasy® mini kit for fibrous tissue (Qiagen). The sample was diluted after homogenisation and a

proteinase-K solution was added. The mixture was incubated at 55ºC for 10 min. Ethanol was added to the supernatant and it was run through the RNeasy Mini spin column containing RNA-binding silica membrane. DNAse digestion was performed on the spin column to remove DNA. After DNase digestion, contaminants were washed away and nuclease-free water was used to elute the RNA. The extracted RNA yield was assessed using the ND-1000 NanoDrop® spectrophotometer.

7.2.4 cDNA synthesis

For cDNA synthesis, the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) was used. A mixture of 1.0 µg of extracted RNA, Oligo $(dT)_{12-18}$ primers and dNTPs was made and incubated at 65ºC for 5 min. The mixture was directly transferred to 50ºC and a master mix containing DEPC-treated water, 10x RT buffer, 25mM $MgCl_2$, 0.1M DTT and a recombinant RNase inhibitor was added. To allow cDNA synthesis the reaction was incubated for 50 min at 50ºC. The reaction was terminated at 70ºC for 15 min and chilled on ice. To remove the RNA, RNase H was added to each tube and incubated for 20 min at 37ºC. Incubation was performed on a Primus96 plus thermocycler (MWG biotech).

7.2.5 Primer design and optimisation

7.2.5.1 Primer design

Primers were designed using the online primer design tool at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/tools /primer-blast) and were commercially synthesised (Invitrogen). Feline mRNA sequences of TLR2, TLR3, TLR4, TLR7, TLR9, IL-1ß, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ and TNF-α were used and primers were selected on the basis of similarity in length and melting temperature. At the time of primer design, the feline mRNA sequence for TLR5 was not accessible and therefore a published primer was used (Ignacio et al., 2005). Primer details are shown in Table 7.1.

Table 7.1: Primers used for amplification of Toll-like receptor and cytokine genes

Primer name	Primer sequence 5'-3'	Product length
GAPDH F	GAGCTGAATGGGAAGCTCAC	100
GAPDH R	CGTATTTGGCAGCTTTCTCC	
IFN-γ F	TGCAGGTCCAGCGCAAAGCAA	120
IFN-γ R	TCGATGCTCTACGGCCTCGAAACA	
IL-1β F	GACGGTTTTGTGTGTGATGC	92
IL-1β R	TCGTATGAGCCAGACAGCAC	
IL-2 F	ACGGTTGCTTTTGAATGGAG	146
IL-2 R	GCACTTCCTCCAGAGGTTTG	
IL-4 F	GCAGCCCCTAAGAACACAAG	102
IL-4 R	TTTGAGGAATTTGGTGGAGC	
IL-6 F	CAGGGCTGTTCGGATAATGT	100
IL-6 R	TCAGTTACATGCCCAGTGGA	
IL-10 F	TGTCTGAGGACAACTGCACC	108
IL-10 R	GCTCGTCCTTGGTTTGAAAG	
IL-12 F	TCAACAGTGTGACTGTGCCA	116
IL-12 R	ATTGATGGTCACTGCACGAA	
TLR2 F	CCTGAAAATGATGTGGGCTT	99
TLR2 R	CGCTCGCTGTAAGACACAAA	
TLR3 F	GACAACAACTTCCCAGGCAT	199
TLR3 R	GACAAGAAAAAGCGCCACTC	
TLR4 F	CTGGAACAGGTGTCCCAAGT	176
TLR4 R	TGCCGTAGTTCTTGCTCCTT	
TLR5 F	TTCCTTCCGCCAGGAGTATTTAGC	217
TLR5 R	GGAGTTCGCACTCACAGATGAACT	
TLR7 F	TTGAGAAGCCCCTTCAGAAA	149
TLR7 R	GGTCACGTGATTGTCTGTGG	
TLR9 F	GAGAGCTCAACCTCAGTGCC	144
TLR9 R	CCAGCAAGAAGTCCACGAAG	
TNF-α F	CTTCCAACTGGAGAAGGGTG	93
TNF-α R	ATCCCAAAGTAGACCTGCCC	

7.2.5.2 Primer efficiency

The efficiency of the primers was tested to validate the use of the Comparative CT method. Each primer was tested with a minimum of three serial dilutions. The CT values were plotted against $\log_{10}$ of the sample volume and the efficiency calculated from the formula:

$$\text{Efficiency} = -1+10^{(-1/\text{slope})}$$

Efficiencies were accepted when between 0.9 and 1.1.

7.2.5.3 Primer optimisation

For each primer a melt curve analysis was performed. To ensure a single product the product needed to yield a single peak. Annealing temperatures were standardised for each primer when a single peak was observed.

7.2.6 Quantitative PCR

Quantitative PCR was performed using SYBR-Green Mastermix (Invitrogen). Each reaction was performed in triplicate in a total volume of 25 µl per well containing 12 µl SYBR green mix, 11 µl H_2O, 1 µl cDNA and 1 µl primer mix. A primer mixture was created of reverse and forward primers at 10 µmol per primer. Reactions were performed in a MX300P™ real time PCR system (Stratagene, Cambridge, UK) with the following cycles: Initial denaturation at 95°C for 10 minutes, 50 cycles of denaturation at 95°C for 30 s, annealing at 55° for 1 min (for TLR2, TLR4, TLR7, TLR9, TNF-α and IL-1ß) or 60°C (for TLR3, IFN-γ, IL-4, IL-6, IL-10 and IL-12A), extension at 72°C for 1 min. Finally a melting curve was created at the end of the assay with a gradient of increasing temperatures until the amplicons were fully dissociated, Thus a unique melt temperature was determined for each primer set. The data was rejected when a peak at the wrong melt temperature, or a wide or stepped peak was found.

7.2.7 Data analysis

Data analysis was performed using Microsoft Excel and SPSS version 18 for windows (IBM, Toronto, USA). All samples were adjusted to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by the 2^{-dCt} method. As all results were in triplicate, the average dCT was used to calculate the relative expression ratio ($R=2^{dCT}$). R was used for statistical analysis.

The data used was continues without a normal distribution, therefore a Mann-Whitney U test was used. The nominal data that had to be compared consisted of more than two groups therefore the Kruskall-Wallis test was performed with the Dunn's comparison test as post-hoc analysis. Differences were considered statistically significant at $p<0.05$. To visualise the combined results, data were normalised to the TLR2 expression in control samples, the barchart shows mean with standard deviation error bars. All box and whisker plots show whiskers for minimum and maximum values. Data graphics were constructed in GraphPad Prism software version 5.

7.3 Results

7.3.1 Sample collection

Biopsies were collected from the oral mucosa lateral to the palatoglossal folds from 31 cats with FCGS and eight orally healthy cats.

7.3.2 RNA extraction

A comparison of different methods was made to determine the best method for extracting total cellular RNA from tissue samples. Three different methods of homogenising the tissue (Section 7.2.2) were investigated:

I. Bead beater

II. Tissue Ruptor

III. Motorised pellet pestle

Methods I and II were not suitable for the tissue used in this study. The tissue structure was too strong to be disrupted by the bead beater and the Tissue Ruptor was not suitable for the small pieces of tissue collected. Method III proved to be the quickest and easiest method.

RNA extraction was performed on all samples. Samples F14, F16 and F26 did not yield sufficiently good quality RNA to be used for cDNA synthesis; the extracted RNA yield was below 50ng/µl and of a substandard quality. RNA was successfully extracted from a total of 28 FCGS and 8 healthy samples and these were used for cDNA synthesis and further analysis.

7.3.3 Primer efficiency

The quality of the product and the efficiency of PCR reactions were validated. For each sample, dissociation curves were analysed to ensure a single product was present at the end of the reaction. Single dissociation peaks with a unique product-dependent melting temperature were obtained for each of the gene products TLR2, TLR3, TLR4, TLR7, TLR9, TNF-α, IL-4, IL-6, IL-10 and IL-12. In contrast, results showed that the primer sets designed to detect the products of the IL-2, TLR5 and IFN-γ genes did not show a single

dissociation peak and therefore were excluded from the study. Two new primers (IFN-γ 2 and IFN-γ 3) were designed and tested for IFN-γ. Melt curves for IFN-γ 2 showed a double peak and therefore the primer was not used further. IFN-γ 3 showed a good dissociation curve and the efficiency was tested. To test the primer efficiency in the PCR reaction, for all primers ten-fold serial dilutions of a cDNA template were made. Some of the primer sets, where the abundance of the template in the sample was low, failed to produce a Ct value distinguishable from the assay threshold at higher dilutions of the cDNA. Where necessary, two- or five- fold dilutions of cDNA were re-tested in PCR reactions. The working concentration was always included. Primer efficiencies are shown in Table 7.2.

The primer efficiency for IL-4 amplification could not be ascertained in the samples used for validation, presumably because the total amount of IL-4 cDNA was too low. No Ct value was produced with the second dilution of the cDNA for IL-4 mRNA expression in the validation sample.

GAPDH proved to be a suitable housekeeping gene by showing a stable expression across both cohorts when samples with a similar cDNA concentration were compared (Figure 7.1). The efficiency was determined to be between 0.9 and 1.1 at both annealing temperatures (Table 7.2).

7.3.4 Toll-like receptor mRNA expression

Expression of TLR2, TLR4, TLR7 and TLR9 genes was increased in the FCGS samples compared to the control. A significant increase was seen in TLR2 ($p=0.01$) and TLR7 ($p=0.047$) mRNA expression (Figure 7.2 and 7.3). A slight decrease was seen in the TLR3 mRNA expression in the FCGS group compared to the control group (Figure 7.2). When the fold-change was calculated a down-regulation was seen in TLR3 mRNA abundance in the FCGS group. All other mRNAs encoding the TLRs were up-regulated in the FCGS group (Figure 7.4).

7.3.5 Cytokine mRNA expression

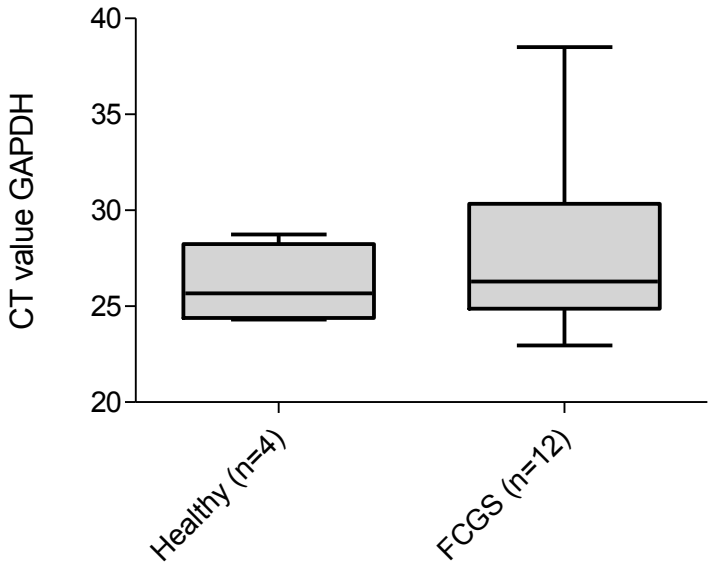
The expression of TNF-α, IFN-γ IL-1ß, IL-4, IL-6 and IL-10 was greater in the FCGS group than in the healthy group. After statistical analysis, the TNF-α ($p=0.032$), IFN-γ ($p=0.017$), IL1-ß ($p=0.017$) and IL-6 ($p<0.0001$) genes showed a significantly higher expression in the FCGS group (Figure 7.3 and 7.5).

Table 7.2: Primer efficiencies

Primer name	Primer sequence 5'-3'	Product length	Primer efficiency
GAPDH F	GAGCTGAATGGGAAGCTCAC	100	93%-99%
GAPDH R	CGTATTTGGCAGCTTTCTCC		
IFN-γ F	TCTAACCTGAGGAAGCGGAA	84	N.A.
IFN-γ R	ATATTGCAGGCAGGACAACC		
IFN- γ 2 F	CTGCAGGTCCAGCGCAAAGC	120	N.A.
IFN- γ 2 R	CGATGCTCTACGGCCTCGAAACA		
IFN-γ 3 F	TGCAGGTCCAGCGCAAAGCAA	120	91%
IFN-γ 3 R	TCGATGCTCTACGGCCTCGAAACA		
IL-1β F	GACGGTTTTGTGTGTGATGC	92	106%
IL-1β R	TCGTATGAGCCAGACAGCAC		
IL-2 F	ACGGTTGCTTTTGAATGGAG	146	N.A.
IL-2 R	GCACTTCCTCCAGAGGTTTG		
IL-4 F	GCAGCCCCTAAGAACACAAG	102	Not determined
IL-4 R	TTTGAGGAATTTGGTGGAGC		
IL-6 F	CAGGGCTGTTCGGATAATGT	100	90%
IL-6 R	TCAGTTACATGCCCAGTGGA		
IL-10 F	TGTCTGAGGACAACTGCACC	108	105%
IL-10 R	GCTCGTCCTTGGTTTGAAAG		
IL-12 F	TCAACAGTGTGACTGTGCCA	116	105%
IL-12 R	ATTGATGGTCACTGCACGAA		
TLR2 F	CCTGAAAATGATGTGGGCTT	99	90%
TLR2 R	CGCTCGCTGTAAGACACAAA		
TLR3 F	GACAACAACTTCCCAGGCAT	199	107%
TLR3 R	GACAAGAAAAAGCGCCACTC		
TLR4 F	CTGGAACAGGTGTCCCAAGT	176	109%
TLR4 R	TGCCGTAGTTCTTGCTCCTT		
TLR5 F	TTCCTTCCGCCAGGAGTATTTAGC	217	N.A.
TLR5 R	GGAGTTCGCACTCACAGATGAACT		
TLR7 F	TTGAGAAGCCCCTTCAGAAA	149	90%
TLR7 R	GGTCACGTGATTGTCTGTGG		
TLR9 F	GAGAGCTCAACCTCAGTGCC	144	112%
TLR9 R	CCAGCAAGAAGTCCACGAAG		
TNF-α F	CTTCCAACTGGAGAAGGGTG	93	108%
TNF-α R	ATCCCAAAGTAGACCTGCCC		

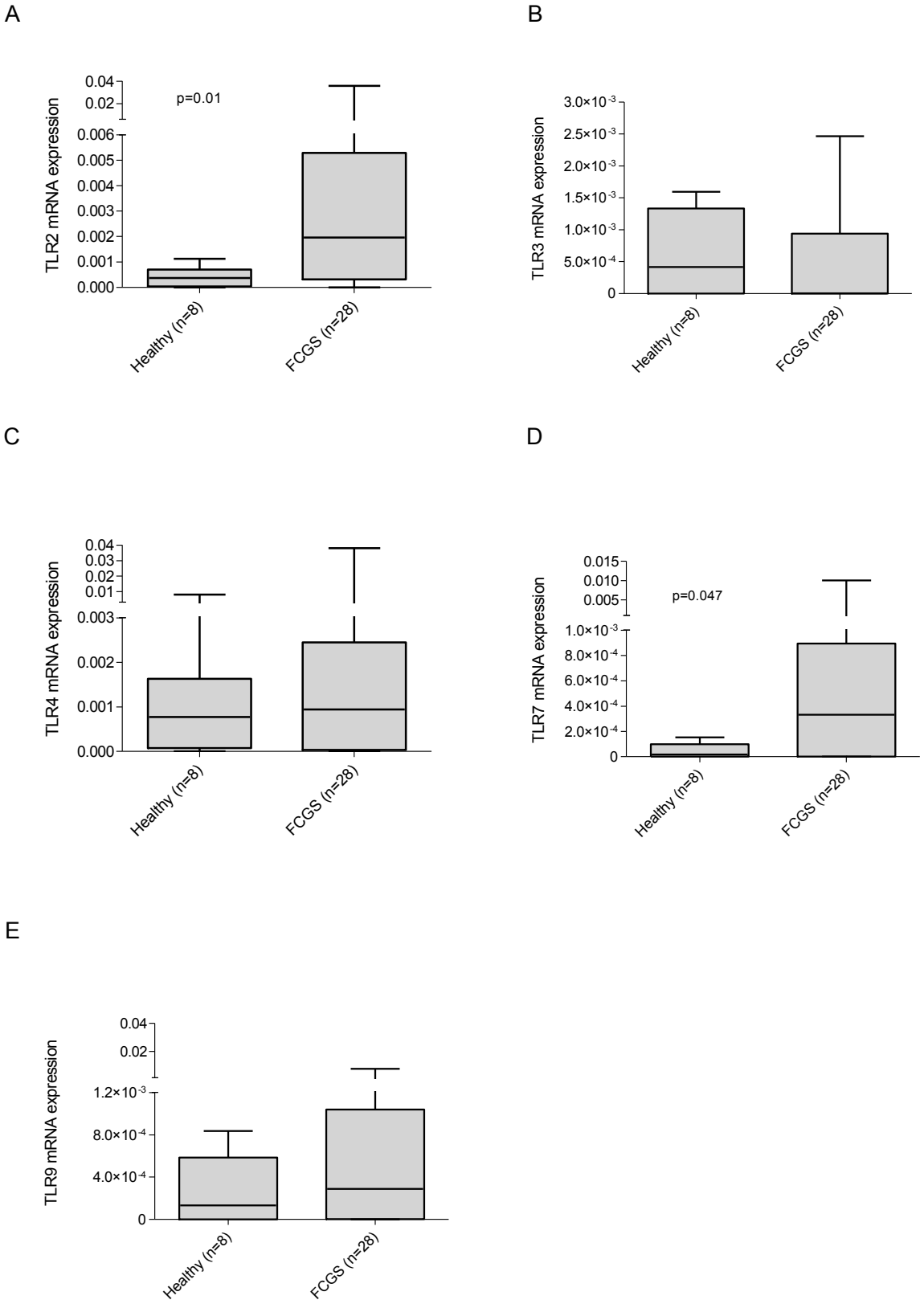
Efficiencies, length and sequence of the primers used and tested.

Figure 7.1: GAPDH Ct value in both cohorts



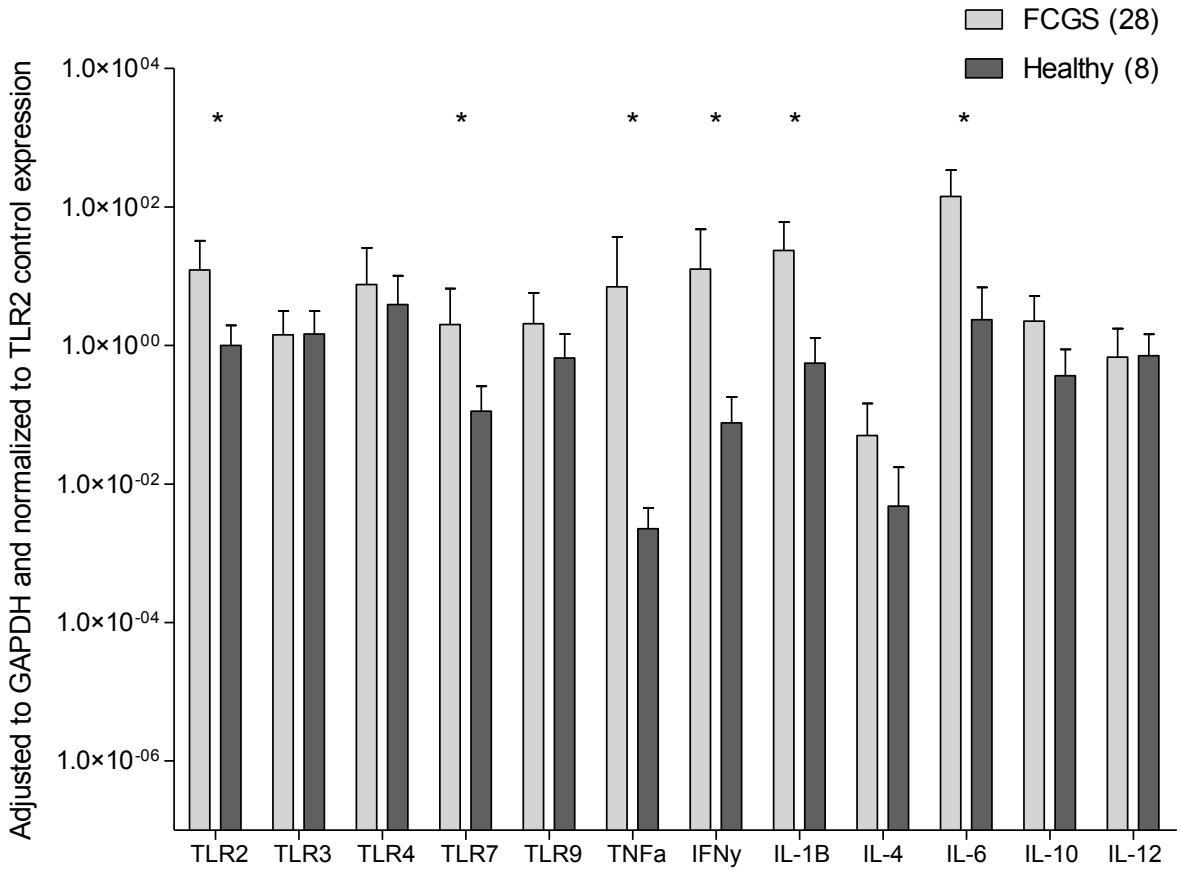
Distribution of GAPDH Ct values between the two groups with a similar cDNA concentration based on the RNA concentration used for synthesis.

Figure 7.2: TLR mRNA expression in FCGS and healthy samples



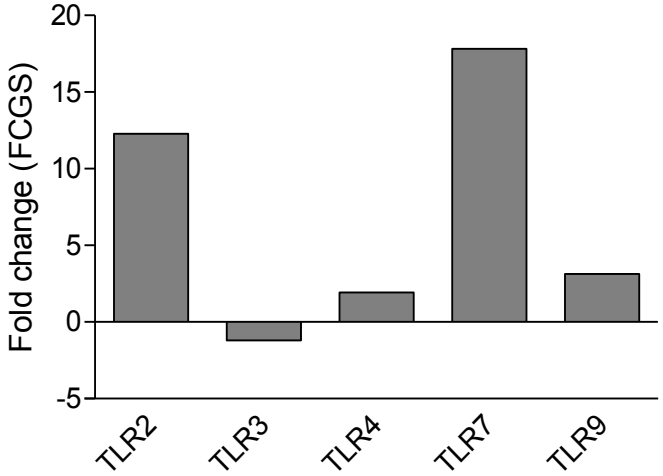
All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: Expression of the TLR2 gene. B: Expression of the TLR3 gene. C: Expression of the TLR4 gene. D: Expression of the TLR7 gene. E: Expression of the TLR9 gene.

Figure 7.3: TLR and cytokine mRNA expression in healthy and FCGS samples



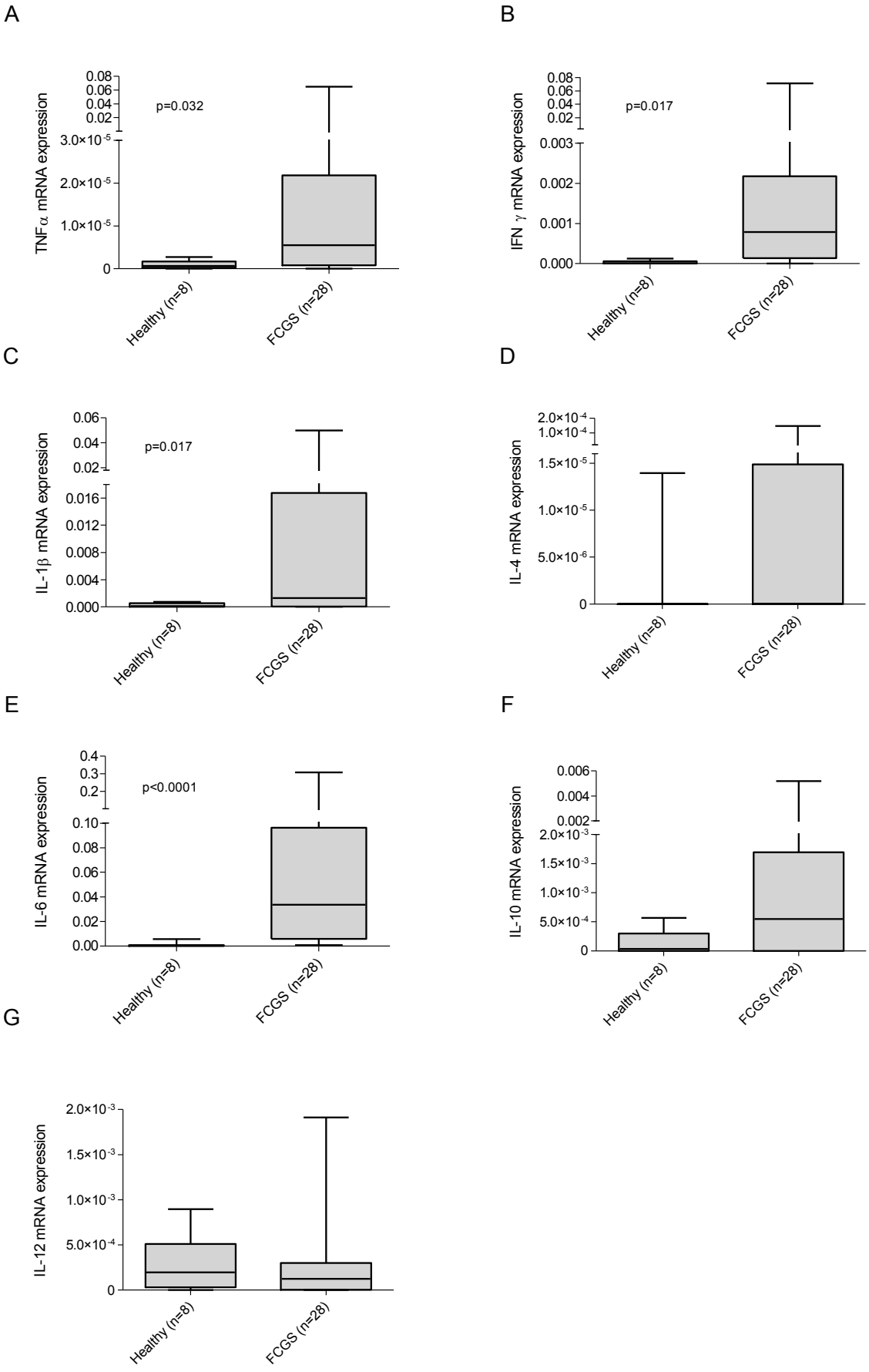
All values were adjusted to GAPDH and normalised to healthy TLR2 gene expression.

Figure 7.4: Fold change in TLR mRNA levels in healthy and FCGS samples



The change in expression levels between cats with FCGS (28) and healthy (8) cats is shown.

Figure 7.5: Cytokine mRNA expression in healthy and FCGS samples



All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TNF-α mRNA. B: IFN-γ mRNA. C: IL-1ß mRNA. D: IL-4 mRNA. E: IL-6 mRNA. F: IL-10 mRNA. G: IL-12 mRNA.

IL-12A mRNA levels showed a slight decrease in the FCGS group, and the increase in IL-4 mRNA was not significant statistically. When the fold change was calculated IL-12A mRNA showed a down-regulation in expression in the cats with FCGS, where all other genes showed an up-regulation of mRNA expression (Figure 7.6).

7.3.6 TLR mRNA expression in cats with and without FCV

Cats from the healthy and FCGS group were taken together and gene expression in tissue biopsies from cats with and without FCV was compared. mRNA expression levels for TLR2, TLR3, TLR4, TLR7 and TLR9 are shown in Figure 7.7. Using the Mann-Whitney U-test, a higher expression of TLR2 mRNA ($p=0.012$) was seen in FCV-positive cats compared to FCV-negative cats. TLR7 mRNA levels were not significantly different ($p=0.057$) but the median expression level was higher in cats positive for FCV. The expression of other TLR genes did not show a significant difference between FCV-positive and FCV-negative cats.

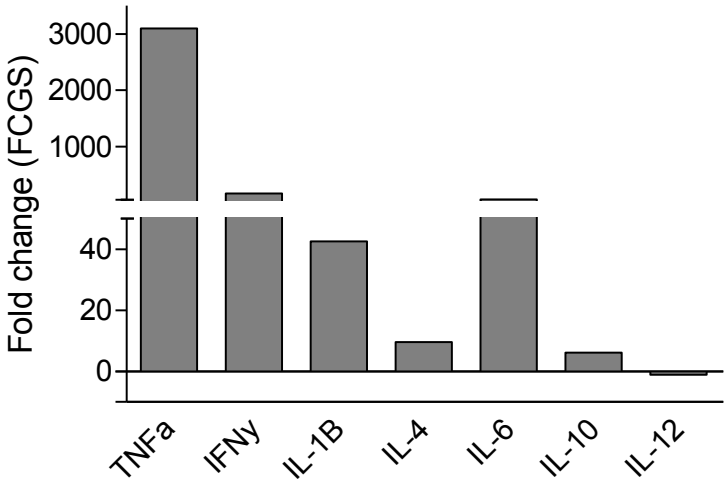
7.3.7 Cytokine mRNA expression in cats with and without FCV

Cytokine gene expression in FCV-positive and FCV-negative cats from the healthy and FCGS populations was also analysed. Expressions of TNF-α, IFN-γ, IL-1ß, IL-4, IL-6, IL-10 and IL-12 genes are shown in Figure 7.8. A significantly higher expression of IFN-γ ($p<0.001$), IL-1ß ($p=0.048$) and IL-6 mRNA ($p=0.008$) was seen in the FCV group.

7.3.8 TLR mRNA expression in cats with and without *T. forsythia*

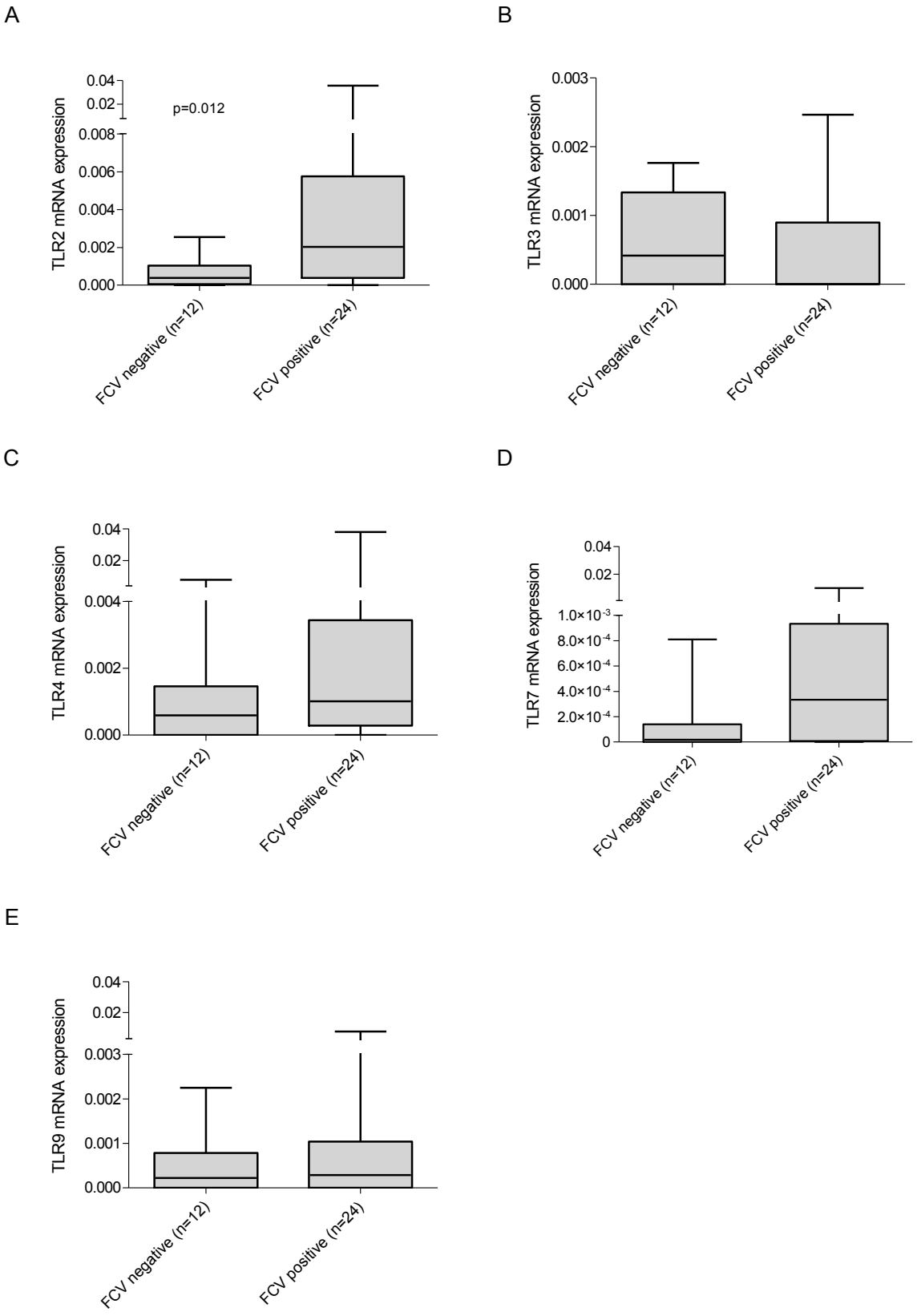
A Mann-Whitney U-test was performed on healthy cats and cats from the FCGS group with and without *T. forsythia*, as detected by 16S rRNA gene sequencing (Section 5.3.2.4) The expression of TLR2 ($p=0.004$), TLR4 ($p=0.005$), TLR7 ($p=0.007$) and TLR9 ($p=0.017$) mRNA was significantly higher in the group where *T. forsythia* was present (Figure 7.9).

Figure 7.6: Fold change of cytokine gene expression in healthy and FCGS samples



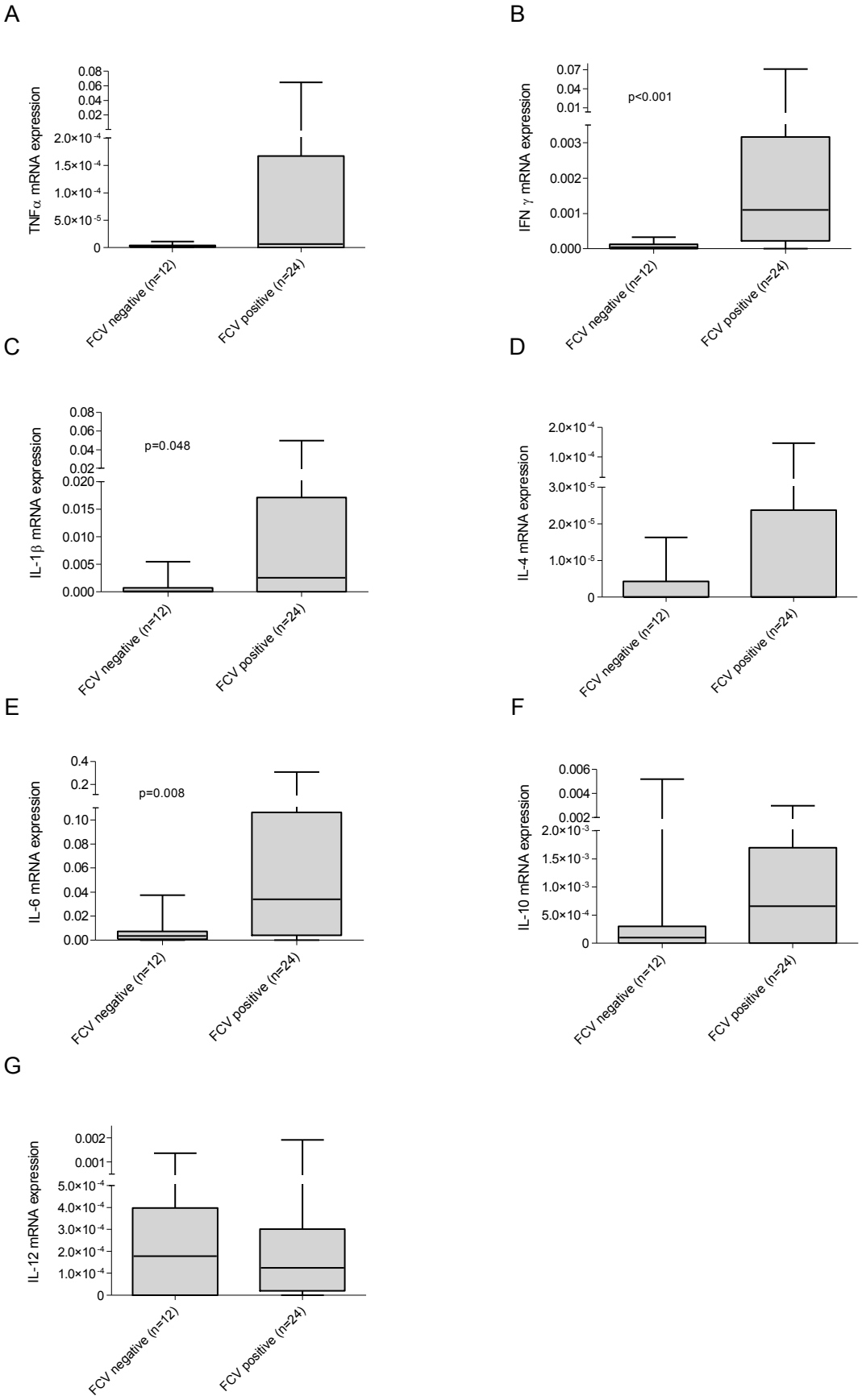
The change in each of the cytokine mRNA levels between cats with FCGS (28) and healthy (8) cats is shown.

Figure 7.7: TLR mRNA expression in cats with and without FCV



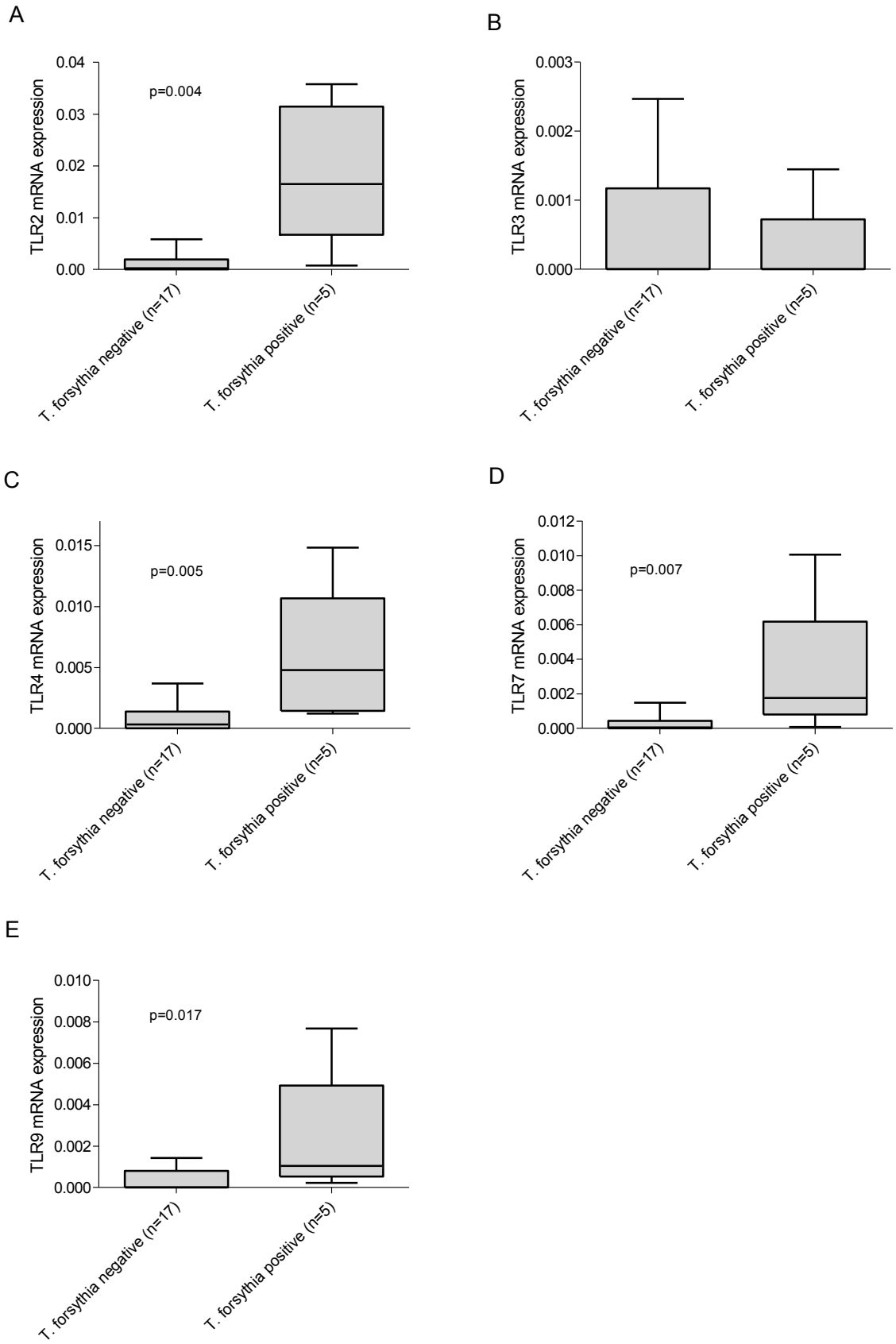
All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. Expression is shown in FCV-positive and FCV-negative cats in the healthy and FCGS group combined. A: TLR2 mRNA. B: TLR3 mRNA. C: TLR4 mRNA. D: TLR7 mRNA. E: TLR9 mRNA.

Figure 7.8: Cytokine mRNA expression in cats with and without FCV



All samples were adjusted to GAPDH by the 2-dCt method and statistical analysis was performed using the Mann-Whitney U-test. Expression is shown in FCV-positive and FCV-negative cats in the healthy and FCGS group combined. A: TNF-α mRNA. B: IFN-γ mRNA. C: IL-1ß mRNA. D: IL-4 mRNA. E: IL-6 mRNA. F: IL-10 mRNA. G: IL-12 mRNA.

Figure 7.9: TLR mRNA expression in cats with and without *T. forsythia*



T. forsythia was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TLR2 mRNA. B: TLR3 mRNA. C: TLR4 mRNA. D: TLR7 mRNA. E: TLR9 mRNA.

7.3.9 Cytokine mRNA expression in cats with and without *T. forsythia*

Expression of cytokine genes was analysed in cats where *T. forsythia* was detected and in cats without *T. forsythia* for healthy and cats with FCGS. A significant increase in expression of TNF-α (p=0.004) and IL-1ß (p=0.017) genes was seen in the group where *T. forsythia* was present (Figure 7.10).

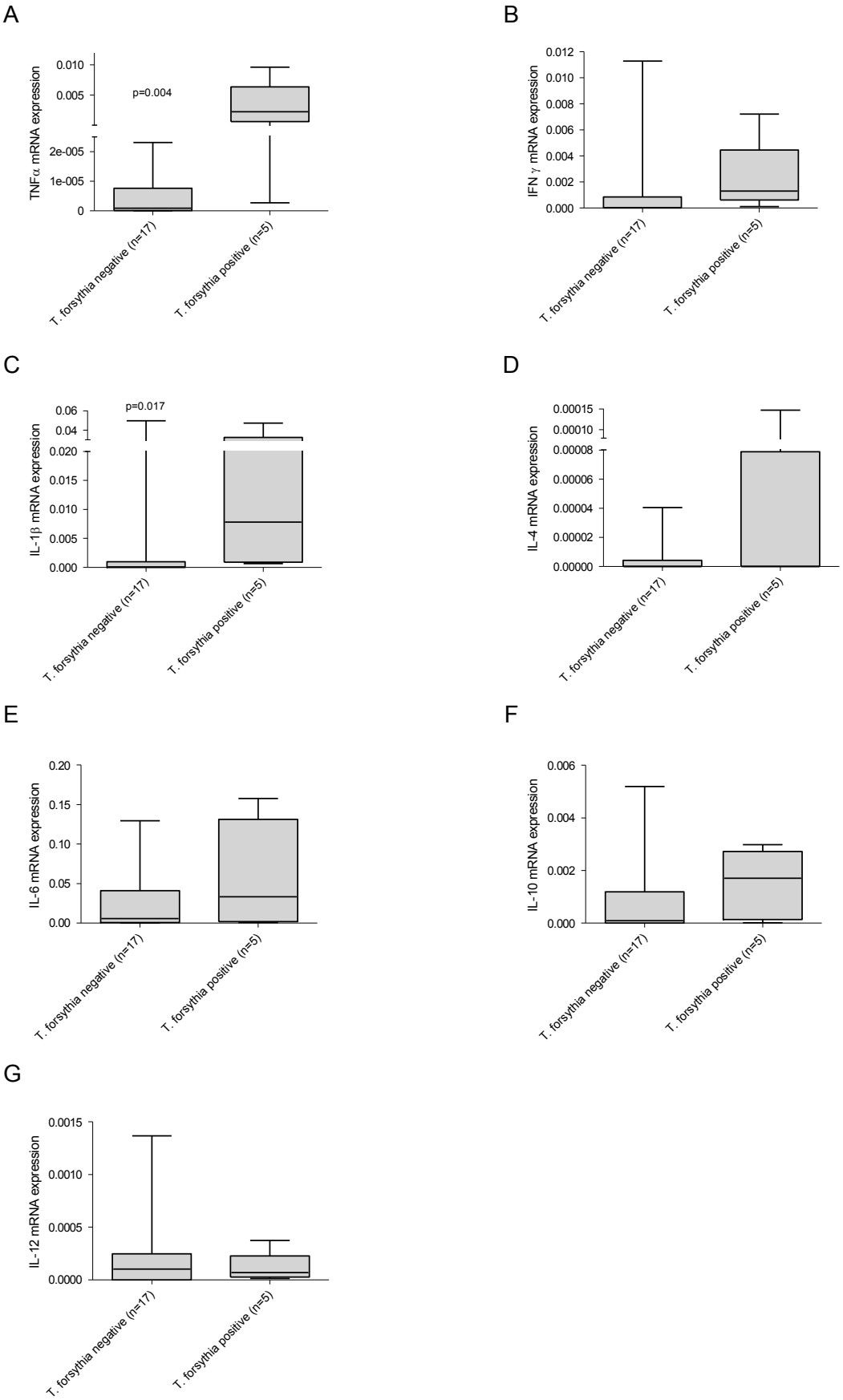
7.3.10 TLR mRNA expression in cats with and without *P. multocida* subsp. *septica*

TLR gene expression was analysed in all cats where *P. multocida* subsp. *septica* was detected by 16S rRNA gene sequencing and compared to the cats where no *P. multocida* subsp. *septica* was detected. TLR7 mRNA expression was significantly higher in the group where no *P. multocida* supsp. *septica* was detected (p=0.024). The other TLR gene products, except TLR3, appeared to show lower expression levels in the *P. multocida* subsp. *septica* group but these differences were not statistically significant when compared to the cats that did not show the presence of *P. multocida* subsp. *septica* (Figure 7.11).

7.3.11 Cytokine mRNA expression in cats with and without *P. multocida* subsp. *septica*

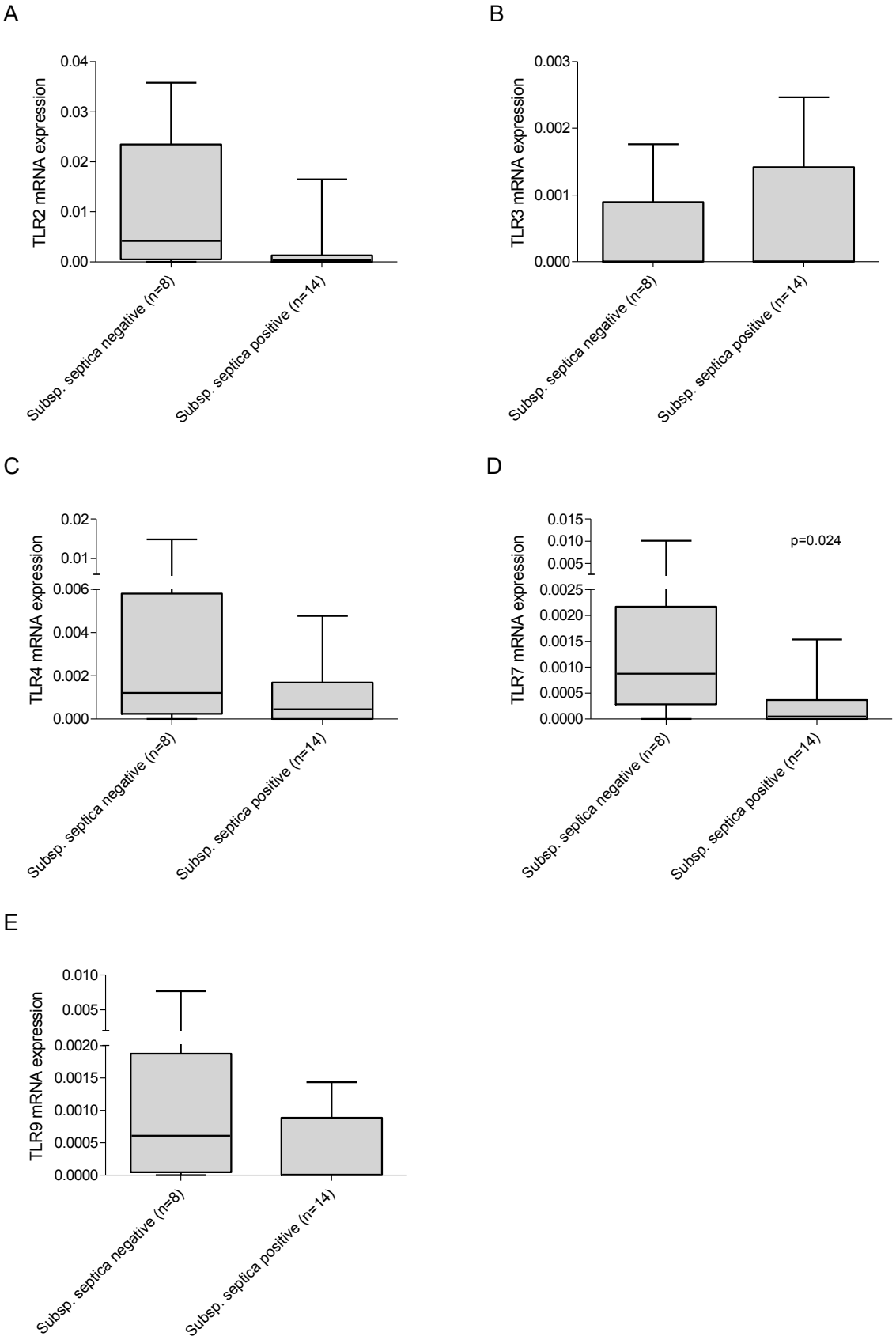
Cytokine gene expression in all cats with and without *P. multocida* subsp. *septica* was analysed. Significantly higher expression was seen in the group that was free of *P. multocida* subsp. *septica* for TNF-α (p=0.017) and IFN-γ mRNA (p=0.040). All other cytokine genes did not show a significant difference in levels of expression (Figure 7.12).

Figure 7.10: Cytokine mRNA expression in cats with and without *T. forsythia*



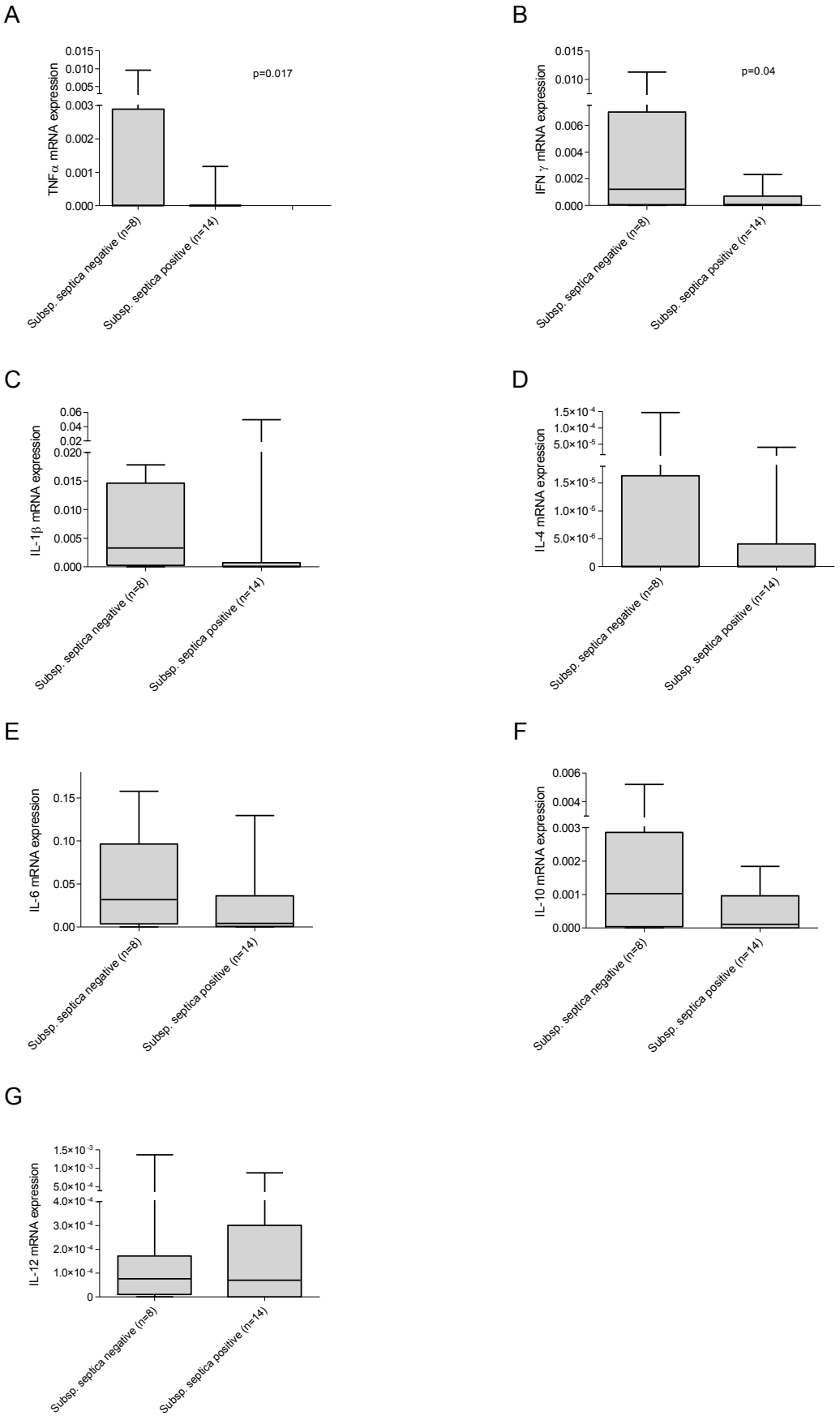
T. forsythia was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U-test. A: TNF-α mRNA. B: IFN-γ mRNA. C: IL-1ß mRNA. D: IL-4 mRNA. E: IL-6 mRNA. F: IL-10 mRNA. G: IL-12 mRNA.

Figure 7.11: TLR mRNA expression in cats with and without *P. multocida* subsp. *septica*



P. multocida subsp. *septica* was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2-dCt method and statistical analysis was performed using the Mann-Whitney U-test. A: TLR2 mRNA. B: TLR3 mRNA. C: TLR4 mRNA. D: TLR7 mRNA. E: TLR9 mRNA.

Figure 7.12: Cytokine mRNA expression in cats with and without *P. multocida* subsp. *septica*



P. multocida subsp. *septica* was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TNF-α mRNA. B: IFN-γ mRNA. C: IL-1Β mRNA. D: IL-4 mRNA. E: IL-6 mRNA. F: IL-10 mRNA. G: IL-12 mRNA.

7.3.12 TLR mRNA expression in cats with and without *P. multocida* subsp. *multocida*

When expression of TLR genes was analysed in cats with and without *P. multocida* subsp. *multocida* it appeared that expression of all TLR genes was higher in the group that did not contain this organism (Figure 7.13). However these differences were not statistically significant.

7.3.13 Cytokine mRNA expression in cats with and without *P. multocida* subsp. *multocida*

When cytokine gene expression was analysed in cats with and without *P. multocida* subsp. *multocida*, no significant changes were seen (Figure 7.14).

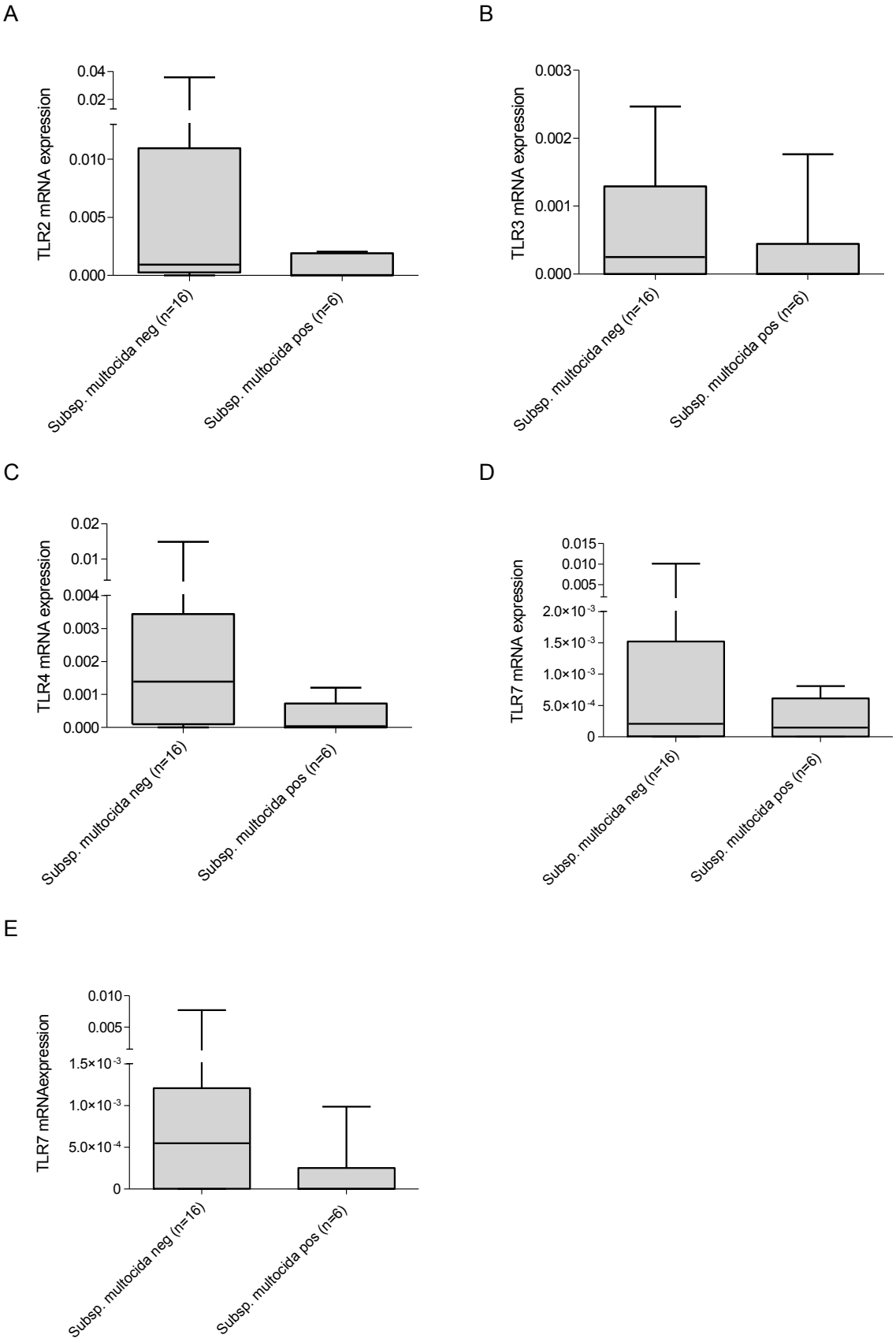
7.3.14 TLR mRNA expression in cats with and without *Pseudomonas* sp.

No significant difference in TLR gene expression was seen between samples containing *Pseudomonas* sp. and samples where no *Pseudomonas* sp. were detected by 16S rRNA gene sequencing (Figure 7.15).

7.3.15 Cytokine mRNA expression in cats with and without *Pseudomonas* sp.

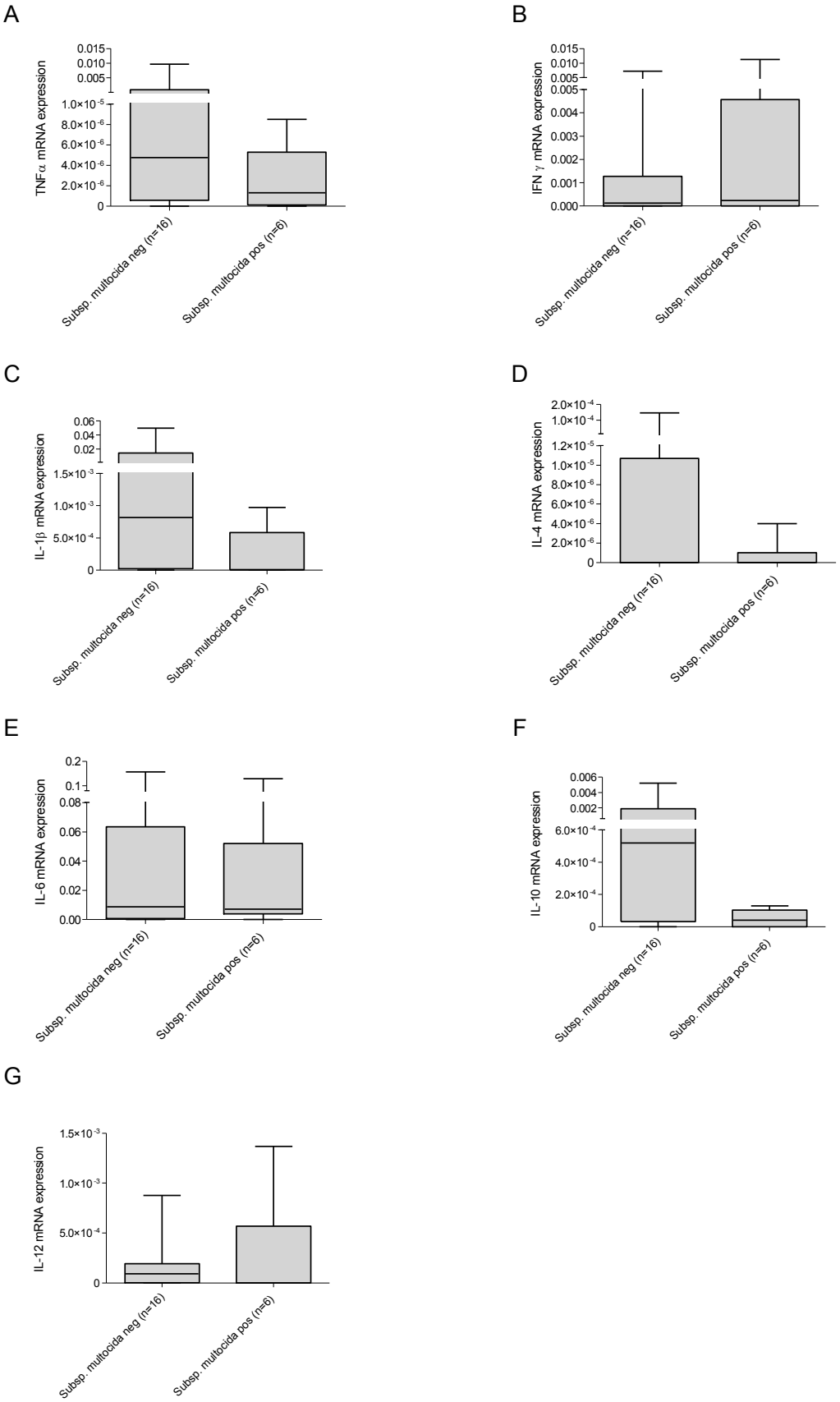
As was seen for TLR mRNA expression, no significant difference was seen in cytokine mRNA expression when cats were divided according to the presence of *Pseudomonas* sp. (Figure 7.16).

Figure 7.13: TLR mRNA expression in cats with and without *P. multocida* subsp. *multocida*



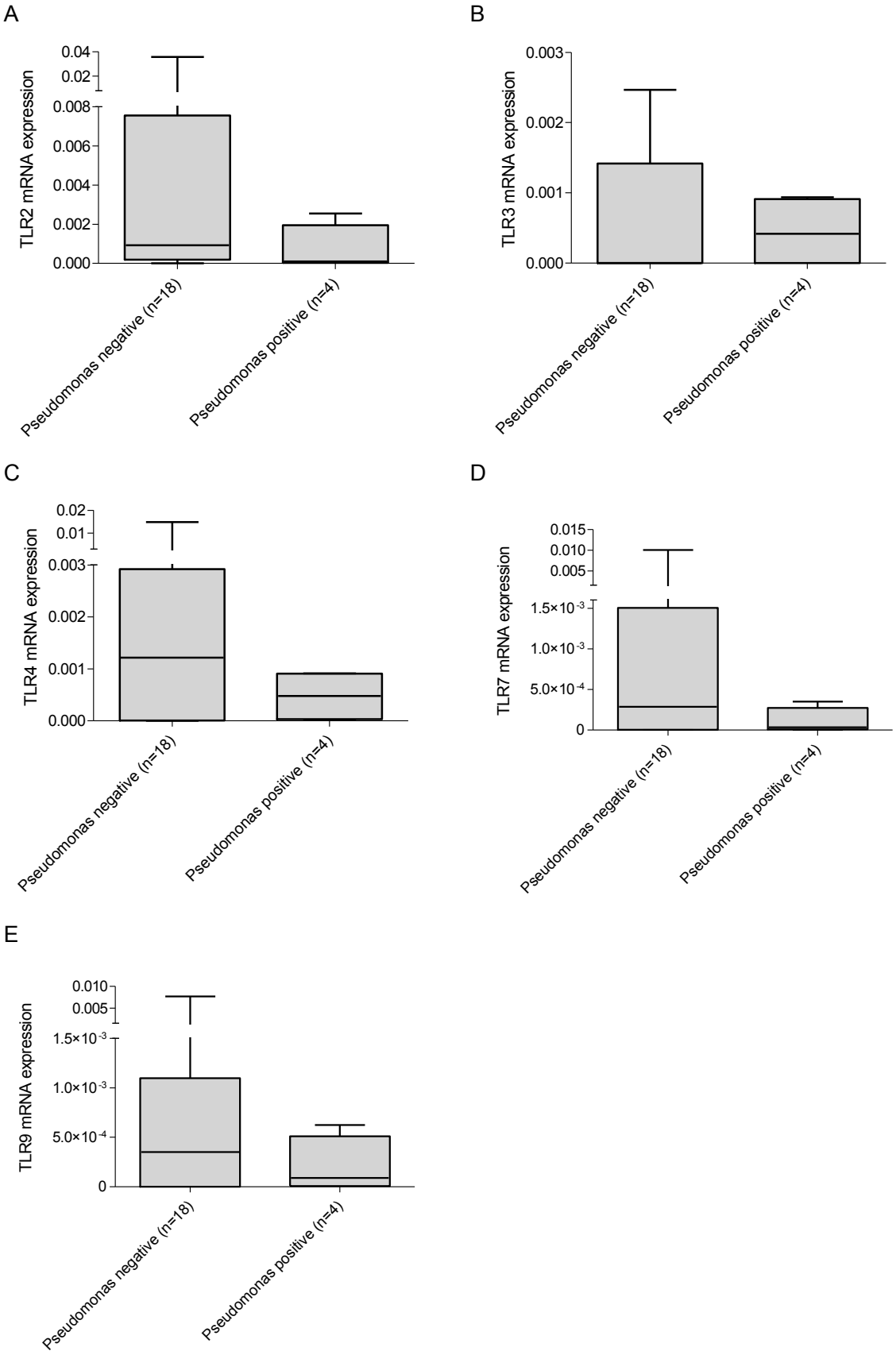
P. multocida subsp. *multocida* was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TLR2 mRNA. B: TLR3 mRNA. C: TLR4 mRNA. D: TLR7 mRNA. E: TLR9 mRNA. Pos: positive, Neg: negative.

Figure 7.14: Cytokine mRNA in cats with and without *P. multocida* subsp. *multocida*



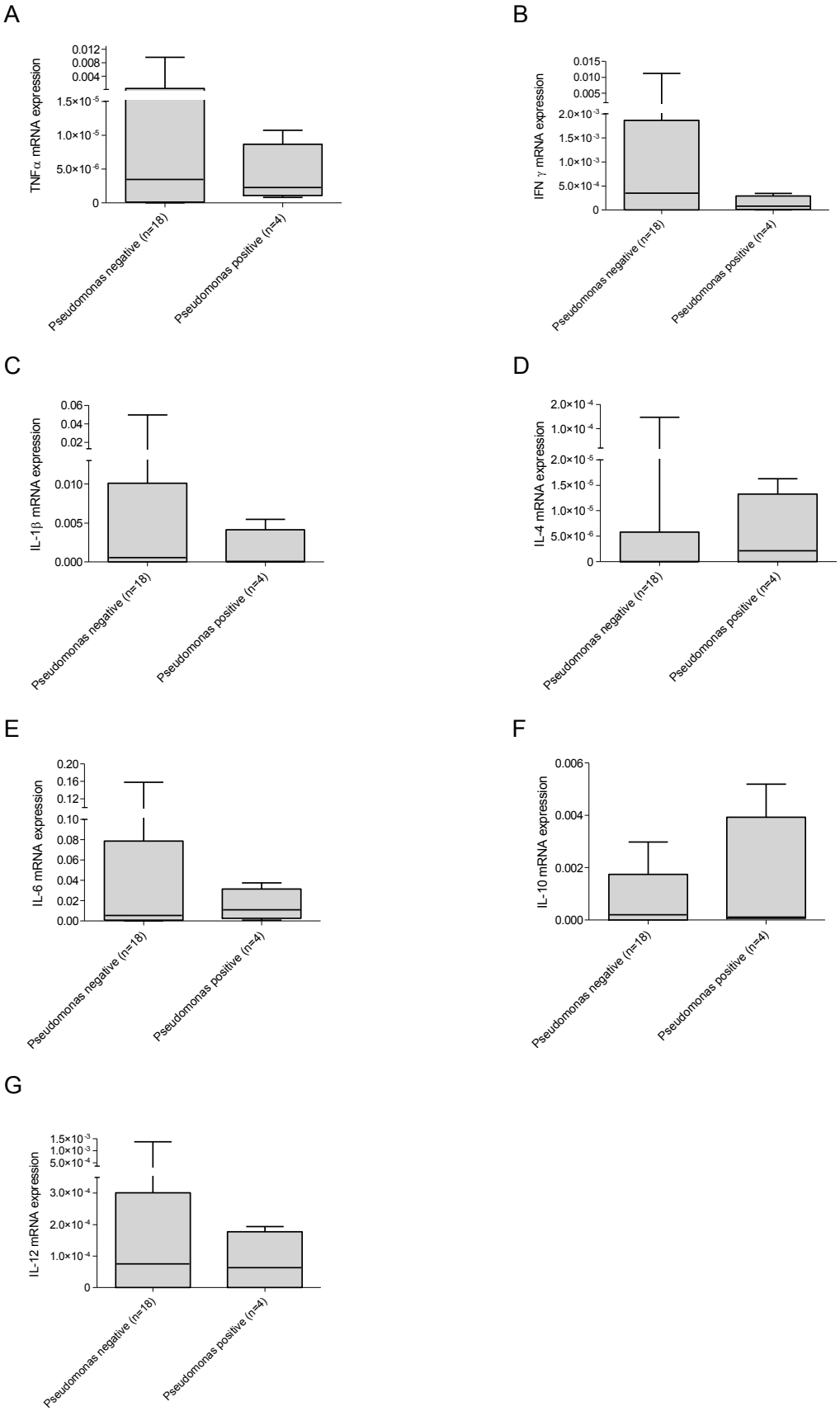
P. multocida subsp. *multocida* was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TNF-α mRNA. B: IFN-γ mRNA. C: IL-1β mRNA. D: IL-4 mRNA. E: IL-6 mRNA. F: IL-10 mRNA. G: IL-12 mRNA. Pos: positive, Neg: negative.

Figure 7.15: TLR mRNA expression in cats with and without *Pseudomonas* sp.



Pseudomonas sp. were detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U-test. A: TLR2 gene. B: TLR3 gene. C: TLR4 gene. D: TLR7 gene. E: TLR9 gene.

Figure 7.16: Cytokine mRNA expression in cats with and without *Pseudomonas* sp.



Pseudomonas sp. were detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TNF-α gene expression. B: IFN-γ gene expression. C: IL-1β gene expression. D: IL-4 gene expression. E: IL-6 gene expression. F: IL-10 gene expression. G: IL-12 gene expression.

7.3.16 TLR mRNA expression in cats with and without *P. circumdentaria*

When cats were divided into two groups according to the presence or absence of *P. circumdentaria* in the oral swabs, a significant difference between the two groups was seen in TLR2 (p=0.039) and TLR3 mRNA expression (p=0.01). The other TLR genes did not show a difference in levels of expression. All TLR genes except TLR3 showed a higher level of expression in the group where *P. circumdentaria* was present (Figure 7.17).

7.3.17 Cytokine mRNA expression in cats with and without *P. circumdentaria*

Comparison of cats with and without *P. circumdentaria* showed a higher level of cytokine mRNA expression in the group where *P. circumdentaria* was present for all cytokines except IL-12. Cytokine mRNA expression in the *P. circumdentaria* group was not significantly different for any of the cytokine genes (Figure 7.18).

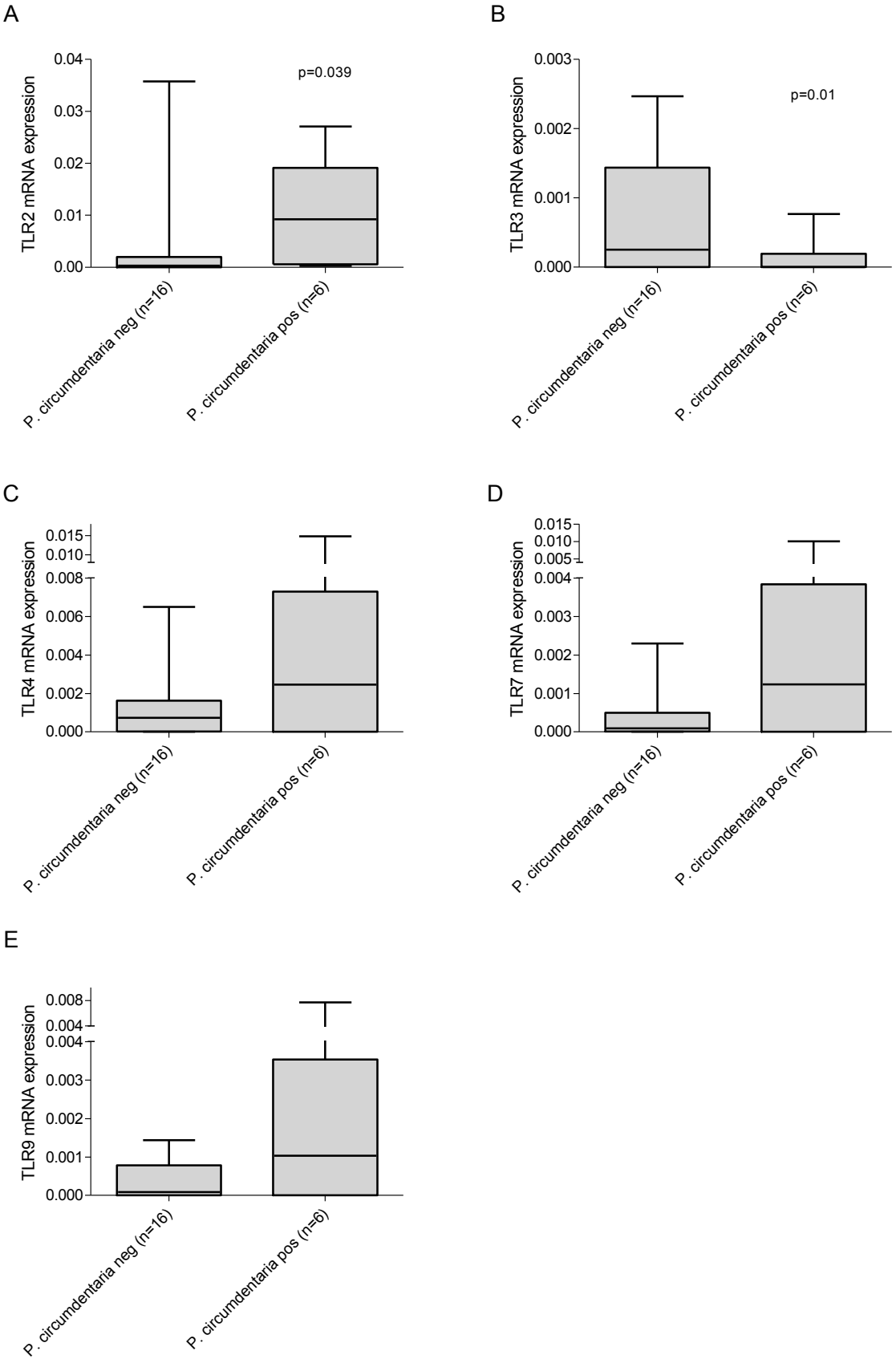
7.3.18 TLR mRNA expression in different histopathological groupings

Gene expression was analysed in cats divided into two different groups according to the type of inflammatory cells present as assessed by histopathological examination (Section 6.3.3). The median levels of the TLR mRNA levels appeared to be greater in cell group 2 (mainly plasma cells). This difference was only statistically significant for TLR3 gene expression (p=0.037) (Figure 7.19).

7.3.19 Cytokine mRNA expression in different histopathological groupings

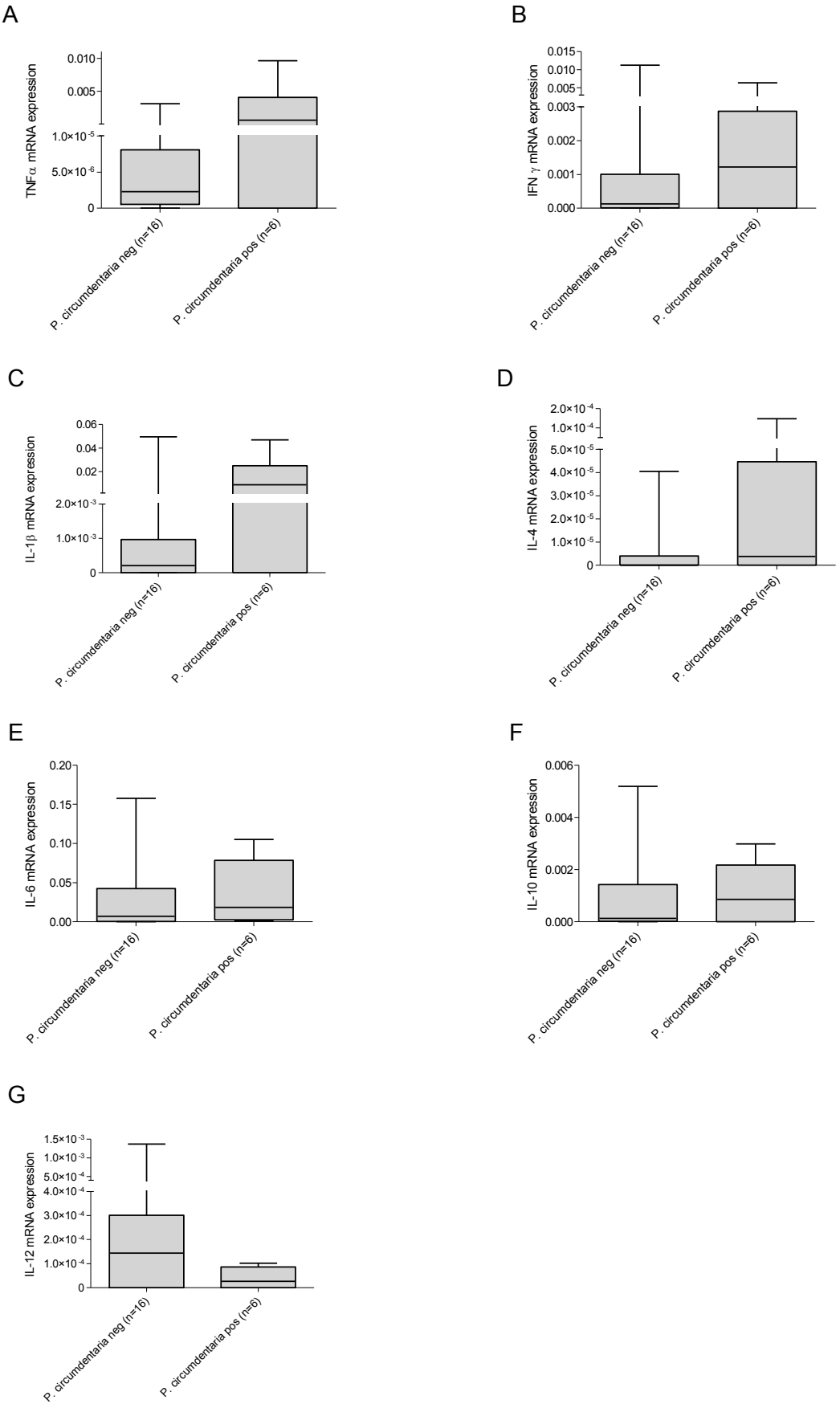
Cytokine gene expression in the two histopathological cell groupings was analysed. The Mann-Whitney U-test showed a statistically significant increase in IFN-γ (0.045), IL-1ß (p=0.021), IL-6 (p=0.045) and IL-10 (p=0.026) mRNA in group 2 (Figure 7.20).

Figure 7.17: TLR mRNA expression in cats with and without *P. circumdentaria*



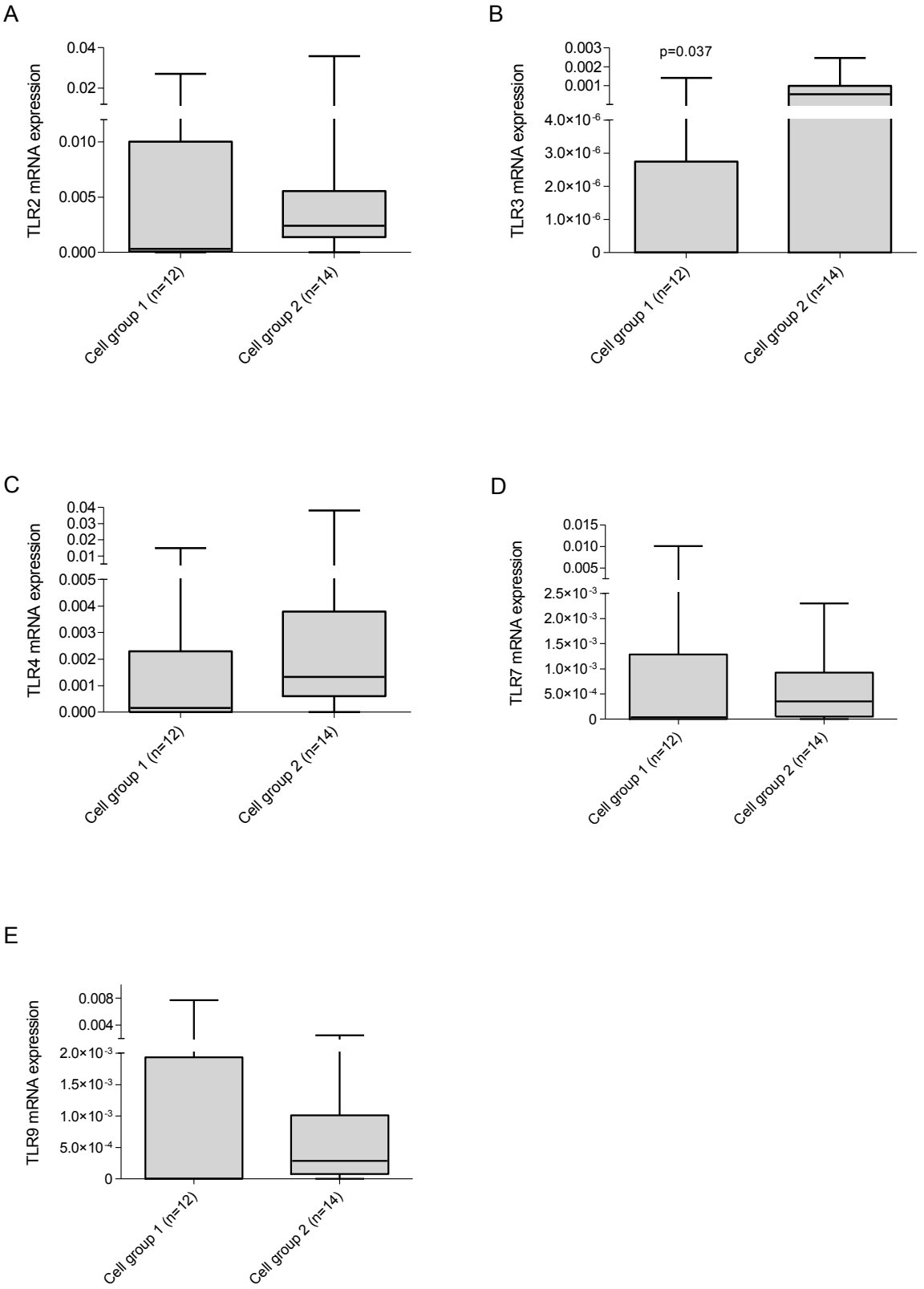
P. circumdentaria was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: Expression of the TLR2 gene. B: Expression of the TLR3 gene. C: Expression of the TLR4 gene. D: Expression of the TLR7 gene. E: Expression of the TLR9 gene. Pos: Positive, Neg: negative.

Figure 7.18: Cytokine mRNA expression in cats with and without *P. circumdentaria*



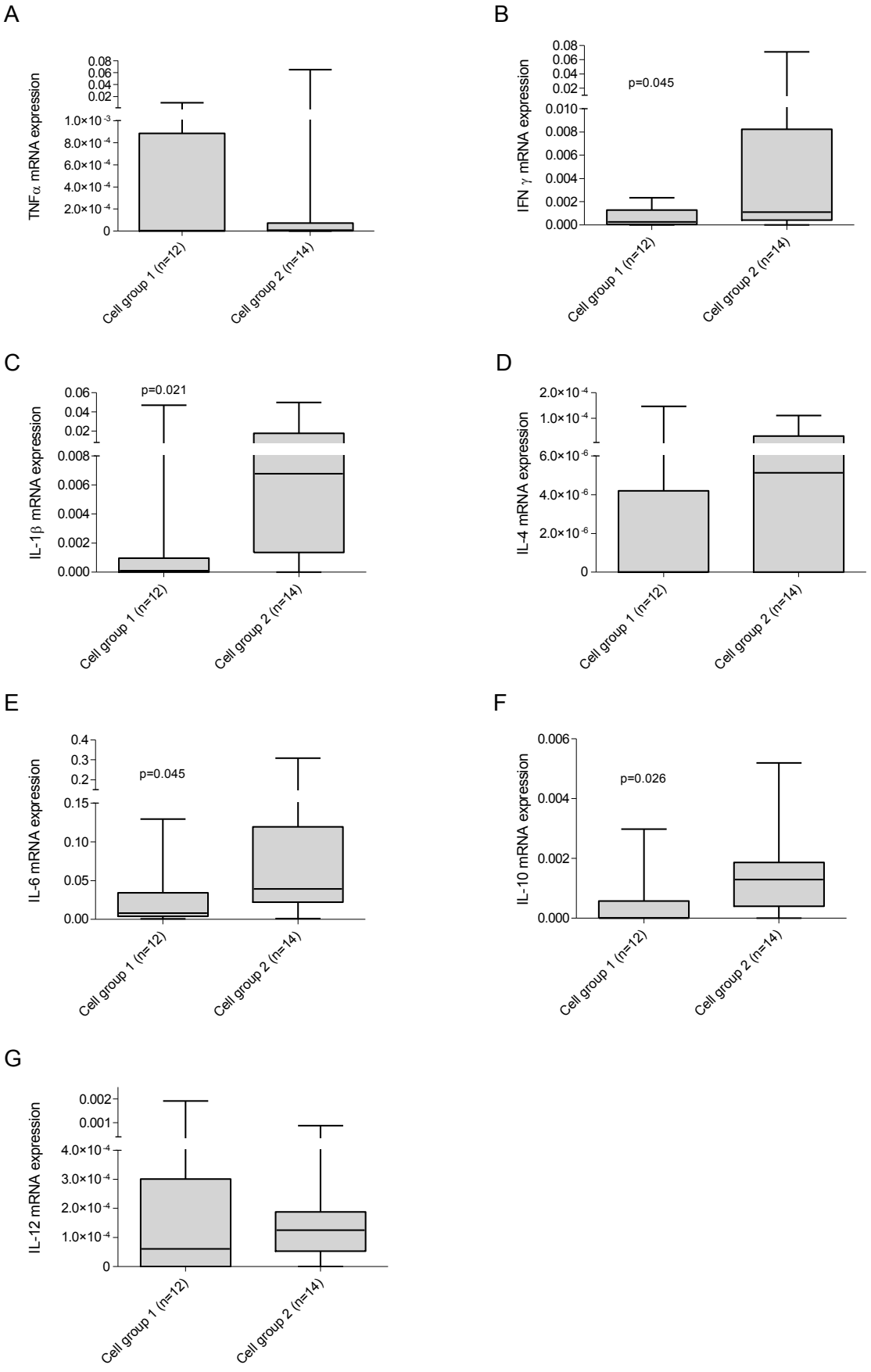
P. circumdentaria was detected by 16S rRNA gene sequencing of bacteria from oral swabs of cats with and without FCGS. All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TNF-α mRNA. B: IFN-γ mRNA, C: IL-1ß mRNA. D: IL-4 mRNA. E: IL-6 mRNA. F: IL-10 mRNA. G: IL-12 mRNA. Pos: positive, Neg: negative.

Figure 7.19: TLR mRNA expression in two histopathological cell groupings



All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TLR2 mRNA. B: TLR3 mRNA. C: TLR4 mRNA. D: TLR7 mRNA. E: TLR9 mRNA.

Figure 7.20: Cytokine mRNA expression in two histopathological cell groupings



All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Mann-Whitney U test. A: TNF-α mRNA expression. B: IFN-γ mRNA expression. C: IL-1ß mRNA expression. D: IL-4 mRNA expression. E: IL-6 mRNA expression. F: IL-10 mRNA expression. G: IL-12 mRNA expression.

7.3.20 TLR mRNA expression according to histopathological severity

Histopathological analysis showed five severity groups (Section 6.3.2). To make group numbers more equal, mild (group 1) and mild to moderate (group 1-2) were taken together. The other categories were moderate (2), moderate to severe (2-3) and severe (3). Differences between the groups were compared with a Kruskal-Wallis test. A significant difference was seen between at least one of the groups analysed for TLR4 gene expression ($p=0.042$). Dunn's multiple comparison test showed a difference between the mild/moderate and moderate/severe group ($p<0.1$) (Figure 7.21). No significant differences were seen in the expression of the other TLR genes.

7.3.21 Cytokine mRNA expression according to histopathological severity

Changes in cytokine mRNA expression between the different severity groups was determined using a Kruskal-Wallis test. A significant difference was shown for IL-1ß mRNA in at least one of the groups ($p=0.003$). A Dunn's multiple comparison test showed differences between the mild/moderate and moderate/severe groups ($p<0.05$) and between the mild/moderate and severe groups ($p<0.05$) No other differences were found to be significant (Figure 7.22).

7.3.22 TLR mRNA expression according to clinical severity

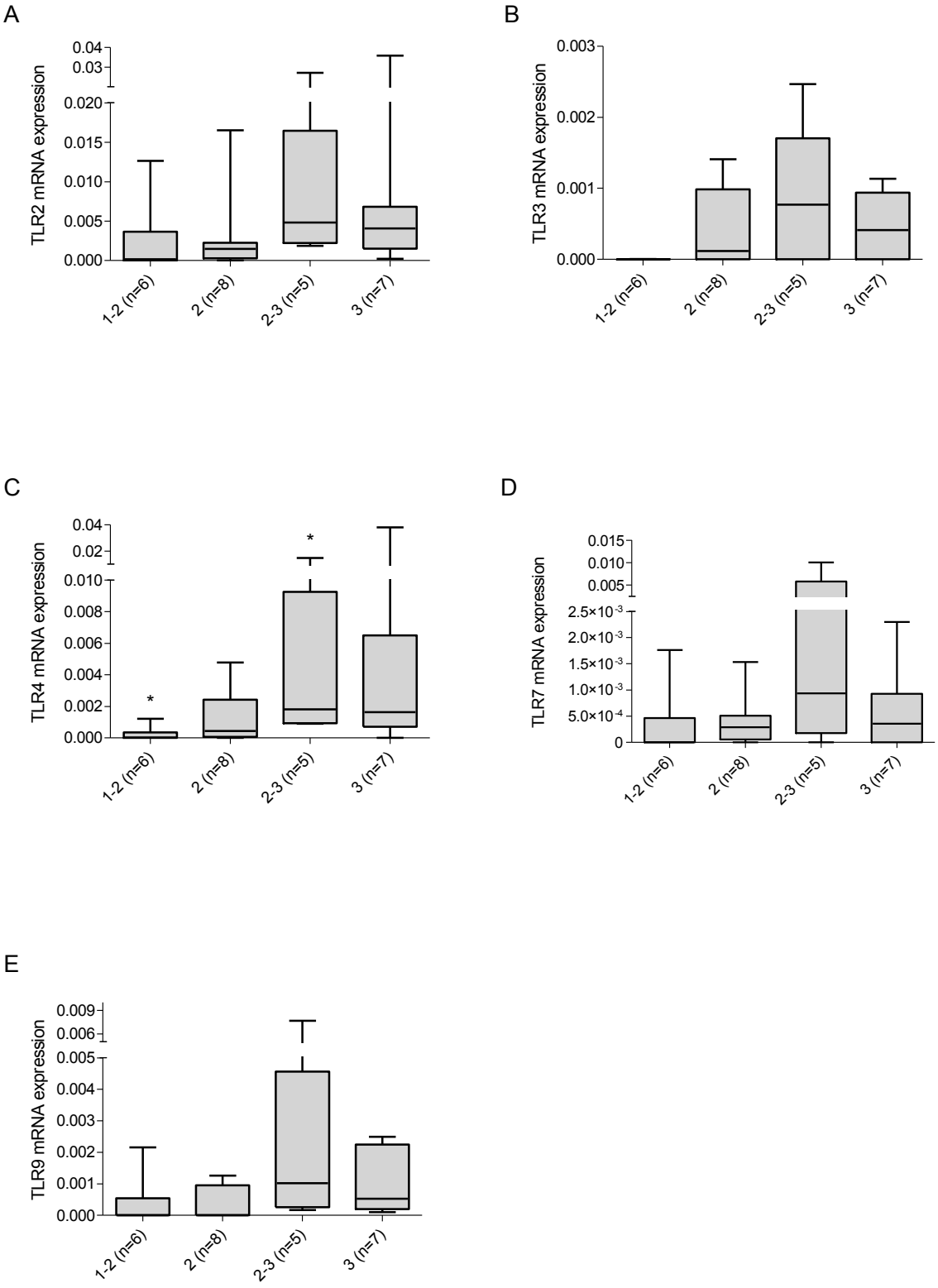
TLR mRNA abundance in the different groups of severity assessed during clinical investigation (Section 3.3.5) was analysed by a Kruskal-Wallis test. Group 1, mild inflammation and group 2, moderate inflammation, were taken together to equalise the group sizes. Group 0; no inflammation, represents the healthy cats. A significant difference in at least one of the groups was seen in TLR2 mRNA expression ($p=0.012$). A Dunn's multiple comparison test showed a difference between group 0 and group 3 in expression of TLR2 mRNA ($p<0.05$) (Figure 7.23).

7.3.23 Cytokine mRNA expression according to clinical severity

The cytokine mRNA levels in the different clinical severity groups was analysed by a Kruskal-Wallis test. A significant difference in at least one of the groups was seen for IFN-γ ($p=0.01$), IL-1ß ($p=0.032$) and IL-6 mRNA ($p=0.0005$). A Dunn's multiple comparison

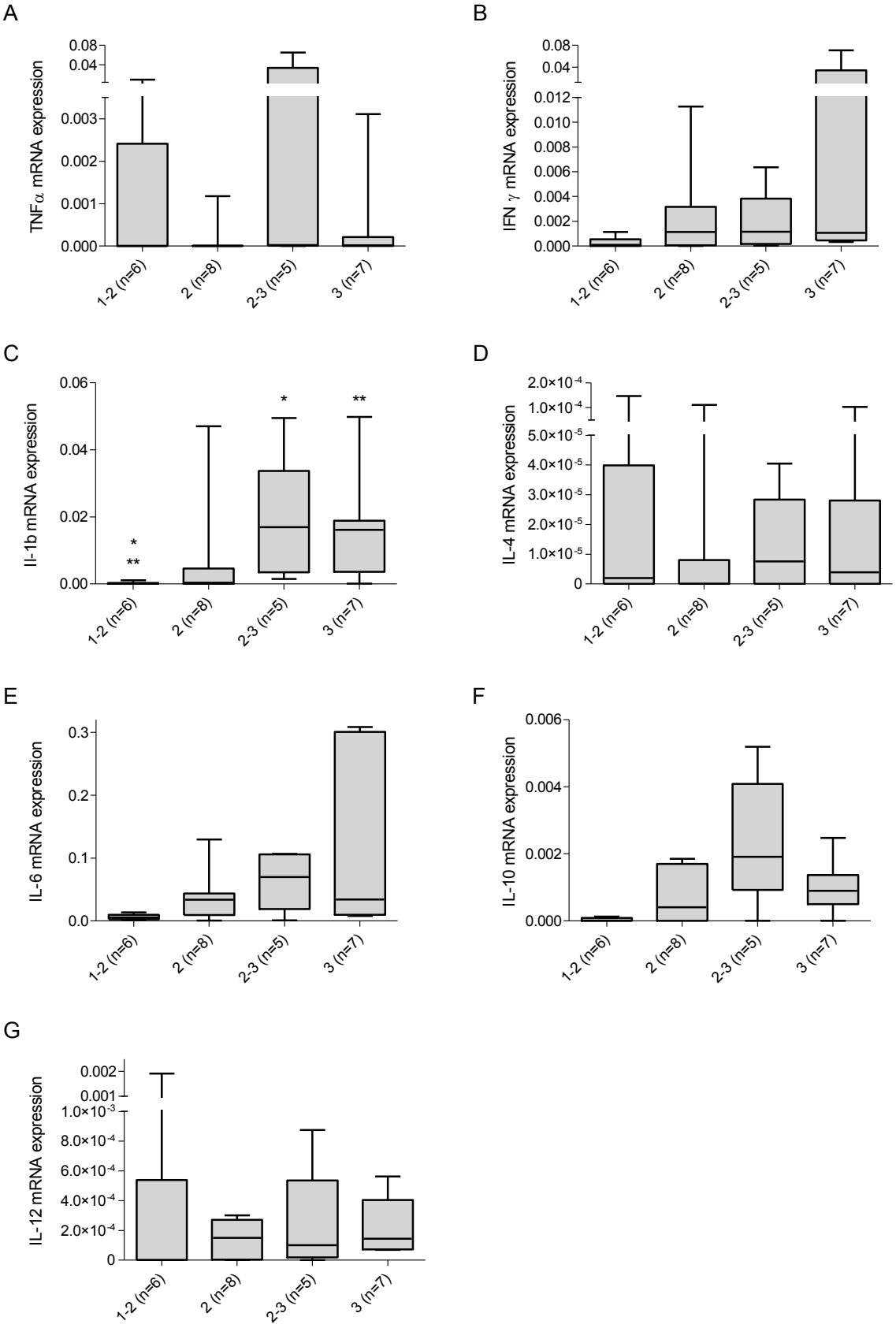
test showed a difference between group 0 and group 3 in the expression of IFN-γ ($p<0.05$), IL-1β ($p<0.05$) and IL-6 mRNA ($p<0.05$) (Figure 7.24).

Figure 7.21: TLR mRNA expression according to histopathological severity



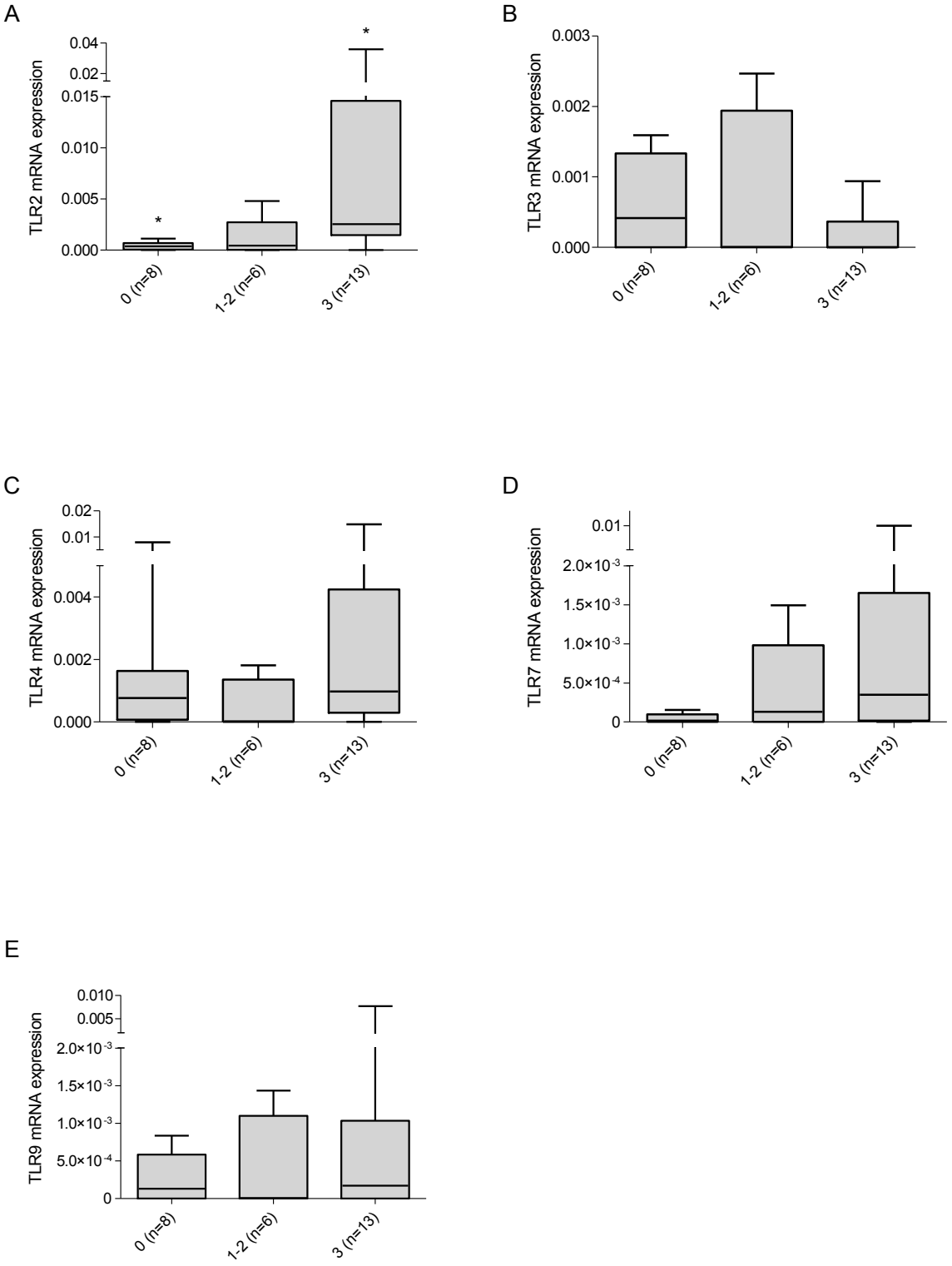
All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Kruskal-Wallis test followed by the Dunn's multiple comparison test. A: Expression of the TLR2 mRNA. B: Expression of the TLR3 mRNA. C: Expression of the TLR4 mRNA * $p<0.1$. D: Expression of the TLR7 mRNA. E: Expression of the TLR9 mRNA.

Figure 7.22: Cytokine mRNA expression according to histopathological severity



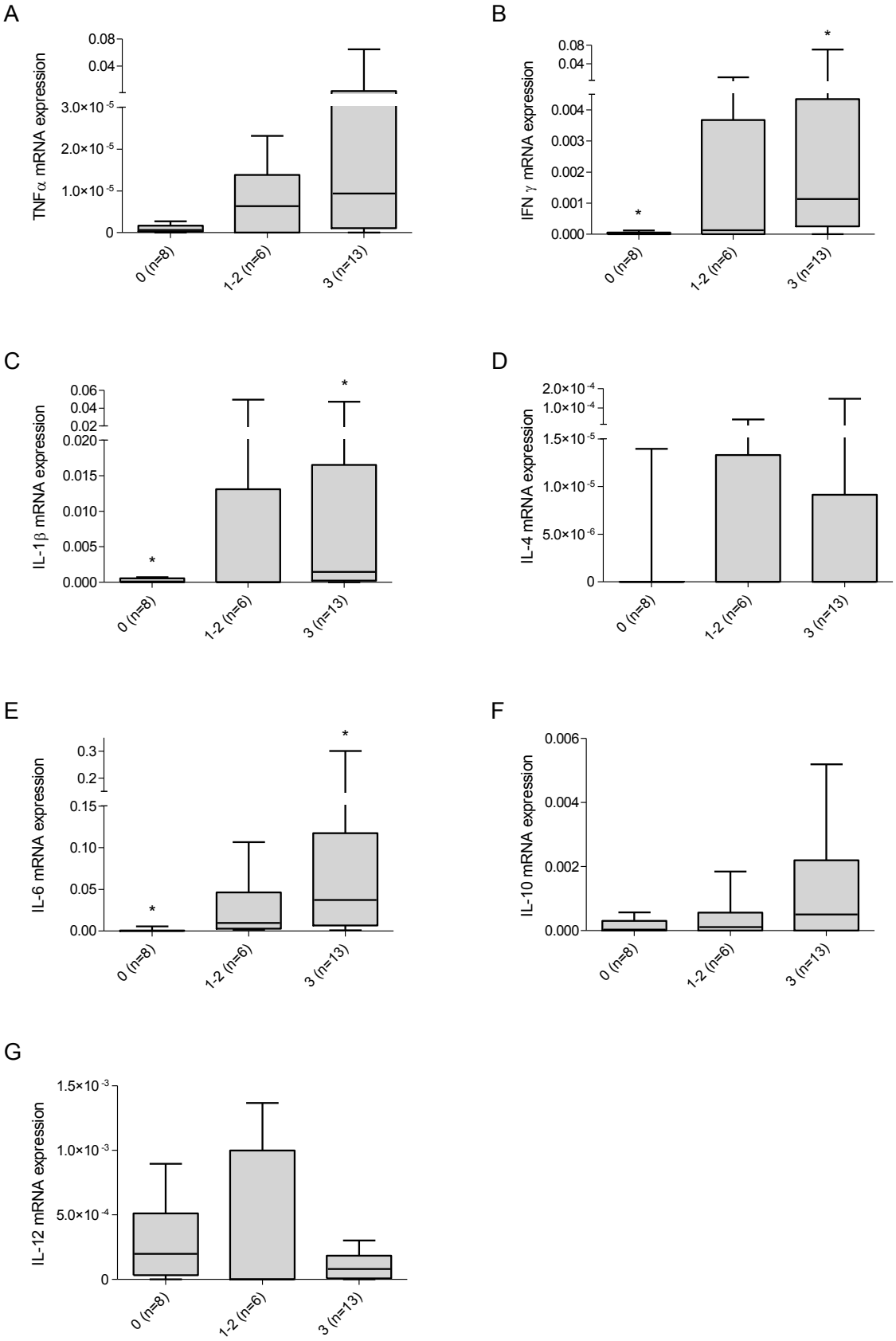
All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test. A: TNF-α mRNA expression. B: IFN-γ mRNA expression. C: IL-1ß mRNA expression * $p<0.05$, ** $p<0.05$. D: IL-4 mRNA expression. E: IL-6 mRNA expression. F: IL-10 mRNA expression. G: IL-12 mRNA expression.

Figure 7.23: TLR mRNA expression according to clinical severity



All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Kruskal-Wallis test, followed by a Dunn's multiple comparison test. A: TLR2 mRNA, * $p<0.05$. B: TLR3 mRNA. C: TLR4 mRNA. D: TLR7 mRNA. E: TLR9 mRNA.

Figure 7.24: Cytokine mRNA expression according to clinical severity



All samples were adjusted to GAPDH by the 2^{-dCt} method and statistical analysis was performed using the Kruskal-Wallis test, followed by a Dunn's multiple comparison test. A: TNF-α mRNA expression. B: IFN-γ mRNA expression, * $p<0.05$. C: IL-1β mRNA expression, *$p<0.05$. D: IL-4 mRNA expression. E: IL-6 mRNA expression * $p<0.05$. F: IL-10 mRNA expression. G: IL-12 mRNA expression.

7.4 Discussion

Quantitative PCR is a reliable technique to assess cytokine and TLR gene expression when using validated primers (Fraga et al., 2008). For the current study, new primers were designed on the basis of feline mRNA sequences for the different genes. Primers used in previous studies were not designed for the real time PCR SYBR-green method as used in this study, since either the product length was not suitable for the purpose of the study or they had been validated in a Taqman assay (Harley et al., 1999; Leutenegger et al., 1999; Ignacio et al., 2005). A well performed SYBR green method is reliable, although there is always the possibility in these fine-tuned techniques for errors. Errors can occur due to pipetting, suboptimal primer efficiency or differences in RNA quality between samples. To ensure accurate results, all primers were tested for efficiency and melt-curve analysis was performed on each run. To reduce the influence of pipetting errors, all samples were run in triplicate and averages were calculated. Primer efficiency was accepted when they were between 90% and 110% (Fraga et al., 2008). The primer efficiency for TLR9 mRNA detection was marginally greater at 112%, but consistent single peak melt curves were obtained. A second product was not observed in the amplification reactions and no amplicons were detected when the template was absent in the reaction. This discrepancy could be explained by experimental error. Primer efficiency for IL-4 could not be obtained because of the small amount of RNA in the samples. Only results with good dissociation curves were accepted, but due to the high number of null results no significant changes were found for this primer between different groups. At the time of primer design, the previously published TLR5 feline mRNA sequence was not accessible online and therefore a published primer set was used (Ignacio et al., 2005). For IL-2, TLR5 and IFN-γ gene analysis, no reliable melt curves could be obtained with the cDNA prepared from the samples, therefore these primers were not used for the final analysis. Due to the lack of RNA that could be extracted from the small tissue samples, there was only enough cDNA to run all the samples again for one new primer set and IFN-γ was chosen. For IFN-γ, two new primer pairs were designed and tested, yielding reliable results with good efficiency.

GAPDH was chosen as a housekeeping gene. GAPDH is commonly used for this purpose and is expressed at high levels in different tissue types (Harley et al., 1999; Kipar et al., 2001; Nguyen Van et al., 2006). Primers for GAPDH were designed and the efficiency of GAPDH was tested for both annealing temperatures used in the study. No substantial difference was found in GAPDH expression between both cohorts (healthy and FCGS) and therefore GAPDH was considered a suitable reference gene for this study. A study

on reference genes for qPCR in feline tissue where a number of different tissues were tested showed that other reference genes might be preferred over GAPDH in some circumstances (Penning et al., 2007). However no feline gingival tissue had been tested in that study and considering the validation tests that have been performed in the current study, GAPDH can be considered valid. A melt curve was made on each run for each primer to ensure a single product with no primer-dimers or DNA contamination.

TLR gene expression was analysed in this study. TLRs activate adapter molecules after binding to their ligand. The activated cascade then leads to induction or suppression of genes that influence the inflammatory response (Akira and Takeda, 2004). The expression of mRNA encoding TLR2, TLR3, TLR4, TLR7 and TLR9 was measured. Two out of the five TLRs tested (TLR2 and TLR7) showed a significant increase in abundance of their respective mRNA in the FCGS group when compared to the healthy group. TLR2 is found on the cell surface. The ligands for TLR2 are derived from a variety of gram-positive and gram-negative bacteria and include lipoprotein, peptidoglycan and glycolipids (Akira and Takeda, 2004). Zymosan is also one of the ligands for TLR2, indicating that TLR2 also recognises fungi. It is likely that host cells could be recognised, since heat-shock protein 70 (HSP70) is seen as a possible ligand. TLR2 mRNA abundance was significantly higher in the biopsies from cats with FCGS, compared to the biopsies from healthy cats. Since activation of TLR2 is known to cause an increase in expression of the TLR2 gene (Weiss et al., 2004) this might indicate a reaction to one of the known ligands. In this study an innate reaction to bacteria mediated by TLR2 is likely since a broad variety of gram-positive and gram-negative bacteria were identified in the oral cavity. TLR2 gene expression was significantly increased in cats harbouring *T. forsythia*. The involvement of TLR2 in *T. forsythia* recognition has been shown previously. Studies on the role of *T. forsythia* in periodontal bone loss have shown a TLR2-dependent pathway for cytokine expression (Myneni et al., 2011). The presence of another bacterial genus known to be important in human periodontal health, (*Porphyromonas* sp.) was also associated with a significant increase in the expression of TLR2. *P. circumdentaria* is from another subspecies, but the inflammatory response in the genus member *P. gingivalis* has been studied and is dependent on TLR2 (Burns et al., 2006). Interestingly, no significant difference was seen in the current study when groups with and without *P. multocida* subsp. *septica*, *P. multocida* subsp. *multocida* and *Pseudomonas* sp. were compared, and these species were detected regularly in cats with FCGS. A possible explanation for the lack of discrimination between gene expression profiles for *P. multocida* subsp. *septica*-positive and *P. multocida* subsp. *septica*-negative cats is the high abundance of the organism in the healthy group.

In the current study, TLR2 mRNA levels demonstrated significantly higher expression in the group positive for FCV when compared to the FCV-negative group, without an obvious explanation. FCV is seen in the majority of cases with FCGS. Severe inflammation has been seen in cats with FCV (Hennet et al., 2011) and the role of bacteria is unclear. However, a stronger reaction to bacteria by innate immunity, as shown by an increase in TLR2 mRNA expression in the current study, suggests that there may be a relationship between the two. Also a higher expression of TLR2 mRNA in the current study was seen in cats with more severe clinical disease in the caudal area of the oral cavity, suggesting that more severe cases show a more intense reaction to the bacteria that are present in the oral cavity.

TLR3 recognises double stranded RNA (dsRNA) (Alexopoulou et al., 2001). Viruses with dsRNA, (reoviruses) were not investigated in this study. However, it has been speculated that TLR3 also recognises single-stranded RNA viruses during the replication process (Jacobs and Langland, 1996; Tabeta et al., 2004). The actual amount of dsRNA in cells infected with single-stranded RNA (ssRNA) viruses is unclear and the exact role of TLR3 in the viral immune response is yet to be elucidated (Edelmann et al., 2004). It has been shown, however, that ssRNA viruses can cause an up-regulation of TLR3 gene expression (Guillot et al., 2005) but this has not been investigated for FCV specifically. The reaction of various TLRs following viral infection also seems to vary between cell types (Ignacio et al., 2005). When comparing healthy cats and cats with FCGS in the current study, no significant difference was observed between the groups for TLR3 expression. A reduction of TLR3 mRNA was seen in the FCGS group when compared to the healthy group. A significantly higher expression of the TLR3 gene was seen in the group where no *P. circumdentaria* was present when compared to the group where the organism was present. One other significant finding was the higher expression in the histopathological cell group 2. Further investigation into the role of TLR3 is needed to explain the changes that have been seen in the current study.

TLR4 recognises lipopolysaccharide (LPS) from several types of Gram-negative bacteria, but also has fusion and envelope proteins as a ligand and is therefore also known to recognise several viruses (Akira and Takeda, 2004). It has been described that host cells can be recognised by several ligands, including HSPs. In the current study, TLR4 mRNA expression appeared to be slightly up-regulated in the FCGS group when compared to the healthy group but no statistically significant difference was seen. A significantly higher expression of the TLR4 gene was found in cats that showed the presence of the Gram-negative bacterium *T. forsythia*, when compared to a group of cats where *T. forsythia* was not present. However, none of the other bacteria were associated with a significant difference in mRNA expression for TLR4. A significant difference in TLR4

mRNA was recorded between groups when divided according to the histopathological severity. The severity was judged on the number of cells present in the tissue samples. The increased numbers of immune cells probably accounted for the increased expression of TLR4 mRNA in the severely inflamed group, but up-regulation of TLR4 gene expression on resident cells could also account for such a change.

TLR7 has ssRNA as a ligand and therefore recognises ssRNA viruses. In the current study, the expression of TLR7 was significantly increased in the cats with FCGS. In some respects this might be expected, since a high percentage of the cats in the FCGS group were found to be positive for the ssRNA virus FCV. Other ssRNA viruses were investigated in this study, namely FIV and FeLV. These viruses, as well as FCV, were found in some healthy cats and FIV was also detected in one cat with FCGS. When healthy and FCGS cats were divided according to their FCV status the difference between the groups in TLR7 mRNA abundance just missed statistical significance using the two tailed Mann-Whitney test. The possibility of false negative results in the FCV test within the FCGS group could be an explanation for this difference. Other factors will play a role but the high expression of TLR7 in the FCGS group suggests that stimulation by viruses is contributing to activation of the immune system in some of the cats with FCGS. Other changes in the TLR7 expression were seen, but without a clear explanation. A significant difference in TLR7 mRNA abundance was seen when groups were divided according to the presence of *T. forsythia*. Cats with *T. forsythia* showed a higher expression of several TLRs and cytokines. Complex interactions between the viral and bacterial pathogens might be present in FCGS and other complex infectious diseases. TLR and cytokine mRNA expression were lower in cats where subspecies of *P. multocida* were identified. A significant difference in TLR7 mRNA levels was found between the cats with and without *P. multocida* subsp. *septica*, with cats without *P. multocida* subsp. *septica* showing a higher mRNA expression. Further investigation into the role of these bacteria in the oral cavity is required in order to explain these changes.

CpG-containing DNA is a specific ligand for the endosomal TLR9, which can therefore recognise, fungi, bacteria and viruses (Hemmi et al., 2001; Ramaprakash et al., 2009). TLR9 mRNA levels appeared to be higher in the FCGS group in the current study but no significant difference was seen. However, a significant increase in expression of TLR9 mRNA was seen in the group with *T. forsythia* present compared to cats where no *T. forsythia* was detected.

The result of TLR ligation in general is the activation of second messenger systems, which share certain components of the pathways that activate nuclear factor κB. This

nuclear factor translocates to the cell nucleus to induce the transcription of many genes, including those encoding cytokines (Akira and Takeda, 2004). Certain aspects of cytokine gene expression were also investigated in this study.

Cytokine gene expression in cats with FCGS has been studied previously (Harley et al., 1999). Reverse transcription PCR was used with GAPDH as a housekeeping gene. An increase was seen in the expression of IL-2, IL-4, IL-6, IL-10, IL-12 and IFN-γ genes in the affected population. A shift from a predominant Th1 response in the healthy population to a combined Th1-Th2 response in the FCGS group was described.

In the current study, a significant increase in cytokine mRNA expression was seen in the FCGS group for TNF-α, IFN-γ, IL-1ß and IL-6. An increase was seen in IL-4 and IL-10 mRNA levels but this was not statistically significant. IL-12 mRNA did show a slight reduction in the FCGS group. An abundance of mRNA encoding for the acute phase cytokines IL-1ß, IL-6 and TNF-α was increased significantly in the FCGS group. The acute phase response induces the production of cortisol and can result in a leukocytosis by the release of high numbers of neutrophils into the bloodstream. Cytokines also increase the speed of neutrophil delivery to the tissues (Paltrienieri, 2008). The response is however not confined to the acute phase reaction since both Th1 and Th2 cytokine gene expression was increased in the current study as described previously (Harley et al., 1999). However, in the current study no significant increase was shown in the abundance of IL-4 and IL-10 mRNA. Furthermore, IL-12 mRNA expression was not increased.

How Th1, Th2 and Th17 cells and cytokines are involved in the pathogenesis of several diseases is still unclear, but in human periodontal disease studies it has been shown that cytokines from these different T cell subsets are expressed together (Preshaw and Taylor, 2011). The present study shows a significant increase in acute phase cytokines. Stimulation of TLRs induces the induction of inflammatory cytokines and LPS is known to be one of the important initiating factors in the host response to infection in human periodontal disease (Preshaw and Taylor, 2011). The disease can also progress by activation of macrophages through breakdown products produced during tissue damage, causing further cytokine secretion.

Unfortunately, we were unable to extend our investigation to the IL-17 family of cytokines, produced by Th17 cells, due to a lack of information on the feline genome. The IL-17 family of cytokines have proven to be important mediators of inflammation involved in man and other species and are involved in a number of inflammatory

diseases in which T cells and B cells are involved (Johnson et al., 2004; Colic et al., 2007; Bougarn et al., 2011).

The high number of plasma cells that have been found in the histopathological analysis in the present study and the high titres of immunoglobulins found in saliva of cats with FCGS (Harley et al., 2003b) supports the involvement of the humoral immune response in the disease. A recent study on the cell populations in the caudal mucosa of cats with FCGS also showed that the predominant cell types identified, CD79a- and IgG-expressing cells, were part of the humoral immune response (Harley et al., 2011).

As with TLR gene expression, cytokine gene expression was compared for groups with and without pathogens that were investigated in the current study. When a comparison was made between cats with and without FCV, a significant increase was seen in the inflammatory cytokines IL-1ß and IL-6 mRNA expression in the group with FCV and also in the abundance of IFN-γ mRNA. IFN-γ is known to be synthesised in response to the activation of T lymphocytes and natural killer cells by virus infected cells (Goodbourn et al., 2000) and is therefore likely to be highly expressed in virus-infected cats.

When divided into cats with and without *T. forsythia* a significant increase in the group with *T. forsythia* was seen in TNF-α and IL-1ß mRNA expression. An increased expression of the acute phase cytokines IL-1ß, TNF-α and IL-6 has been seen in human monocytes stimulated with DNA from *T. forsythia* (Sahingur et al., 2010). Therefore it is not surprising that the acute phase cytokines are increased in cats when this micro-organism is present. However, other bacteria, (*P. multocida* subspecies, *Pseudomonas* sp. and *P. circumdentaria*) were not associated with a higher expression of cytokine genes. One possible reason for this is that a large proportion of healthy cats also harboured some of these bacteria. In a group of cats where no *P. multocida* subsp. *septica* was isolated, a higher level of mRNA expression was detected for TNF-α and IFN-γ. For the groups with *P. multocida* subspecies and *Pseudomonas* species a lower (but not statistically significant) abundance of mRNA was seen for most of the cytokines. In the groups where *T. forsythia* and *P. circumdentaria* were isolated, expression of most cytokine genes were higher but in many cases this did not reach statistical significance.

For the two different histopathological cell groups, plasma-lymphocytic (cell group 1) and plasmacytic (cell group 2), significant differences were observed in the abundance of mRNA in IFN-γ, IL-1ß, IL-6 and IL-10, with a higher expression of these cytokines in cell group 2. High numbers of plasma cells were seen in most samples, but in cell group 2 the cells were predominantly plasma cells. Consequently, the numbers of lymphocytes and neutrophils were lower.

When the cats were divided according to the histopathological severity, a significant difference in IL-1ß gene expression was seen between the groups. The acute phase cytokine also showed a significant difference between groups when subdivided according to clinical severity. Very low expression of the cytokine was seen in the mildly inflamed groups and a higher expression was seen in the more severely inflamed groups. IFN-γ and IL-6 also showed a significant difference between the clinical severity groups. An increase in expression of the different cytokines in the more severe cases concurs with the findings of Harley et al. (1999).

The results in this chapter show that a multifactorial aetiopathogenesis of FCGS is most likely. Bacteriological studies show that opportunistic bacterial infection is likely to play a role (Sims et al., 1990; Dolieslager et al., 2011). In the present study, the bacteria that seem to show the most powerful impact on the measured immune system parameters are *T. forsythia* and *P. circumdentaria*. These bacteria induce significantly higher expression of certain cytokine and TLR genes and therefore seem to play a role in the inflammatory process. A greater level of expression of gene transcripts encoding a number of cytokines and TLR genes was also seen in the cats infected with FCV. Environmental factors, bacterial infection and the host response to infection may act in combination with viral infection to influence the disease process (Tenorio et al., 1991; Addie et al., 2003).

Analysing cytokine and TLR gene expression *in vivo* has the advantage that the possible link between TLR stimulation and cytokine production is maintained and can be investigated. Many factors involved in the process, including some features of the immune system, are not yet understood and require further investigation. Therefore, it is important to also investigate the possible reaction of each putative pathogen individually and in combination. This should involve studies on feline cells or a feline cell line in an *in vitro* model system. Although this will provide useful information on mechanisms involved in the induction of the feline innate immune response it would not address the translational problems involved in relating an *in vitro* model to an *in vivo* situation.

Chapter 8 Putative risk factors for FCGS

8.1 Introduction

Possible risk factors for FCGS that have been described are the signalment (breed, age), diet and environmental factors that cause stress (Frost and Williams, 1986; Diehl and Rosychuk, 1993; Addie et al., 2003). Stress factors in the cat's environment that have been described include the presence of other cats in the household, changes that have been taking place within the environment and no opportunity to roam outdoors (Amat et al., 2009; Herron, 2010). Relevant changes in the environment could include a change in household members, redecoration and/or new (neighbourhood) pets. In Section 3.3.1 the signalment of cats with FCGS and healthy cats included in the current study were compared. More data was collected by the use of a questionnaire-based study.

Feline viruses, in particular FCV, have been implicated in the aetiopathogenesis of FCGS (Gaskell and Gruffydd-Jones, 1976; Knowles et al., 1989; Reubel et al., 1992) and thus vaccination or lack of vaccination for FCV may be a possible risk factor.

8.2 Materials and methods

The questionnaire was prepared on the basis of possible risk factors for FCGS. A paper version of the questionnaire was sent out to veterinary surgeons that were a member of the British Veterinary Dental Association. An online version of the questionnaire was created and uploaded on the website on feline diseases (http://www.dr-addie.com/stomatitis.html) created by Dr. D.D. Addie, BVMS, PhD (Institute Comparative Medicine, University of Glasgow) The complete questionnaire is presented in the Appendix. Contingency tables were made and Fisher's exact tests were performed to analyse the possible risk factors. Mean age was compared by an independent samples t-test. All statistical analysis was performed in GraphPad Prism version 5.0.

8.3 Results

A total of 72 questionnaires were received for cats with FCGS and 25 for healthy cats.

8.3.1 Signalment

8.3.1.1 Breed

When the breeds of 72 cats with FCGS were compared to the breeds of 25 healthy cats, a total of 16 (22.2%) cats in the FCGS group were purebred cats, compared to a total of 3 (12.0%) in the healthy group. Four (5.6%) and one (4.0%) of cats, respectively, were purebred crosses and the remaining cats were domestic short hair (DSH), domestic long hair (DLH) or domestic medium hair (DMH). Although a higher percentage of purebred cats was found in the FCGS group, there was no statistically significant difference between the FCGS and healthy cats with regard to number of domestic and purebred cats (Figure 8.1).

8.3.1.2 Age

The mean age of the 72 cats with FCGS was 7.0 years. Most cats (37.5%) fell into the age category of 5-10 years old (61-119 months). The mean age for the 25 healthy cats was 5.7 years. Most cats (45.5%) from the healthy group were part of the 1-5 years old (0-60 months) group (Figure 8.2). No significant difference in mean age was seen between the healthy cats and cats with FCGS.

8.3.1.3 Sex

Sex was recorded for 72 cats with FCGS and 25 healthy cats; 34 (47.2%) of the cats with FCGS were male (32 neutered) and 38 (52.8%) female (29 neutered). In the healthy group, 14 (56.0%) were male (11 neutered) and 11 (44.0%) female (6 neutered) Figure 8.3). No significant difference was seen between the healthy cats and cats with FCGS.

Figure 8.1: Breed distribution of 72 cats with FCGS and 25 healthy cats

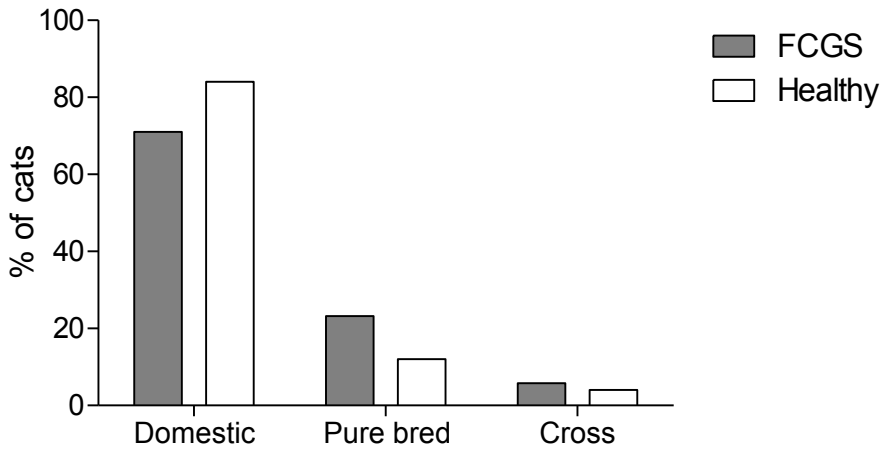


Figure 8.2: Age distribution of 72 cats with FCGS and 25 healthy cats

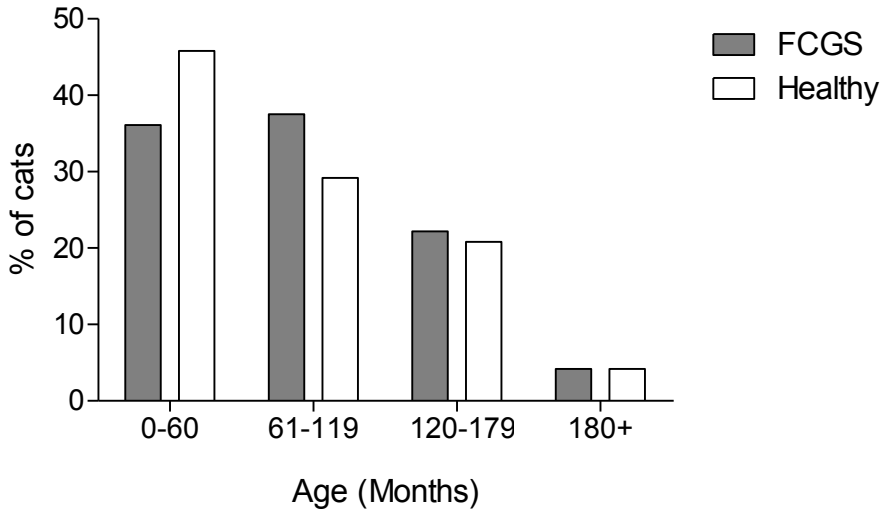
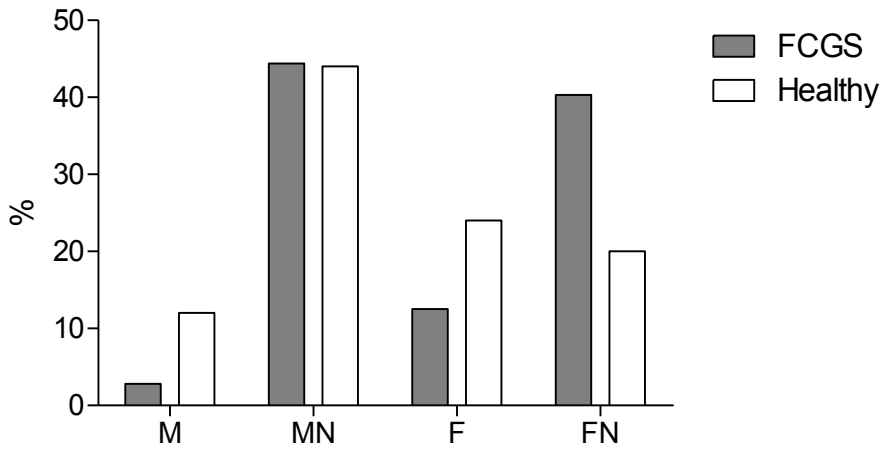


Figure 8.3: Sex distribution in 72 cats with FCGS and 25 healthy cats.



M:Male, MN: Male neutered, F: Female, FN: Female neutered

8.3.2 Possible risk factors

8.3.2.1 Single versus multi-cat households

The cats from both groups were compared according to the presence of other cats in the household. The question on the presence of other cats was answered for a total of 66 cats with FCGS and for 23 healthy cats. Of 66 cats with FCGS, 9 (13.6%) had no other cats present in the household and in 57 (86.4%) of the cases there were other cats present in the household (Figure 8.4). For the healthy group of 23 cats, no other cats were present in the household for 13 (56.5%) of the cases and in 10 (43.5%) of the cases other cats were present in the household. A Fisher's exact test showed a significant difference between the healthy cats and cats with FCGS when the presence or absence of other cats in the household was compared (p=0.0001).

8.3.2.2 Indoor versus outdoor environment

The ability of cats to go outside was also compared in the FCGS group and the healthy group (Figure 8.5). Answers for this particular question were given for 63 cats with FCGS and 24 healthy cats. Of 63 cats with FCGS, 32 (50.8%) were indoor cats and 31 (49.2%) were outdoor cats. In the 24 healthy cats, four (16.7%) were indoor cats and 20 (83.3%) were outdoor cats. A Fisher's exact test showed a significant difference between the healthy cats and the cats with FCGS when the ability to roam outdoors was compared (p=0.004).

8.3.2.3 A combination of 'stress factors' within the household

When cats with and without the ability to roam outdoors and the presence of other cats in the household were compared between the FCGS and healthy groups (Figure 8.6), 28 (48.3%) cats in the FCGS group were unable to roam outdoors with other cats in the household, 4 (6.9%) cats were unable to roam outdoors without other cats, 26 (44.8%) were able to roam outdoors with other cats in the household and none were able to roam outdoors without other cats. In the healthy group, 1 (4.3%) of the cats came from an indoor-multi cat household. 3 (13%) were unable to roam outdoors cats without other cats present, 9 (39.1%) of the cats were able to roam outdoors with the presence of other cats and 10 (43.5%) were able to roam outdoors cats without other cats present in the household. The numbers were too small for statistical analysis.

Figure 8.4: Percentage of cats with and without FCGS living in a single cat or a multi-cat household.

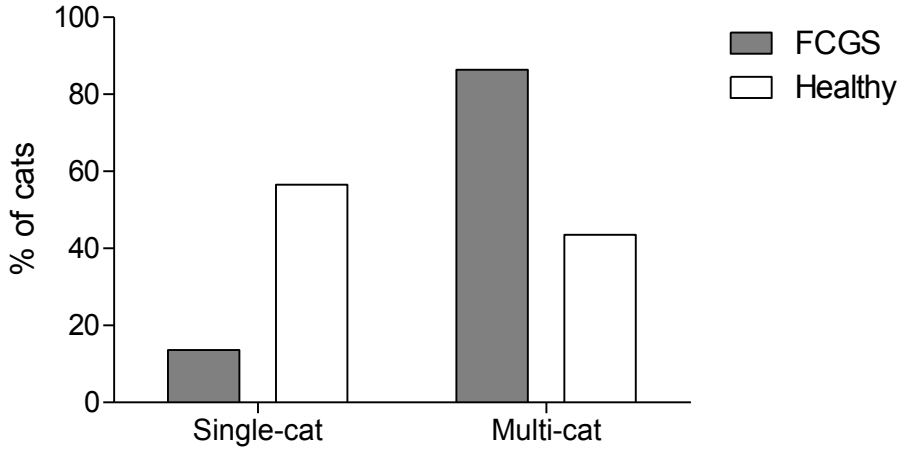
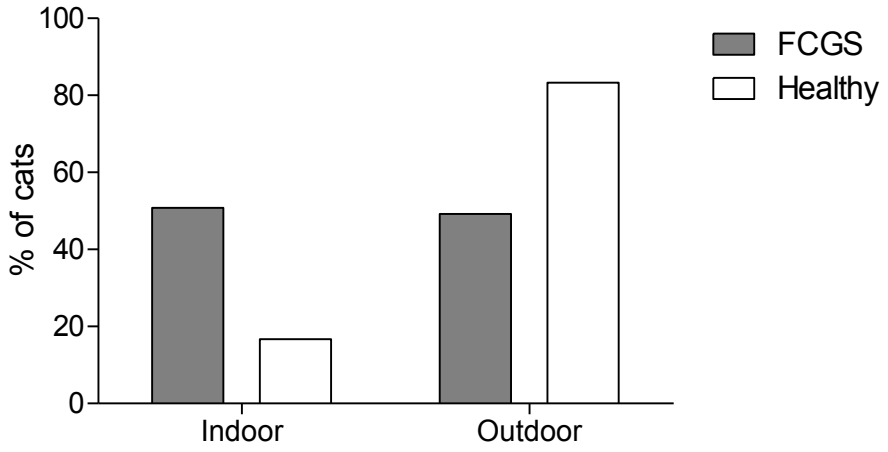
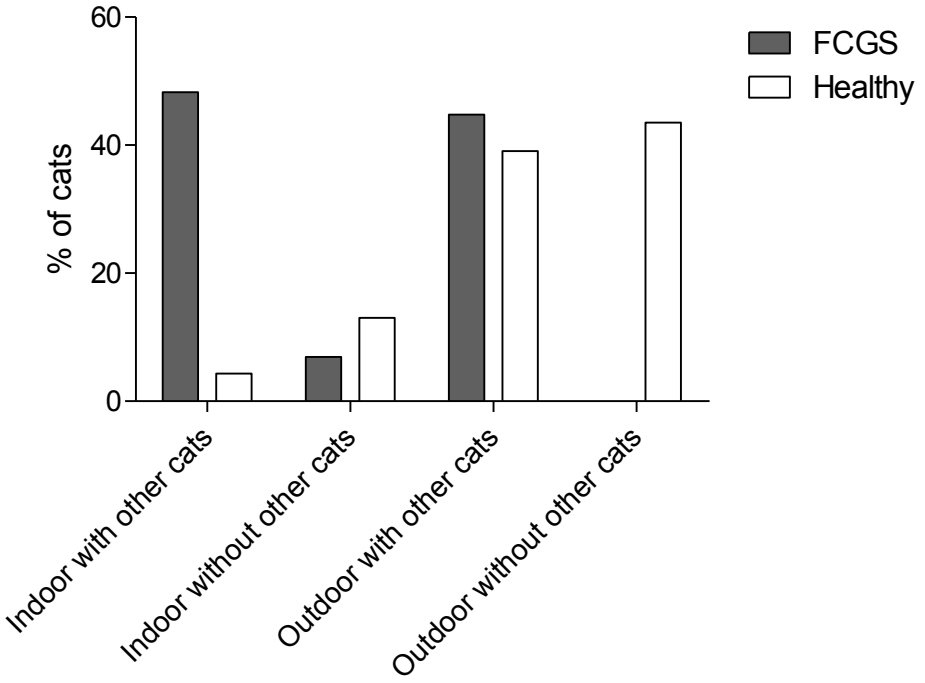


Figure 8.5: Percentage of cats with and without FCGS able and unable to roam outdoors.



Indoor: cats unable to roam outdoors, outdoor: cats able to roam outdoors

Figure 8.6: Percentage of cats able and unable to roam outdoors, with or without other cats in the household.

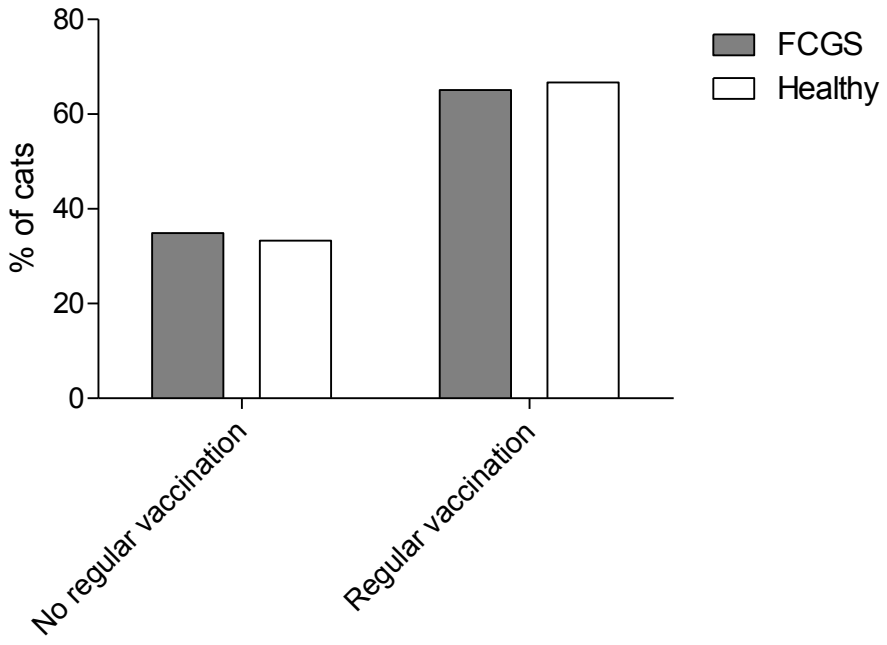


Indoor: cats unable to roam outdoors, outdoor: cats able to roam outdoors

8.3.2.4 Vaccination status

When the vaccination status was compared in 63 cats with FCGS and 21 healthy cats, no difference was seen. Forty-one (65.1%) of the cats with FCGS and 14 (66.7%) of the healthy cats received regular vaccinations (Figure 8.7).

Figure 8.7: Percentage of cats with and without FCGS receiving regular vaccinations.



8.4 Discussion

In the cats used for sample collection and those included in the questionnaire results, no difference was seen in breed between the healthy and FCGS groups. A higher percentage of purebred cats was seen in the FCGS group but no significant difference was found. Suggestions that certain breeds are predisposed for FCGS have been made (Frost and Williams, 1986; Diehl and Rosychuk, 1993) but this was not supported in a study by Healey et al. (2007). Also, no link between FCGS and age or sex could be shown in the current study.

It is possible that stress might play a role in the aetiopathogenesis of FCGS. An unpredictable captive environment has been shown to elevate urine cortisol concentration in cats (Carlstead et al., 1993). Animals that are not able to perform natural behaviour have higher stress levels. Idiopathic cystitis is an example of a disease in cats that has been studied intensively for its association with environmental stress factors (Cameron et al., 2004; Westropp et al., 2006; Defauw et al., 2011).

From the questionnaire results it was found that a significantly higher proportion of the cats with FCGS were living in the company of other cats when compared to the healthy group. Also, a difference was seen between cats confined to staying indoors and having the possibility to go outdoors, with a higher proportion of the cats in the FCGS group being indoor cats. When these two factors which can be seen as stress factors in the cats' environment (Amat et al., 2009) were taken together, a clear division was seen. The proportion of cats kept outdoors while living with other cats in the household was similar in both groups. A difference was seen in the large proportion of cats with FCGS that were held indoors, together with other cats, compared to the healthy cats where the largest group were outdoor cats from a single-cat household.

We can also speculate that if these stress factors are part of the aetiopathogenesis of FCGS, the higher proportion of purebred cats that is seen in the FCGS group is connected to the possibility that these cats are often held indoors and possibly live more often with other cats in the household. This should however be investigated further and a more in-depth epidemiological study is therefore required.

Assessment of the diet covers a large part of the questionnaire. As described in Section 1.3.1.1 diet may be of importance in the development of FCGS. All cats in the current study have at least a mild form of periodontal disease and as described before, diet can play a role in preventing the accumulation of plaque. Dried foods reduce plaque

accumulation when compared to wet foods. Another possibility is the presence of hypersensitivity towards certain food additives. Therefore a thourough investigation into the feeding history of cats with FCGS is of importance.

Not all the answers from the questionnaire have been analysed in this chapter. A greater number of completed questionnaires are needed to be able to carry out more extensive statistical analyses. Answers to questions with too many variables could not be analysed because of low numbers. Some of the questions in the current questionnaire could be modified. As far as diet is concerned, it is important to specifically ask what the cat was being fed at the time FCGS was diagnosed. The same should be the case for dental prophylaxis, since these factors are likely to change as oral disease progresses. In addition to checking for other cats in the household, it would also have been interesting to check for the presence of dogs in the household and if there were any other cats or dogs in the close neighbourhood. A modified version of the questionnaire is produced and results are currently being collected.

The data presented represents preliminary results of a study that has the potential to help us better understand the possible aetiopathogenesis of FCGS. More questionnaires should be analysed in order to make a wider statistical analysis possible. More questionnaires are being collected to expand the data for this study.

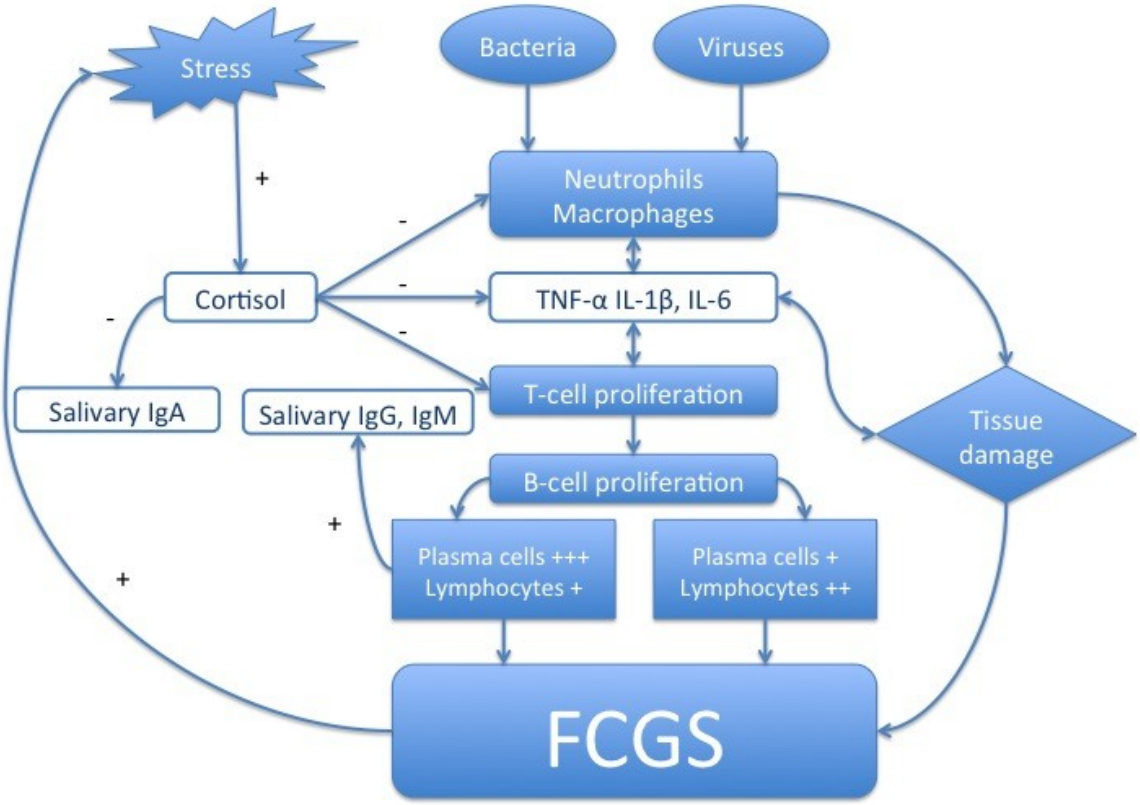
Chapter 9 General discussion

FCGS is a severe and frustrating disease, which unfortunately has been very poorly described in literature. It is not only a painful and stress-inducing disease for the cat but because of the poor response to different treatment methods, it can be very worrying and challenging for owners. Studies have investigated possible causes of FCGS but the exact aetiopathogenesis remains unclear. Implicated pathogens are bacteria and viruses but immune-related causes have also been suggested. In the current study different aspects of FCGS and possible links with the immune system were investigated in an attempt to improve our knowledge of the aetiopathogenesis of FCGS. A summary of the factors that may contribute to the development of FCGS is shown in Figure 9.1.

The clinical definition of FCGS has been poorly described as pointed out in Section 1.2.1 of this thesis. The distribution of lesions in the cats studies for this thesis has been recorded by a board certified specialist in veterinary dentistry. Similar to the description in more recent studies (Hennet et al., 2011), inflammation of the tissue lateral from the palatoglossal folds, and the attached gingiva is described in all cats in the current study and should therefore be included in the definition of the disease. The severity scale used in the study with distributions of the lesions is a good guideline for further clinical trials when a scale is needed to grade improvement of clinical signs.

Previously, a connection between FCGS and signalment factors such as age and breed have been suggested (Frost and Williams, 1986; Diehl and Rosychuk, 1993; Healey et al., 2007). In Chapter 3 of this thesis, these possible risk factors were taken into consideration. A relatively high percentage (18%) of affected animals were purebred cats, compared to what is expected in a normal population (Healey et al., 2007). Since the number of cats was relatively low, the signalment was also recorded via a questionnaire-based study. No significant differences between 25 healthy and 72 affected cats were shown for age, breed and sex and no evidence was found for a role of any of these factors in the disease aetiopathogenesis. Therefore it seems unlikely that a simple genetic factor is involved in the aetiopathogenesis. However, it has been suggested that stress may play a role in the aetiopathogenesis of FCGS (Diehl and Rosychuk, 1993) and this is where the suggested higher presence of FCGS in purebred cats may be of importance - purebred cats are often held indoors and commonly in combination with other cats, which could be a reason for a higher prevalence.

Figure 9.1: A model for FCGS aetiopathogenesis



In the current study it was shown that stress factors may be of importance in the development of FCGS. The presence of other cats and the inability to roam outdoors are thought to be stress inducers in the cat (Amat et al., 2009; Herron, 2010). Preliminary data from the questionnaire designed in the current study shows that a significantly higher number of cats with FCGS had no access to outdoors and significantly more cats with FCGS were from multi-cat households compared to the healthy group. Although this is suggestive of stress-inducing factors playing a role, a large-scale epidemiological study is required in order to gain a better insight as to how these factors are possible contributors to the development of FCGS (Figure 9.1). A decrease in salivary IgA has been shown in cats with FCGS (Harley et al., 2003a). A possible cause for this is cortisol increase during stress. Several stress-factors can play a role however, stress caused by FCGS itself, should not be overseen. Clinical measurements of cortisol and cathecholamine levels in serum, saliva or urine are good parameters for stress, however the reason of an increase needs also be investigated. Measuring these clinical parameters in healthy cats and diseased cats would ideally be accompanied by measurements after changing the possibly stress-factors.

Viruses that have previously been implicated in the aetiopathogenesis of FCGS (FeLV, FIV, FHV-1 and FCV) were assessed in the current study (Chapter 4). Although previous studies have investigated the presence of feline viruses in FCGS (White et al., 1992; Lommer and Verstraete, 2003; Quimby et al., 2008), conflicting data on the presence of viruses in cats with FCGS has been published. Most studies show the prevalence of FCV in cats with FCGS to be above 50% but the prevalence of other viruses varied widely (Knowles et al., 1989; Bellei et al., 2008). As seen in other studies, the prevalence of FCV is significantly higher in cats with FCGS when compared to the healthy group in the current study. This concurs with the hypothesis that FCV carriage is associated with FCGS (Thompson et al., 1984). The question remains in what way FCV plays a role in inducing the lesions typically seen in cats with FCGS. It has been suggested that only certain strains of FCV would induce FCGS, this however could not be confirmed when SPF cats were infected with FCV strains isolated from cats with FCGS (Knowles et al., 1991; Reubel et al., 1992). Severe inflammation lateral to the palatoglossal folds could be induced but was self-limiting in all cases. FCV shedding and FCGS is well correlated, but a number of cats with FCGS lesions did not test positive for FCV in the current study. FCV seems not to be the only factor involved in the aetiopathogenesis but must be considered as playing a major role.

The potential effect of viruses on the host immune response to FCGS is discussed in Chapter 7. The FCGS group showed a significant increase in the TLR7 mRNA gene expression. The immune response is reacting to a viral stimulus since TLR7 recognises

single-stranded RNA, which is a common feature of viral genomes. However, when the healthy and diseased cats were taken as a total and divided according to the presence or absence of FCV, TLR7 gene expression was not significantly increased in the FCV group. This could be explained by the possibility of false negative FCV test results in the FCGS population. A higher expression of IL-1ß, IL-6 and IFN-γ is also seen in the FCV positive cats. The ability of FCV to stimulate an immune response could be investigated using a cell-culture based *in vitro* model, although such a model cannot examine the complex immune response created by pathogens *in vivo*. The immune response seems to react to the presence of the virus and more research on this subject will possibly help to explain how the presence of FCV influences the development of FCGS.

Bacteriological studies on samples of cats with or without oral diseases have previously focussed on standard culture methodology or on the detection of pre-selected bacteria (Mallonee et al., 1988; Love et al., 1989; Norris and Love, 1999b; Booij-Vrieling et al., 2010). In this thesis, culture-independent methods have been used that enable the identification of a much wider range of bacteria present in the feline oral cavity. 16S rRNA gene sequencing has been used previously to identify bacteria in a variety of diseases and is a proven method to identify uncultivable bacteria (Hutter et al., 2003; Siqueira and Rocas, 2005; Woo et al., 2008).

The culture-independent technique used in this study, 16S rRNA gene cloning and sequencing, was used in tandem with bacterial culture to optimise bacterial detection. The bacteria identified by 16S rRNA gene sequencing were not all identified by culture methods. Fastidious species are not able to grow when standard culture methods as used in the current study are performed. Conversely, some bacteria isolated by culture methods were not detected by 16S rRNA gene sequencing. One possible reason for this is primer bias, which can occur if an unequal amplification of PCR product results in a higher amplification of some bacterial DNA and under-representation of other species (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). Therefore the combination of culture-dependent and culture-independent methods gives the most complete view of the bacterial oral flora.

The obtained 16S rRNA gene sequences were compared to known sequences in the online public access database and identified by matching with the database sequence that gave the highest percentage identity. In some cases, matches occurred with more than one database sequence, which prevented a totally accurate bacterial identification. This is one of the few limitations of 16S rRNA gene sequencing, which is unable on occasion to distinguish between species with very similar 16S rRNA gene sequences (Woo et al., 2009). The percentage identity required with a database

sequence for an unknown sequence to be matched and identified as the corresponding bacterial species varies, with values between 97% and 99.5% having been used (Stackebrandt and Goebel, 1994; Janda and Abbott, 2007) Percentage identity values below this cut-off point are highly indicative of potentially novel phylotypes, which can be confirmed as such by sequencing of the entire 16S rRNA gene. In the current study, a cut-off value of 98% was used.

On the basis of the results from this study, the most suitable antibiotic therapy would be cefavecin. Antibiotic treatment is not the first choice of treatment however antibiotics are often useful in cases with periodontal disease before surgical treatment. *P. multocida* is sensitive to cefovecin as well as a wide variety of anaerobes. Another reason for cefovecin to be a good choice of treatment is the injectable formula that acts up to 14 days, as tablets or capsules often result in reduced owner compliance in cats with a painful oral cavity.

While Sanger 16S rRNA gene sequencing has been a valuable tool in aiding our understanding of the bacteria associated with oral diseases, it gives limited coverage of the oral microbiome due to the small number of clones (usually 50) sequenced from the clone libraries generated for each sample. Recent rapid advances in gene sequencing technologies have occurred with the advent of high throughput (next generation) sequencing (Pozhitkov et al., 2011). This approach can give millions of gene sequencing reads per sample and at a fraction of the cost of Sanger sequencing. Therefore future studies on the oral microbiome associated with FCGS and feline oral health should utilise high throughput sequencing to obtain the most accurate assessment of the bacteria that are important in oral health and disease. Consequently this will provide new information on the use of antimicrobial therapy and the possible pathogens that play a role in FCGS.

Since cats with FCGS can initially improve with anti-bacterial treatment (Frost and Williams, 1986), the involvement of bacteria in the disease is highly likely. In the present study a change was identified in the composition of the phyla between cats with FCGS and healthy cats. The microbes in the oral cavity are known to interact with each other and form a complex microflora (Kuramitsu et al., 2007). An imbalance of the bacterial flora is likely to occur in FCGS. Two of the bacterial species that were often identified in cats with FCGS, *T. forsythia* and *P. circumdentaria*, also appeared to be involved in stimulating a host immune response. In samples in which these bacteria were present, a significant increase in mRNA expression of TLR and cytokine genes was seen. TLR2 was significantly increased in the cats harbouring *T. forsythia* or *P. circumdentaria*. Additionally, cats harbouring *T. forsythia* showed a significant increase

in the expression of TLR4, TLR7, TLR9, TNF-α and IL-1β genes when compared to cats where this microorganism was not identified. *P. multocida* species failed to show a change in the immune response although they were found in high numbers in cats with FCGS. However, these bacteria were also commonly present in the healthy oral cavity. There is a strong possibility that lesions initiated by FCV are colonised by bacteria that are present in the oral cavity and which will cause further tissue damage. The presence of pathogenic bacteria such as *T. forsythia* and *P. circumdentaria* is likely when concurrent periodontal disease is present, and the current study showed that non-pathogenic bacteria as well as pathogenic bacteria were able to colonise the lesions.

In Chapter 6 of this thesis, samples were analysed on a histopathological basis to assess cell infiltrate and severity of disease. Two infiltration types could be identified on this basis. The first is a plasmacytic and lymphocytic infiltrate and the second is a mainly plasmacytic infiltrate. It could be speculated that these represent a different phase of development of the disease; however no difference in duration of the disease was seen when the two cell infiltrates were compared. This explanation therefore seems unlikely but it has to be realised that the time since onset of the disease is not an exact measure. This factor is variable and dependent upon the cat owners' interpretation. Often a disease will only be addressed when serious clinical signs occur i.e. when the cat stops eating or grooming. However a significant correlation was shown between the severity grade of the histopathology and the cell-type infiltrate, which suggests the possibility that the cell-type groups represent different phases of FCGS development. The more severe cases of FCGS did show a predominantly plasmacytic infiltrate, compared to a combined plasmacytic lymphocytic infiltrate in less severe cases. It is not certain if the severity reflects the duration of the disease; however if this was the case and severe pathology indicated long-standing disease, it is tempting to suggest that the bacterial colonisation is indeed secondary since TLR4 mRNA expression, mostly known for recognising LPS, was significantly higher in the more severe cases.

Plasma cell infiltrates are often seen in diseases with an immune-mediated background (Perry et al., 1997; Dias Pereira and Faustino, 2003). It has been suggested that the high salivary levels of immunoglobulins in cats with FCGS can be caused by the high plasma cell concentration within the oral tissues The most predominant plasma cells in cats with FCGS are of the IgG type (Harley et al., 2011) The salivary IgG concentration in cats with FCGS is much higher than in healthy cats and a moderately higher IgM concentration has also been shown (Harley et al., 2003b). However, it is not known how these concentrations change according to disease severity.

In the current study cytokine and TLR mRNA gene expression levels were investigated for each cat with FCGS and compared to a group of healthy cats Previously, only cytokine expression in cats with FCGS was assessed by reverse transcriptase PCR (Harley et al., 1999); expression of cytokines IL-2, IL-4, IL-6, IL-10, IL-12 and IFN-γ was significantly higher in cats with FCGS when compared to a group of healthy cats. TLR gene expression has not previously been assessed in cats with FCGS.

Significantly higher gene expression in FCGS samples was seen for TLR2 and TLR7 and for the cytokines TNF-α, IFN-γ, IL-1β and IL-6 in the present study. This suggests an immune reaction in the oral mucosa that may be stimulated by a combination of bacterial and viral agents. Moreover, in cats harbouring *T. forsythia* and *P. circumdentaria* gene expression of several TLRs and cytokines was increased suggesting that these bacteria are pathogenic, compared to bacteria found extensively in the healthy oral cavity such as *P. multocida* species, that did not show an obvious immune response. This strengthens the suggestion that a secondary bacterial colonisation of lesions is playing a part in FCGS, and when no pathogenic bacteria are present the lesions still persist. To investigate the influence of bacteria and viruses on the feline immune response, future studies could use feline cells or cell lines stimulated with different pathogens. It would be useful to analyse in particular, gene expression of the Th17 family of cytokines, which have been shown to be important in the immune response to human oral diseases (Gaffen and Hajishengallis, 2008). However, no gene sequences for the feline Th17 cytokine family are currently available.

So far no treatment method for FCGS has proven successful. In a multi-factorial disease it is a challenging task to address different initiating factors in order to successfully treat the disease. So far treatments that have given the highest success rate have been influencing different factors in addressing bacterial burden by hygienic measures in combination with either glucocorticoid administration or treatment against FCV with IFN-ω (Hennet et al., 2011). The treatment to reduce the bacterial load in the oral cavity shows the importance of the role bacteria play in the disease aetiopathogenesis. Periodontal disease was present in all cases of FCGS seen in the current study. Periodontal disease provides a source of pathogenic bacteria, which can cause secondary infections. Eliminating this source by removing all affected teeth, even those with mild gingivitis, has been one of the most successful treatments so far (Hennet, 1997; Girard and Hennet, 2005; Bellei et al., 2008). In the current study, it was shown that the lesions can be colonised by different bacteria, pathogenic and non-pathogenic, and the immune reaction reflects the influence of a combination of bacteria and viruses. Also, when initiating treatment, it is important to assess environmental factors particularly with a view to reducing any possible stress situations. Further investigations

into the immune response could lead to the development of immune therapy. It is important to assess the possibility of an inappropriate immune response. Immune modulating drugs like glucocorticoids, in an anti-inflammatory dosage, give a good clinical improvement (Hennet et al., 2011). Other immune modulators could be tried for the treatment of FCGS. Recently ciclosporin was registered for cats with atopic dermatitis. Ciclosporin has been shown to decrease the expression of IL-2, IL-4, IFN-γ and TNF-α in feline mononuclear cells (Kuga et al., 2008). It is of great importance that the involvement of pathogenic agents is ruled out or eliminated before the start of any immunomodulating therapy. The different factors involved in the aetiopathogenesis should be targeted including stress and pathogens. IFN-ω treatment is currently being used (Mihaljevic and Klein, 1998; Mihaljevic, 2003; Southerden and Gorrel, 2007; Hennet et al., 2011), against FCV infection in FCGS. Targeting specific TLRs with agonists or antagonists to activate or partly de-activate the response to certain pathogens could be investigated further (O'Neill et al., 2009). More specific research into the immune response against the variety of pathogens is needed. Inhibiting or activating the immune response could raise potential concerns and targeting one TLR by inhibition could influence other TLRs through their pathways.

The work in this thesis adds valuable information to the current knowledge on the aetiopathogenesis of FCGS. It provides information on possible predisposing factors and agents that may be of importance and contribute to the development of FCGS. This thesis is an important step towards a better understanding of the aetiopathogenesis of FCGS and provides a basis for future studies on this important feline disease.

Appendix

QUESTIONNAIRE FELINE CHRONIC GINGIVITIS STOMATITIS

Feline Chronic Gingivitis Stomatitis (FCGS) is an extremely painful and frequently seen condition. It is often frustrating to treat with little understood about its underlying aetiology. This study aims to investigate the initiating causes and putative risk factors with the goal of improving treatment and preventive measures. The objective of this questionnaire is to try and identify possible risk factors by comparing affected and unaffected cats. We would therefore appreciate your cooperation in always including a completed questionnaire for an unaffected cat with that of the affected cat.

QUESTIONNAIRE INSTRUCTIONS

- Thank you for taking the time to fill this out. It will require input from both the veterinary surgeon/nurse and the owner but we very much appreciate your willingness to help.
- It will take approximately 15 minutes to complete each questionnaire (an affected and an unaffected/ control cat)
- ***Please select affected cats who:***
  - ***are older than 18 months***
  - ***are showing signs of this condition i.e. persistent /severe inflammation of the oral/pharyngeal/lingual mucosa, extending beyond attached gingiva of teeth.***
  - ***are undergoing GA for treatment for these condition***
- ***Please select unaffected cats who:***
  - ***Are older than 18 months***
  - ***Have no oral inflammation (mild inflammation of the gingival margin is acceptable)***
- Fill in this questionnaire **using the clinical records** available for this cat.
- Please try to answer **all** questions
- If you have any questions/queries, there are contact details at the end of the questionnaire.
- Please send this questionnaire to the following address:

Glasgow Dental Hospital & School
Sanne Dolieslager, MacFarlane Room, level 9
378 Sauchiehall Street
Glasgow
G23JZ

PLEASE WRITE TODAY'S DATE

Day	Month	Year

YOUR CLINIC REFERENCE

Owner name	Practice reference no

PRACTICE DETAILS

Name	
Address	
Postcode	
City	
Telephone	

Questionnaire Feline Chronic Gingivitis Stomatitis

SECTION 1

Signalment

1.1 Age ____ years ____ months

1.2 Breed *(please specify)* ____

1.3 Sex *(please tick the appropriate box)*

Male entire	Male neutered	Female entire	Female neutered

1.4 Weight *(if known)* ____ kg

1.5 Body Condition Score *(please tick appropriate box)*

1. Emaciated ☐
2. Thin ☐
3. Moderate ☐
4. Overweight ☐
5. Obese ☐

1.6 When was this cat first seen by your practice?

Day	Month	Year

SECTION 2

Environment

2.1 Where did the owner acquire the cat *(please tick appropriate box)*

- Breeder ☐
- Kitten from single cat household ☐
- Kitten from a multicat household ☐
- Shelter ☐
- Don't know ☐
- Other *(please specify below)* ☐

____

2.2 Is the cat: *(please tick appropriate box)*

Indoor	Outdoor	Indoor/Out door	Don't Know

2.3 Are there other cats in the household? *(please tick appropriate box)*

Yes	No	Don't know

If Yes :
how many other cats are in the household? ____

Have any of these cats ever been in a rescue/re-homing facility

Yes	No	Don't know

2.4 Are there people in the household smoking? *(please tick appropriate box)*

Yes	No	Don't know

2.5 Please write down any recent changes in the cats environment e.g. Moving house, new furniture, re-decoration, different floor/furniture cleaner, new pets, change in members of the household, new neighbour incl. pets etc.

Change	Date

2.6a What is the owners **country**?
2.6b What is the owners **postcode**?

2.7 In what environment has the cat been most of the time (please tick appropriate box)

Rural	Urban	City	Don't Know

2.8 Is the cat fed on a dry commercial diet? *(please tick appropriate box)*

- Every day
- 4-6 times a week
- 2-3 times a week
- Once a week
- 1-4 times a month
- Never

2.9 Is the cat fed on a wet commercial diet? *(please tick appropriate box)*

- Every day
- 4-6 times a week
- 2-3 times a week
- Once a week
- 1-4 times a month
- Never

2.9a In what form is the wet food presented? *(please tick appropriate box)*

- Pouch
- Foil Tray
- Can with poplid
- Can with ordinary lid

2.10 Is the cat fed on a raw/homemade diet? *(Please tick appropriate box)*

- Every day
- 4-6 times a week
- 2-3 times a week
- Once a week
- 1-4 times a month
- Never

2.11 What are the usual food brands the cat receives?

2.12 How many times a day is the cat fed? (*Please circle appropriate answer*)

1x	2x	3x	4x or more	Ad libitum	Don't know

2.13 Does the cat receive food treats or any nutritional supplements? (*Please tick appropriate box)*

Yes	No	Don't know

If **YES** please specify

2.14 What does the cat drink? (*Please tick appropriate boxes. More than one answer possible)*

- Tapwater
- Rainwater
- Cow milk
- (Whiskas) Cat Milk
- Evaporated milk
- Other (*please specify below)*

2.15 Does the cat hunt and catch prey (birds, mice etc) *(Please tick appropriate box)*	Yes	No	Don't know

If **YES** ; please specify all known prey species.

2.16 What type of litter is used in the litter tray?

SECTION 3

Preventive health

3.1 When were cat wormers last given to the owner? (Day/Month/Year) *(Leave blank if unknown)*	Day	Month	Year

3.2 What kind of wormers have been used by the owner? *(please tick appropriate box)*

- Spot on ☐
- Paste ☐
- Tablets ☐
- Injection ☐
- Other *(please specify below)* ☐

Please give name of any products used if known:

3.3 Does the owner give wormers from other sources e.g pet shops , internet etc	Yes	No	Don't know

3.4 When were cat flea products last given to the owner? (*leave blank if unknown)*	Day	Month	Year

3.5 What kind of flea products were given to the owner? *(please tick appropriate box)*

- Spot on ☐
- Tablet ☐
- Spray ☐
- Injectable ☐
- Other *(please specify below)* ☐

Please give names of any products used if known:

3.6 Does the owner give flea products from other sources e.g pet shops , internet etc	Yes	No	Don't know

3.7 Does the cat regularly receive a vaccine (e.g every 12–18 months)?	Yes	No	Don't know

If NO : please write the date of the last known vaccination	Day	Month	Year

3.8 What vaccine(s) types has the cat received previously? *Please write the date of last vaccination (if known) next to the relevant vaccine name*

Feligen RCP		Katavac Eclipse		Nobivac Rabies		Purevax RCPFeLV	
Felocell CVR		Leucogen		Nobivac Tricat		Quantum Cat CVRP	
Fevaxyn FeLV		Leukocell 2		Purevax FeLV		Quantum Cat FeLV	
Fevaxyn iCHP		Nobivac BB for cats		Purevax RC		Quantum Rabies	
Fevaxyn iCHPChlam		Nobivac Ducat		Purevax RCP		Rabisin	
Fevaxyn Pentofel		Nobivac FeLV		Purevax RCPCh		Other (please specify)	
Katavac CHP		Nobivac Forcat		Purevax RCPCh FeLV			

SECTION 4

Dental prophylaxis

4.1 What dental prophylactic measures are carried out regularly? *(please tick all that apply)*

- **NONE** ☐
- Toothbrushing ☐
- Chlorohexidine solution/paste ☐
- Dental treats/chews ☐
- Toothpaste minus brushing ☐
- Prescription dental diets (*if yes please answer 4.1a*) ☐
- Other (please specify below) ☐

_____

Since what date has the prophylactic measure been used? _____

4.1a If a dental prescription diet is given, what is the brand of this diet? *(please tick appropriate box)*

- Hill's T/D ☐
- Royal Canin Dental ☐
- Other (*please specify below*) ☐

_____

SECTION 5

Duration

5.1 Since when has the cat signs of FCGS? *(please write date of onset)*

Clinical Signs

5.2 What clinical and behavioural signs is the cat presently showing? *(please tick the appropriate box for each sign)*

- Anorexia/Inappetance ☐
- Excessive salivation ☐
- Halitosis ☐
- Dysphagia ☐
- Change in fur/coat ☐
- Pawing at mouth ☐
- Weight loss ☐
- Vomiting/Retching ☐
- Changes in grooming behaviour ☐
- Pyrexia ☐
- Change in drinking behaviour ☐
- Local facial swelling ☐
- **NONE** ☐

5.2a Please tick the statement(s) which best describes the **cat's appetite**?

- Eating normally ☐
- Not eating ☐
- Eating more than normal ☐
- Eating less than normal ☐
- Eats wet food on his/her own but can't eat dry food ☐
- Eats dry food but does not eat wet food ☐
- Eats only pureed food, or only eats when hand fed ☐
- Don't know ☐

5.2b Pleas tick the statement(s) which best describes the **cat's drinking behaviour**?

- Drinking normaly ☐
- Drinking less than normal ☐
- Drinking more than normal ☐

5.2c Please tick the statement(s) which best describes the **cat's grooming behaviour**?

- Grooming normally ☐
- Grooming excessively ☐
- Grooms occasionally but not as much as before ☐
- Will not groom ☐
- Don't know ☐

5.2d Please tick the statement(s) which best describes the **cat's toilet behaviour**?

- Normal toileting ☐
- Refuses to go outside as before ☐
- Change in location within the house ☐
- Missing the litter tray ☐
- Change in frequency ☐
- Don't know ☐

5.2e Please tick the statement(s) which best describes changes in the **cat's personality**?

- No changes in personality ☐
- More friendly than normal ☐
- More shy/timid than normal ☐
- More nervous/anxious than normal ☐
- More aggressive than normal ☐
- Other *(Please specify below)* ☐

5.2f Please tick the statement(s) which best describes the **cat's activity level**?

- Normal activity level ☐
- Plays/interacts spontaneously but not as frequently as before. ☐
- Low activity level but will play occasionally when engaged by people or other pets ☐
- No interest in people or pets, spends much of the time asleep. ☐
- Don't know ☐

5.3 Does the cat display any unusual behaviour/habits which you think may be associated with oral disease? E.g eating soil, chewing plants etc

Yes	No	Don't know

If YES; please state behaviours/habits

5.4 Does the owner think the cat is in discomfort? Please tick the statement which applies.

- No discomfort ☐
- Mild discomfort ☐
- Moderate discomfort ☐
- Severe discomfort ☐
- Don't know ☐

SECTION 6

Signs on physical exam

6.1 What signs were detected on initial physical exam? *(please tick all that apply)*

- **Unable to carry out physical exam** ☐
- Hyperaemia ☐
- Inflammation→swollen/red/warm/pain ☐
- Oral ulceration ☐
- Pain when attempting to open mouth during exam ☐
- Enlarged lymph nodes ☐
- Enlarged tonsils ☐
- Grossly visible plaque ☐
- Calculus ☐
- Other (please specify below) ☐

SECTION 7

History

7.1 Has the cat received any previous treatment in the last 18 months (for any condition including FCGS) e.g. antibiotics/anti inflammatories/antivirals?

Yes	No	Don't Know

If NO: Go to **Question 7.4**

If YES:

7.2 Please write in all antibiotics/antivirals used in the last 18 months (Include date given/prescribed, dosage, injectable or tablet/suspension,and length of treatment)

Date	Medication	Dosage	Duration

7.3 Please write in all antinflammatories used in the last 18 months. (Include date given/prescribed, dosage, injectable or tablet/suspension,and length of treatment)

Date	Medication	Dosage	Duration

7.4Please write in any other drugs/treatments used in the last 18 months. (Include date given/prescribed, dosage, injectable or tablet/suspension,and length of treatment)

Date	Medication	Dosage	Duration

7.5 Has the cat been diagnosed/suspected to have any concomitant illness/injuries in the last 18 months?	Yes	No	Don't Know

If YES:
Please give brief details of suspected diagnoses with dates

Date	Diagnosis

7.6 Has the cat had any previous dental work?	Yes	No	Don't Know

If YES, give details of when and what procedures have been carried out ?	

7.7 Has the cat ever been tested for **FIV** (to your knowledge) ?	Yes	No

If **Yes,** what test (s) were used and what was the result (s)? (*Please circle appropriate answer (s)*)

	Please write in test name and lab used if known	**Date of test**	**Result**	
In house kit			Positive	Negative
External lab test			Positive	Negative

7.8 Has the cat ever been tested for **FeLV**?	Yes	No

If **Yes,** what test (s) were used and what was the result (s)? *(Please circle appropriate answer (s))*

	Please write in test name if known	Date of test	Result	
In house kit			Positive	Negative
External lab test			Positive	Negative

7.9 Has the cat ever been tested for Feline Corona virus? | Yes | No |

If **YES**: please give the date of sampling and results

7.10 Has the cat ever been tested for Bartonella? | Yes | No |

If **YES**: please give the date of sampling and results

7.11 Has any viral isolation, PCR or bacterial culture been carried out on the oral cavity? | Yes | No |

If **Yes :** please give the date of sampling and results

SECTION 8

Oral Exam under GA

8.1 Tick the box which best reflects the average grade of **dental calculus** present

Grade 0 None ☐
Grade 1 Supragingivial with slight subgingival ☐
Grade 2 Moderate subgingival ☐
Grade 3 Abundant supragingivial and/or subgingivial ☐

8.2 Tick the box which best reflects the average grade of **dental plaque** present

Grade 0 None ☐
Grade 1: Mild ☐
Grade 2: Moderate ☐
Grade 3: Severe ☐

8.3 Tick the box that best describes **the severity of the inflammation** listed below

	None	Mild reddened	Moderate Severe congestion	Severe Ulceration/Proliferation/granulation
Maxillary buccal mucosal inflammation				
Mandibular buccal mucosal inflammation				
Maxillary attached gingival inflammation				
Mandibular attached gingival inflammation				
Inflammation lateral to palatoglossal folds				
Molar salivary gland inflammation				
Oropharyngeal inflammation				
Lingual and/or sublingual inflammation				

8.4 Tick the box that best describes the tonsillitis present

None	☐
Mild	☐
Moderate	☐
Severe	☐

8.5 Tick the box that best describes the haemorrhage from the oral mucosa when gently swabbed/probed

Grade 0 None	☐
Grade 1 Some bleeding	☐
Grade 2 Spontaneous bleeding without swabbing	☐

8.6 Tick the box which best describes the **submandibular lymph nodes**

Grade 1 Normal	
Grade 2 Left or right enlarged (*please circle which one)*	**R L**
Grade 3 Both enlarged	

8.7 Were any FTRLs (Feline Tooth Resorptive Lesions) detected on exam?	Yes	No
8.8 If yes, how many are present?		
8.9 Is there any other oral pathology present?	Yes	No

If **YES** please specify

8.10 Was dental radiography carried out?

- ☐ No
- ☐ Yes on all teeth
- ☐ Yes on some teeth

If **YES** please state any abnormalities

8.11 Is periodontal disease present? (*Please tick appropriate boxes*)

- ☐ Grade 0 Healthy
- ☐ Grade 1 Gingivitis
- ☐ Grade 2 Initial
- ☐ Grade 3 Moderate
- ☐ Grade 4 Severe

Please specify the periodontal disease below

Are periodontal pockets present?	Yes	No
Is Gingival hyperplasia or recession present	Yes	No
Is furcation exposure present	Yes	No
Is there tooth mobility?	Yes	No

8.12 Use the diagrams below to mark on the following lesions/problems.

Inflammation/Ulceration	*Draw as a hatched area on* ***Diagram 1***
FTRLs (Feline Tooth Resorptive Lesions)	*Circle affected tooth on* ***Diagram 2***
Missing/Extracted teeth	*Cross over missing tooth* ***Diagram 2***

DIAGRAM 1

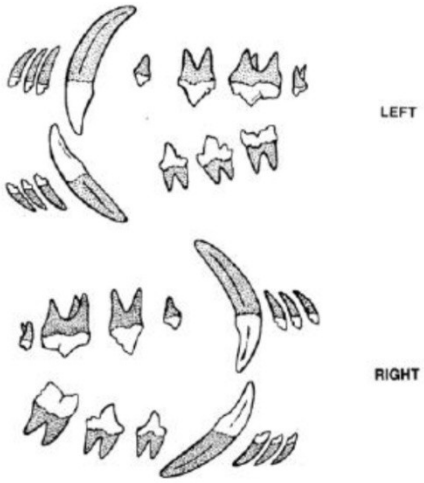


DIAGRAM 2

For advice/help/information about any aspect of the questionnaire, please email Sanne Dolieslager: s.dolieslager.1@research.gla.ac.uk (0141) 2119762

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[Available http://www.avdc.org/dental-charts.pdf] (Accessed 1/3/08)

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