Use of unbiased metagenomic and transcriptomic analyses to investigate the association between feline calicivirus and feline chronic gingivostomatitis in domestic cats

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OBJECTIVE
To identify associations between microbes and host genes in cats with feline chronic gingivostomatitis (FCGS), a debilitating inflammatory oral mucosal disease with no known cause, compared with healthy cats and cats with periodontitis (control cats).

ANIMALS
19 control cats and 23 cats with FCGS.

PROCEDURES
At least 1 caudal oral mucosal swab specimen was obtained from each cat. Each specimen underwent unbiased metatranscriptomic next-generation RNA sequencing (mNGS). Filtered mNGS reads were aligned to all known genetic sequences from all organisms and to the cat transcriptome. The relative abundances of microbial and host gene read alignments were compared between FCGS-affected cats and control cats and between FCGS-affected cats that did and did not clinically respond to primary treatment. Assembled feline calicivirus (FCV) genomes were compared with reverse transcription PCR (RT-PCR) primers commonly used to identify FCV.

RESULTS
The only microbe strongly associated with FCGS was FCV, which was detected in 21 of 23 FCGS-affected cats but no control cats. Problematic base pair mismatches were identified between the assembled FCV genomes and RT-PCR primers. Puma feline foamy virus was detected in 9 of 13 FCGS-affected cats that were refractory to treatment and 5 healthy cats but was not detected in FCGS-affected cats that responded to tooth extractions. The most differentially expressed genes in FCGS-affected cats were those associated with antiviral activity.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested that FCGS pathogenesis has a viral component. Many FCV strains may yield false-negative results on RT-PCR–based assays. Coinfection of FCGS-affected cats with FCV and puma feline foamy virus may adversely affect response to treatment. (*Am J Vet Res* 2021;82:381–394)

Feline chronic gingivostomatitis is a chronic inflammatory mucosal disease that affects 0.7% to 12% of domestic cats.[1–3] Cats with FCGS typically develop extensive inflammatory lesions throughout the oral cavity, especially in the area lateral to the palatoglossal folds.[1,4] These lesions are often associated with severe signs of pain and altered behavior such as hyporexia, poor grooming, and irritability and can be life-threatening if unresolved.[1,5,6] Some cats with FCGS are euthanized because of insufficient response to or lack of resources for treatment. Despite the severity and prevalence of FCGS in the domestic cat population, the etiology of the disease remains elusive.

ABBREVIATIONS

FCGS	Feline chronic gingivostomatitis
FCV	Feline calicivirus
FFV	Feline foamy virus
GBP	Guanylate-binding protein
KCNB2	Potassium voltage-gated channel subfamily B member 2 gene
LIMK2	LIM domain kinase 2 gene
mNGS	Metatranscriptomic next-generation RNA sequencing
MSC	Mesenchymal stem cell
NCBI	National Center for Biotechnology Information
nr	RNA database
nt	Genomic database
PFFV	Puma feline foamy virus
RT-PCR	Reverse transcription PCR

Current evidence suggests that FCGS is caused by an inappropriate immune response to chronic antigenic stimulation, with possibly multiple inciting causes.[3,5] Mucosal lesions associated with FCGS have high expression of inflammatory cytokines. The oral mucosa of clinically normal cats has a predominantly type 1 helper T-cell cytokine expression profile; however, FCGS oral lesions typically shift from a type 1 to a mixed type 1 and 2 helper T-cell cytokine expres-

sion profile as the disease progresses.[7] Infiltration of the FCGS-affected tissue by plasma cells and lymphocytes (and to a lesser extent neutrophils, macrophage-like cells, and mast cells) contributes to the inflammatory response.[8,9] The predominance of CD8+ T cells within FCGS lesions suggests that the inflammation might be caused by a viral infection.[9,10] The possibility of respective associations between FCGS and multiple infectious agents, including FCV, FeLV, FIV, feline herpesvirus type 1, and *Bartonella* spp, has been investigated.[11–23,a,b] Despite evidence suggesting a possible association between FCV and FCGS, results of multiple studies[12–16,18,19,21,23,24,a,b] are inconsistent, and the role of FCV in FCGS remains uncertain.

In the absence of a definitive link between FCV and FCGS, treatment of affected cats has focused on alleviation of inflammatory lesions and associated clinical signs. The current standard of care is either extraction of all teeth or extraction of all premolar and molar teeth.[1,4] Extraction of teeth results in a successful outcome for 60% to 80% of treated cats, with successful outcome defined as complete resolution of FCGS lesions or substantial clinical improvement with mild to moderate inflammation remaining.[1,4,25] No single treatment has achieved a clinical success rate close to 100%, and cats that fail to respond to treatment require lifelong medical management or euthanasia.

Medical management of cats with FCGS that do not respond to tooth extraction (refractory FCGS) may include antimicrobials, analgesics, and immunomodulatory and immunosuppressive agents and is associated with varying success and the potential for substantial adverse effects.[4,5,26] The immunomodulatory action of MSCs has also shown promise for the treatment of cats with refractory FCGS.[27–29] The positive responses to immunomodulatory treatments strongly support the role of an aberrant immune response, either on its own or in reaction to an exogenous stimulus (ie, pathogen), in the pathogenesis of the disease.

Broad screening for microbes and host factors is necessary to better understand the ambiguous and potential multifactorial etiology of FCGS. The variable response of affected cats to tooth extraction and inconsistent association of various pathogens with FCGS might represent multiple disease entities that appear clinically similar or identical. Multiple pathogens might interact synergistically or separately to cause disease, or the disease may have an infectious origin in some patients and autoimmune or immune-mediated origin in other patients. Results of a study[11] in which a few hundred 16S rRNA gene amplicons from 9 FCGS-affected cats were sequenced failed to suggest an association between FCGS and any specific bacterium. Although more comprehensive microbiome analyses could be performed by means of shotgun sequencing, that method is generally limited to metagenomic sequencing of bacteria. Moreover, traditional methods, such as microbiological culture, serology, or targeted DNA- or RNA-based testing, have limited ability to identify novel infectious agents and are susceptible to various biases that create false-negative and false-positive calls.[30–33]

Currently, mNGS is the 1 assay that can simultaneously and comprehensively assess the presence and activity of all known microbes. It identifies and counts both microbial- and host-expressed gene sequences by unique match of shotgun sequencing reads, which are amplified by use of unbiased random primers in RNA library preparations.[33–35] Metagenomic next-generation RNA sequencing has been used to identify putatively causal pathogens from every domain of life (ie, prokaryote, virus, and eukaryote) in vertebrate hosts such as snakes, birds, and humans.[31,32,36–50] By means of de novo assembly of raw reads, mNGS can also identify novel microbes with distant similarities to known genomes and enumerate genetic variants.

The primary purpose of the study reported here was to use mNGS to compare transcriptomic signatures of microbes and host genes in cats with FCGS with those in healthy cats and cats with periodontitis (control cats). Swab specimens of the oral mucosa were collected for RNA isolation from cats with or without periodontitis or with lesions histologically confirmed as FCGS. Because FCGS might be a multifactorial disease process, the relative abundances of microbes and host gene expression were compared between cats with FCGS that responded to tooth extraction (responsive FCGS) and cats with refractory FCGS and between cats with refractory FCGS that did and did not respond to MSC treatment in an attempt to identify factors associated with treatment response. Identification of an association between a specific microbe and FCGS treatment success or failure would help inform treatment decisions and thereby improve patient outcomes and facilitate prioritization of research on preventive measures and nonsurgical treatment.

Materials and Methods

Animals

All study procedures were reviewed and approved by the University of California-Davis Institutional Animal Care and Use Committee. Forty-two client-owned cats were recruited for the study from 3 veterinary dental specialty services at different locations in California: University of California-Davis (Davis, Yolo County), Aggie Animal Dental Center (Mill Valley, Marin County), and VCA San Francisco Veterinary Specialists (San Francisco, San Francisco County). Consent for study enrollment was obtained from the owner of each cat prior to study initiation. The study population included 14 clinically normal cats, 5 cats with mild to moderate periodontitis, and 23 cats with FCGS (**Supplementary Table S1**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381).

A diagnosis of FCGS was made on the basis of moderate to severe inflammation of the oral mucosa

lateral to the palatoglossal folds, with or without gingivitis, and was confirmed by histologic evaluation of oral lesions. Cats with severe periodontitis, osteomyelitis of any oral structure, and evidence of oral neoplasia were excluded from the study, as were cats undergoing immunosuppressive treatment (eg, chemotherapy) and cats with immunocompromising diseases (eg, diabetes mellitus).

Sample collection

All samples were collected by 1 of 3 board-certified veterinary dental specialists (BA [University of California-Davis], MS-R [VCA San Francisco Veterinary Specialists], and MJL [Aggie Animal Dental Center]). For each cat, a sterile cotton-tipped applicator was used to swab the oral mucosa lateral to the palatoglossal folds. The swab was placed in a sterile conical tube that contained 250 µL of guanidinium thiocyanate.[c] The tube and its contents were then vortexed before being frozen and stored at –20°C until transported on dry ice to a laboratory, where the specimen was stored frozen at –80°C until processing (**Supplementary Figure S1**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381).

For each healthy cat and cat with periodontitis, an oral mucosal swab specimen was obtained during routine periodontal treatment. For cats with FCGS, swab specimens of the oral mucosal lesions were obtained immediately before tooth extraction (n = 12) or initiation of MSC therapy after it was determined that the condition had not responded to tooth extraction (10), or they were obtained only once 6 months after tooth extraction without disease resolution (1). For each of 4 cats (1 healthy cat and 3 cats with FCGS), swab specimens from the left and right side of the oral cavity (ie, paired samples) were obtained at the same time and processed separately. For 4 cats (1 cat with responsive FCGS from which paired samples were collected and 3 cats with refractory FCGS that underwent MSC therapy), oral mucosal swab specimens were obtained before and 6 months after tooth extraction to allow time to determine whether the condition was going to respond to tooth extraction.

mNGS library preparation

Swab specimens were processed and prepared as described.[33,51] To each thawed oral mucosal swab specimen, 1 mL of guanidinium thiocyanate was added to extract RNA. The specimen was briefly vortexed, and 600 µL of the specimen fluid (sample) was transferred to a plastic tube. The sample was mechanically homogenized[d] with 2.8-mm ceramic bashing beads[e] for 3 minutes at 30 Hz, then centrifuged at 16,000 × *g* and 4°C for 5 minutes. The supernatant was transferred to another plastic tube and underwent RNA extraction with DNase[f] and was then eluted into 20 µL of water and snap frozen in liquid nitrogen. A miniaturized 384-well mNGS library preparation[g] was used to prepare RNA sequencing libraries as described.[34] The protocol included the use of robotic[h] and sonic[i] liquid handlers, size selection with magnetic beads,[j] quantity and quality assessment of double-stranded DNA with a quantitative PCR assay and chip-based capillary electrophoresis,[k] initial sequencing[l] for sample pooling calculations, and sample tracking with in-house custom oligomer barcodes. Final pooled samples underwent 125 nucleotide paired-end sequencing.[m]

Bioinformatics pipeline

An open-source cloud-based platform[n] was used as described[52] to facilitate pathogen detection. That platform is a rapid computational pipeline developed to analyze mNGS reads for potential pathogens by the DeRisi Laboratory and Chan-Zuckerberg Biohub, both of San Francisco.[43,52] Briefly, reads were filtered for the host genome; reagent sequences; quality, complexity, and redundance; and a diverse set of metazoan genomes, then assembled and filtered again and aligned to the NCBI nonredundant genomic (nt) and transcriptomic (nr) databases. The process was repeated without the host genome filter at the beginning and aligned at the end instead of to the host transcriptome. The *Felis catus* genome (RefSeq GCF 000181335.3, version 9.0) was used as the host genome.

Statistical analysis

Aligned read counts were taken as the raw relative abundances of each observed gene or microbe. Read count profiles for paired specimens were manually compared with each other for process validity and averaged together as 1 sample in statistical analyses. Genes and microbes were analyzed for significant differences with a generalized linear model assuming a negative binomial distribution and shrinkage estimation for dispersions.[o] The method used incorporated data for all microbes into a background model to contextualize the comparisons, which is especially relevant for a zero-inflated data set (ie, most microbes observed in any sample are not observed in most samples).[53]

The relative abundance of each observed gene and microbe was compared between cats with FCGS (n = 23 cats; 24 samples) and healthy cats (14 cats; 14 samples) and cats with periodontitis (5 cats; 5 samples) combined (control cats) and between cats with responsive FCGS (9 cats; 9 samples) and cats with refractory FCGS (13 cats; 14 samples). For the comparison between cats with FCGS and control cats, results only for oral mucosal samples obtained prior to tooth extraction were analyzed with 2 exceptions: a postextraction sample was analyzed for 1 cat with refractory FCGS for which a pretreatment sample was unavailable and for 1 cat with refractory FCGS that appeared to also be unresponsive to MSC treatment. For the comparison between cats with responsive FCGS and cats with refractory FCGS, results for all oral mucosal samples obtained from cats with active lesions and for which the outcomes were

known were analyzed. Results for samples obtained after successful disease resolution were not included in the comparison. Additionally, results for oral mucosal samples obtained from cats without active FCGS lesions were excluded from the background model. For each comparison, counts from the nt (ie, genomic hits) and nr (ie, mRNA expression) were analyzed separately for microbial abundance.

Significant differences in the relative abundance of each observed gene and microbe were evaluated by multiplying the number of comparisons made and assessed for a false discovery rate of < 0.001. Owing to false statistical inflation that can occur in omics comparisons such as these and the potential of real global shifts in gene expression and microbiota following the onset of disease, we focused on outliers in terms of *P* values and relative abundance that emerged beyond shifts in the group in an attempt to differentiate driver from passenger microbes. To account for statistical inflation from a few outlier samples, the distribution of read counts by sample and comparator was plotted and assessed. Additionally, potential false-positive microbes were manually assessed by the preparation and review of genomic coverage maps (data not shown). Those maps were evaluated on the basis of the concept that reads hitting only 1 locus broadly conserved across many organisms tend to be invalid while reads that are distributed across a genome and vary between samples tend to be real.

The presence or absence of each microbe was also tested for correlation with FCGS or with response to tooth extraction by the Fisher exact test and adjusted for multiple comparisons.

Phylogenetic analysis

Phylogenetic analysis was used to assess the relationship among FCV isolates detected in oral mucosal samples. The FCV genomes were assembled within an open-source cloud-based platform[n] as described.[52] A multiple-sequence alignment was created from the sequences of 11 nearly (> 90%) complete and reference FCV genomes obtained from the NCBI, Bethesda, Md, by use of an open-source multiple-sequence alignment software program[p] as described.[54] Only reference genomes with a length > 50% that of the FCV RefSeq genome were included in the analysis. The evolutionary model that provided the best fit for the data was selected by use of another open-source software program[q] as described.[55] A phylogenetic tree was built with 200 bootstraps by use of commercially available software[r] as described.[56,57] The relationship between genomes was determined on the basis of their proximity on the resulting phylogenetic tree. The tree was rooted at the phylogenetic midpoint.

Assessment of FCV RT-PCR primers from previous studies

To explore the effect that detection accuracy of FCV in previous PCR assay–based studies[12,13,16,21,24] might have on the association between FCV and FGCS, the RT-PCR primers used in those studies were compared with the FCV genomes assembled in this study. These FCV genomes were aligned by use of a progressive pairwise alignment tool.[s] The results were analyzed for base pair mismatches by means of simple counting and predicting the changes in primer annealing temperature on the basis of thermodynamic contributions of the Watson-Crick free-energy change for the mismatches by use of a bioinformatics server[t] as described.[58,59]

Results

Sequencing

The mean ± SD number of total reads per sample prior to quality control and filtering was $9.9 \times 10^7 \pm 2.6 \times 10^7$ total reads. A mean ± SD of $7.9 \times 10^6 \pm 1.7 \times 10^6$ reads mapped to the External RNA Control Consortium spike-in controls. A mean ± SD of 67.55 ± 19.53% of reads remained after removal of the host gene sequences and low-quality reads. Following all filtering steps, a mean ± SD of $1.1 \times 10^7 \pm 1.3 \times 10^7$ total reads remained, which represented 11.00 ± 13.68% of initial reads identified.

The water-only (ie, no template) negative control contained few background sequences. Of the 7.9×10^6 total reads sequenced, 7.6×10^6 reads were the spike-in controls, and only 4,958 (0.62%) total reads passed all the filter steps.

Comparison of microbe abundance between FCGS-affected cats and control cats

Of the microbes detected through mNGS, only FCV was found to be significantly ($P = 6.0 \times 10^{-42}$) associated with and abundant in FCGS-affected cats relative to control cats ($\log_2$ difference = 15.3; **Figure 1**) according to protein-encoding (nr) read counts. Feline calicivirus was detected in 22 of 24 (92%) oral mucosal samples analyzed from FCGS-affected cats and was not detected in any of the samples obtained from control cats (healthy cats and cats with periodontitis). *Comamonadaceae bacterium* NML970147 was the bacterium most uniquely abundant in control cats according to nr read counts ($\log_2$ difference = −25.5; $P = 1.6 \times 10^{-46}$) and was detected in 9 of 19 (47%) samples analyzed from control cats and in no samples from FCGS-affected cats.

When mNGS results were compared on the basis of nt read counts (which included non–protein-encoding regions), FCV remained the most significantly ($P = 9.4 \times 10^{-40}$) abundant microbe in FCGS-affected cats relative to control cats ($\log_2$ difference = 15.0; **Supplementary Figure S2**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381). There was no significant difference in the abundance of *C bacterium* NML970147 between FCGS-affected and control cats when mNGS results were compared on the basis of nt read counts.

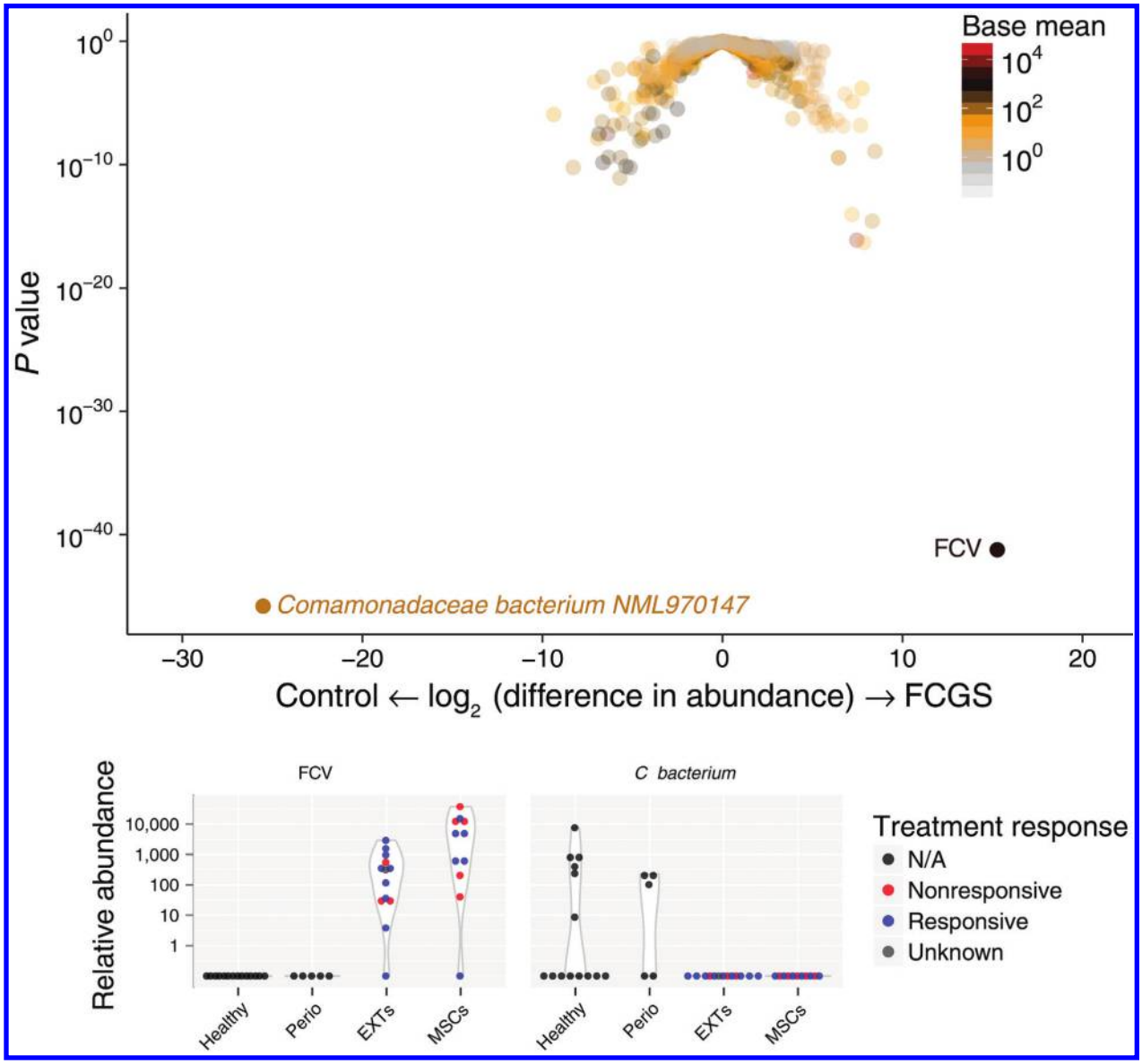

Figure 1—Plot of the differential abundance of microbes between control cats (healthy cats [n = 14] and cats with mild to moderate periodontitis [5]) and FGCS-affected cats (23) on the basis of counts of mNGS-filtered reads mapped to each microbe to the NCBI nr. Each dot represents the statistical analysis of read counts for a unique microbe. For each microbe, the difference in abundance between control and FCGS-affected cats is plotted on a logarithmic scale on the basis of the statistical significance (*P* value). Negative differential abundance values indicate that the microbe was found in greater abundance in control cats relative to FCGS-affected cats, and positive values indicate that the microbe was found in greater abundance in FCGS-affected cats relative to control cats. The color of each dot represents the mean read count for the microbe across all samples (ie, the higher the color mapping of the dot, the greater the mean abundance in all samples). Outlier microbes are individually identified. Plots of the relative abundance (ie, normalized read counts) for each experimental group (healthy cats, cats with periodontal disease [Perio], FCGS-affected cats that were sampled immediately prior to tooth extraction [EXTs], and cats sampled immediately prior to MSC therapy [MSCs]) for outlier microbes are also provided.

Although various viruses commonly identified in cats were sporadically observed in oral mucosal samples obtained from FCGS-affected and control cats, no significant association was identified between FCGS and any virus other than FCV that has been previously investigated for a possible relationship with FCGS **(Figure 2)**. Feline immunodeficiency virus was identified in only 2 of the 23 (9%) FCGS-affected cats and was not identified in any of the control cats. Both of the FIV-infected cats were coinfected with FCV and did not respond to tooth extraction. Neither feline herpesvirus type 1 nor FeLV was identified in any of the cats evaluated in this study.

Comparison of microbe abundance between cats with responsive FCGS and cats with refractory FCGS

When mNGS results were compared on the basis of nr read counts, the abundance of 4 microbes differed significantly between FCGS-affected cats

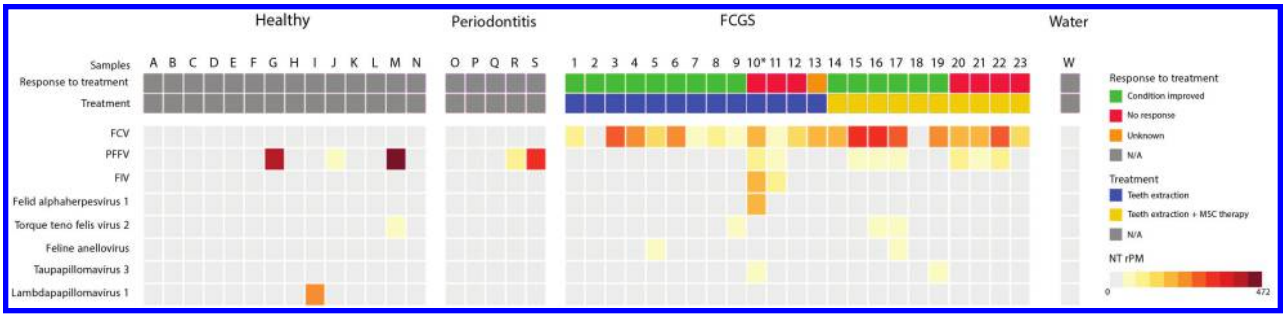

Figure 2—Heat map that depicts the normalized nt read counts for viruses identified by mNGS analysis of oral mucosal samples obtained from healthy cats (n = 14 cats; 15 samples), cats with mild to moderate periodontitis (5 cats; 5 samples), and cats with FCGS that did (responsive FCGS; 9 cats; 12 samples) and did not (refractory FCGS; 13 cats; 15 samples) respond to tooth extraction. Results are provided only for viruses with at least 1 read matching by both nr and nt and that was > 10 SDs above the mean nt read count for all microbial hits in at least 1 oral mucosal sample. Healthy cats and cats with periodontitis are identified by uppercase letters; FCGS-affected cats are identified by numbers. Paired oral mucosal samples (1 from the left side and 1 from the right side of the mouth) were analyzed from 1 of the 14 healthy cats and 2 of the 23 cats with FCGS. Paired oral mucosal samples (1 obtained before and 1 obtained 6 months after tooth extraction) were analyzed from another cat with FCGS. For the cats with FCGS, all oral mucosal samples were obtained immediately before tooth extraction unless otherwise indicated. Notice that FCV was detected only in cats with FCGS. Puma feline foamy virus was detected in 3 healthy cats, 2 cats with periodontitis, and 8 of 13 cats with refractory FCGS; it was not detected in any of the cats with responsive FCGS. N/A = Not available. NT = NCBI nt. rPM = Number of reads hitting the organism per million total quality and host-filtered reads. *Sample obtained after treatment.

with clinical resolution following primary treatment with extractions (responsive) and cats with refractory FCGS **(Figure 3)**. *Cardiobacterium valvarum* ($\log_2$ difference = 18.9; $P = 2.9 \times 10^{-7}$) and PFFV ($\log_2$ difference = 20.6; $P = 3.9 \times 10^{-22}$) were significantly more abundant in cats with refractory FCGS than in cats with responsive FCGS. Puma feline foamy virus was detected in 9 of 15 oral mucosal samples analyzed from cats with refractory FCGS but was not detected in the 9 samples analyzed from cats with responsive FCGS. Interestingly, *C valvarum* was detected in only 1 of the 15 oral mucosal samples analyzed from cats with refractory FCGS and 9 of 19 samples analyzed from control cats. *Moraxella pluranimalium* ($\log_2$ difference = −19.0) and *Tessaracoccus aquimaris* ($\log_2$ difference = −6.3) were significantly ($P = 9.3 \times 10^{-7}$ for both) more abundant in cats with responsive FCGS than in cats with refractory FCGS. *Moraxella pluranimalium* was detected in only 1 of 9 samples analyzed from cats with responsive FCGS and 7 of 19 samples obtained from control cats. *Tessaracoccus aquimaris* was detected in 6 of 9 cats with responsive FCGS, 5 of 15 cats with refractory FCGS, and all 19 control cats.

When mNGS results were compared on the basis of nt read counts, among the organisms previously mentioned only PFFV was significantly ($P = 3.4 \times 10^{-17}$) more abundant in cats with refractory FCGS, compared with cats with responsive FCGS ($\log_2$ difference = 23.1; **Supplementary Figures S3 and S4**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381).

FCV phylogenetic analyses

A phylogenetic tree was built by the use of the nearly complete FCV genomes assembled in the present study and those in the NCBI database **(Figure 4)**. Eleven genomes were sequenced during the present study, of which 1, 2, and 8 were isolated from cats that were examined at the Mill Valley, San Francisco, and University of California-Davis practices, respectively. Two of the genomes sequenced in this study were identical and were obtained from the left and right sides of the mouth at the same time from an FCGS-affected cat. Two other genomes were closely related (ie, sequences were 98.5% identical) and were obtained from 2 unrelated cats with refractory FCGS. The remaining 7 genomes were diverse, sharing 78% to 82% sequence identity (**Supplementary Table S2**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381). Only 1 cat was positive for FCV after disease resolution. That cat had FCGS that was refractory to tooth extraction but had disease resolution following MSC treatment. One FCV genome was sequenced from that cat before tooth extraction and another after disease resolution following MSC treatment. The sequences of those 2 genomes were quite different, which suggested resolution of the initial FCV infection concordant with clinical resolution, followed by reinfection with a different strain of the virus. No phylogenetic patterns on the basis of response to FCGS treatment were identified for the 11 FCV genomes assembled in this study.

Comparison between assembled FCV genomes and RT-PCR primers used in other studies

Evaluation of nucleotide base pair mismatches between the 10 unique FCV genomes sequenced from oral mucosal samples of cats with FCGS that did (n = 1) and did not (9) respond to tooth extraction in the present study and 4 RT-PCR primer pairs used in other studies[12,13,16,21,24] was conducted to investigate the relationship between FCV and FCGS

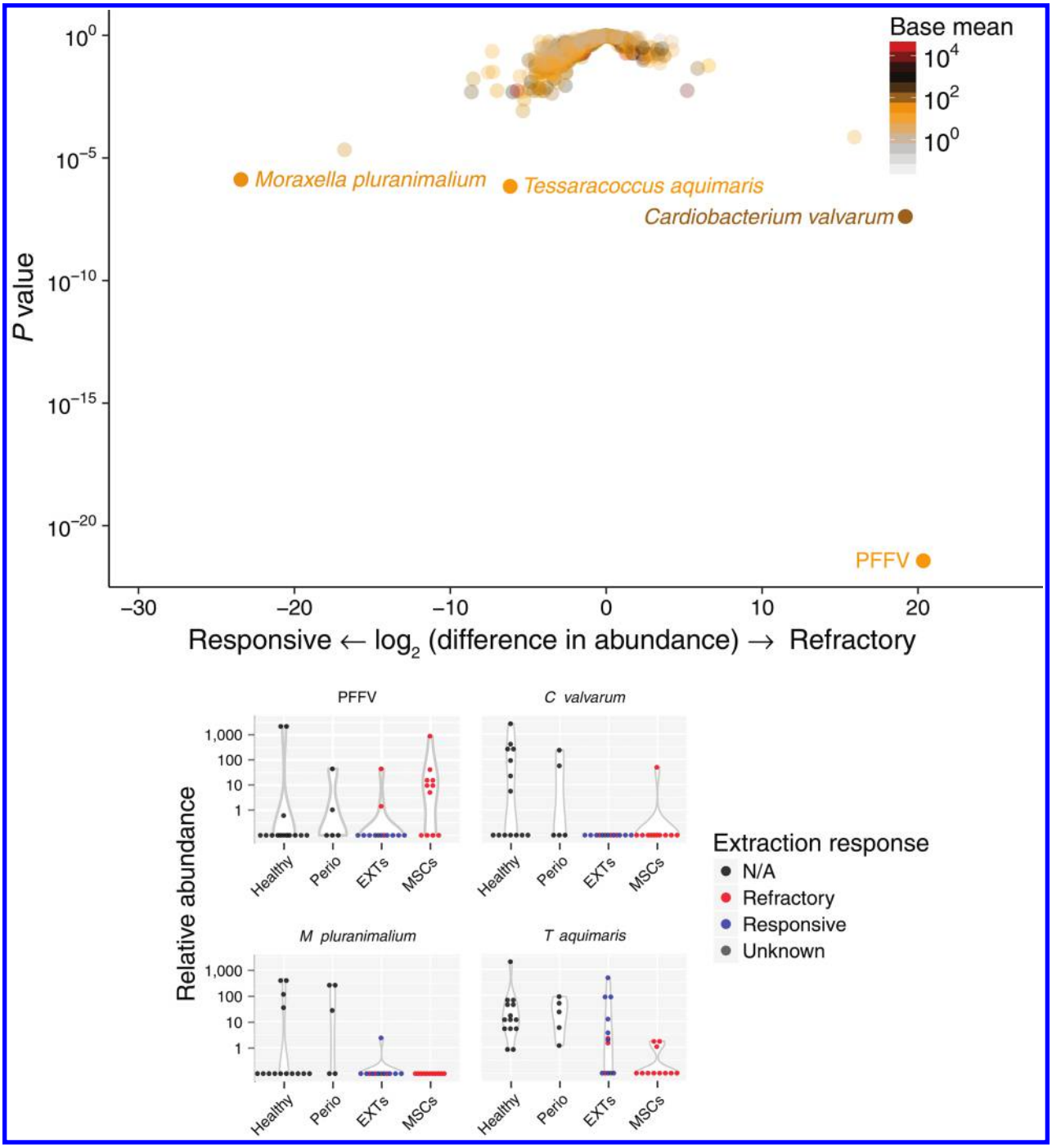

Figure 3—Plot of the differential abundance of microbes between cats with responsive FCGS and cats with refractory FCGS on the basis of nr read counts. Negative differential abundance values indicate that the microbe was found in greater abundance in cats with responsive FCGS relative to cats with refractory FCGS, and positive values indicate that the microbe was found in greater abundance in cats with refractory FCGS relative to cats with responsive FCGS. ***See*** Figure 1 for remainder of key.

(Figure 5). Eight of the 10 genomes had at least 2 base pair mismatches with the H_8 primer pair and M_Cali primer pair. All 10 genomes had at least 2 base pair mismatches with the S_Calcap primer pair. Only 1 genome had 2 base pair mismatches with the Wilhelm primer pair, and the target region of the Wilhelm primers was not represented in 1 incomplete genome. Four, 3, 7, and 0 genomes had base pair mismatches within 3 nucleotides of the 3' end of the target region for the H_8, M_Cali, S_Calcap, and Wilhelm primer pairs, respectively. Ten, 4, 8, and 0 of the FCV genomes assessed had a > 10°C difference in the estimated PCR annealing temperature owing to base pair mismatches between the target region of the genome and the H_8, M_Cali, S_Calcap, and Wilhelm primer pairs, respectively.

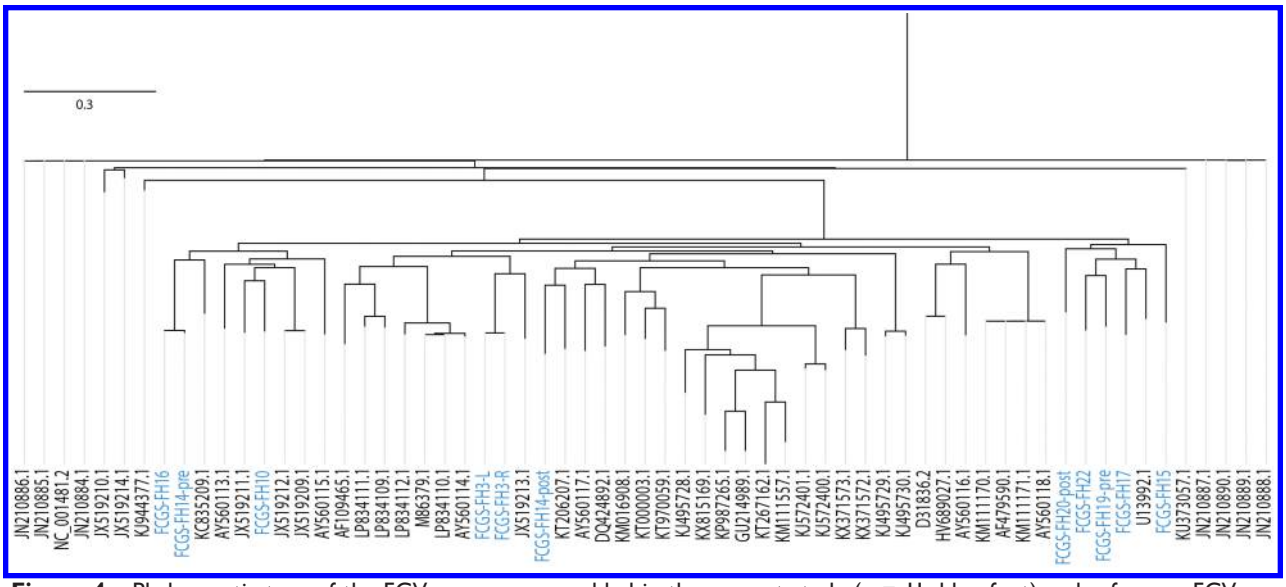

Figure 4—Phylogenetic tree of the FCV genomes assembled in the present study (n = 11; blue font) and reference FCV genomes obtained from the NCBI. The FCV genomes assembled from 2 samples obtained at the same time from the left (FCGS-FH3-L) and right (FCGS-FH3-R) sides of the mouth of 1 FCGS-affected cat were identical. Two FCV genomes obtained from a cat with refractory FCGS before (FCGS-FH14-pre) and 6 months after (FCGS-FH14-post) tooth extraction varied substantially, which suggested the cat cleared the original FCV infection and was subsequently reinfected with another strain of the virus. Genomes that are in close proximity on the phylogenetic tree are more similar genetically than are genomes that are farther apart on the tree. The tree was rooted at the phylogenetic midpoint. The bar depicts the scale of substitutions per genomic position (nucleotide), which is a measure of the evolutionary time between 2 nodes.

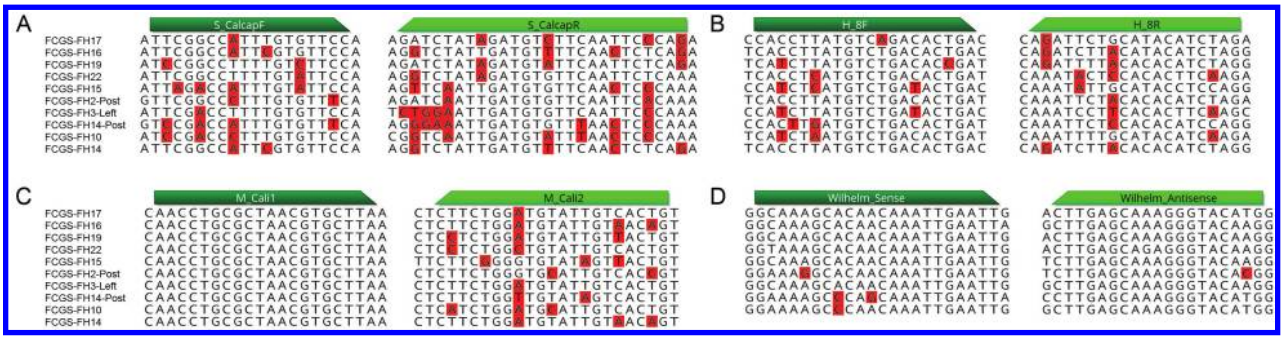

Figure 5—Nucleotide base pair mismatches between the 10 unique FCV genomes sequenced from oral mucosal samples of cats with responsive (n = 1) and refractory (9) FCGS in the present study and the RT-PCR primers used in other studies[12,13,16,21,24] conducted to investigate the relationship between FCV and FCGS. Each row represents the nucleotide sequence for a genome isolated from a cat of the present study within the target region of the 4 sets of RT-PCR primers used in studies by Dowers et al[12] and Quimby et al[21] (S_CalcapF and S_CalcapR; A), Rolim et al[24] (H_8F and H_8R; B), Nakanishi et al[16] (M_Cali1 and M_Cali2; C), and Belgard et al[13] (Wilhem_Sense and Wilhelm_Antisense; D). The identification of the genome sequenced in the present study is provided in the leftmost column, and mismatches or inconsistencies with the RT-PCR primers are highlighted in red. The target region of the Wilhem primers (D) was not represented in the FCGS-FH14 genome. The target nucleotide sequences for the RT-PCR primers were chosen because they were believed to be conserved across FCV strains. Nucleotide mismatches between the infecting FCV strain and the RT-PCR primers can lead to false-negative results when RT-PCR assays are used to evaluate samples for the presence of the virus. For each primer sequence, the 3' end is to the left and the 5' end is to the right.

Evaluation of the multiple-sequence alignment of the FCV genomes assembled from the cats of the present study revealed a region that is highly conserved with little genetic variation. That region is located at the border of the first and second open reading frames of the FCV reference genome M86379, between bases 5,307 and 5,345. That region would be an ideal location for 1 primer of an RT-PCR primer pair and is already targeted by the M_Cali1 forward primer, for which no nucleotide mismatches were identified when it was compared with the 10 FCV genomes assembled from the FCGS-affected cats of the present study.

Comparison of host gene expression between FCGS-affected cats and control cats

Comparison of host gene expression between FCGS-affected cats and control cats revealed that the expression of 140 genes was significantly greater in control cats than in FCGS-affected cats and expression of 422 genes was significantly greater in FCGS-affected cats than in control cats **(Figure 6)**. Our analysis focused on the furthest statistical outliers (ie, genes with values of $P < 10^{-8}$ in control cats [n = 5] and $P < 10^{-9}$ in FCGS-affected cats [8]). The furthest outly-

ing gene was *GBP-1* ($P = 6.6 \times 10^{-21}$), which was expressed in all cats; however, its relative abundance in FCGS-affected cats was 39 times that in control cats. Four genes within the GBP family were among the 8 genes that had the most significantly greater expression in FCGS-affected cats than in control cats. Those 4 genes (*GBP-1*, *GBP-5* [$P = 1.7 \times 10^{-13}$], *GBP-6* [$P = 3.9 \times 10^{-11}$], and an unannotated gene genomically located between *GBP-1* and *GBP-6* [Loc109502446, which we putatively assigned as a *GBP*; $P = 1.7 \times 10^{-13}$]) were not more highly expressed in cats with periodontitis than in healthy cats, which suggested that they were specifically upregulated in cats with FCGS. Other genes with a significantly higher relative abundance in cats with FCGS than in control cats included aconitate decarboxylase I (*CAD*; $P = 3.9 \times 10^{-11}$), *LIMK2* transcript variant X3 ($P = 1.9 \times 10^{-10}$), cluster of differentiation 28 (*CD28*; $P = 4.9 \times$

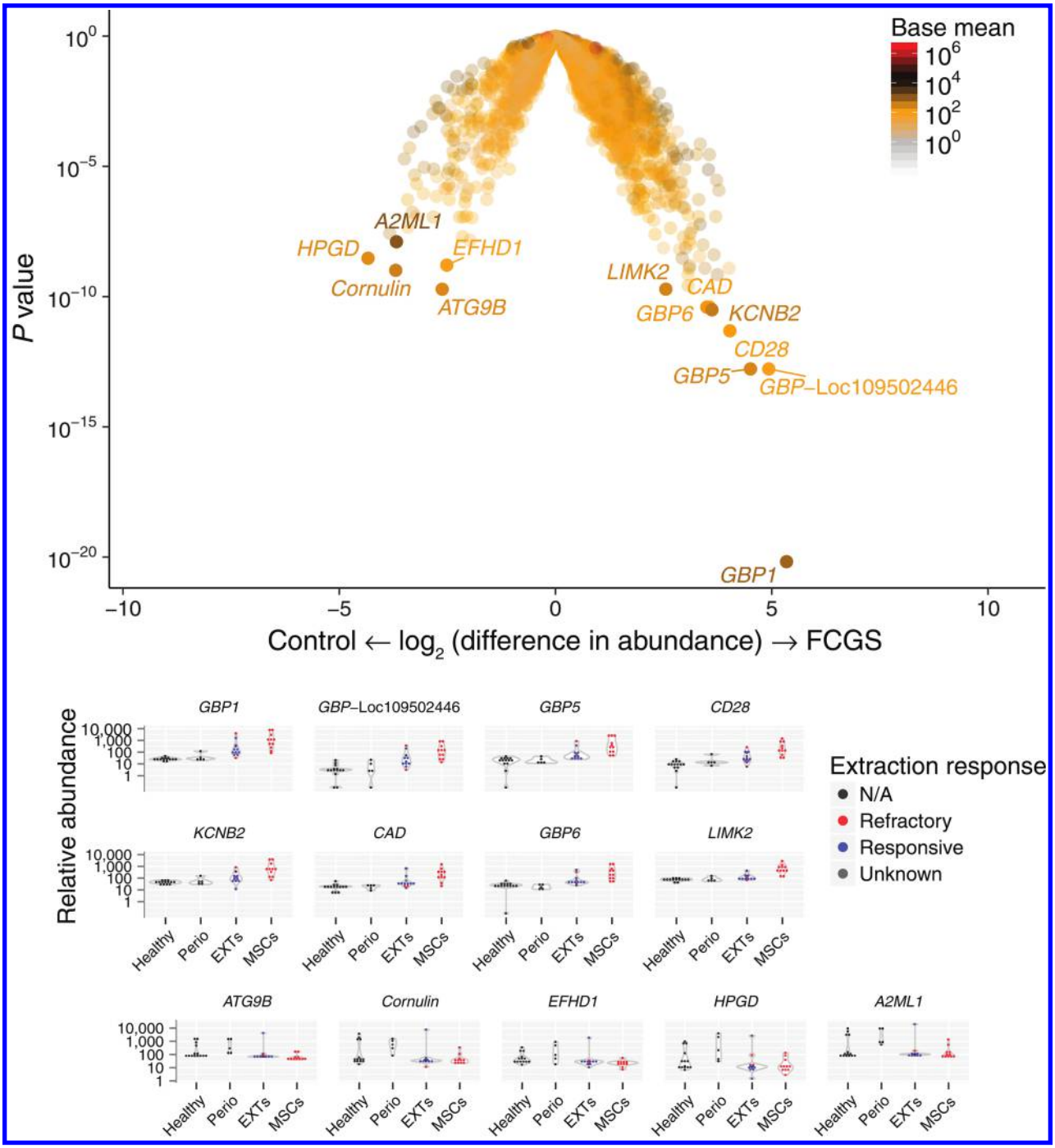

Figure 6—Plot of the differential abundance of cat gene expression between FGCS-affected cats and control cats on the basis of mNGS read hits. Four of the 8 outlier genes identified belonged to the GBP family, which has various antimicrobial and specific antiviral activities. **See** Figure 1 for remainder of key.

10^{-12}), and *KCNB2* ($P = 3.1 \times 10^{-11}$). Genes with a significantly higher relative abundance in control cats than in cats with FCGS included autophagy-related 9B (*ATG9B*; $P = 1.9 \times 10^{-10}$), cornulin ($P = 1.0 \times 10^{-9}$), EF-hand domain-containing protein D1 (*EFHD1*; $P = 1.6 \times 10^{-9}$), 15-hydroxyprostaglandin dehydrogenase (*HPGD*; $P = 3.0 \times 10^{-9}$), and α-2-macroglobulin-like 1 (*A2ML1*; $P = 1.3 \times 10^{-8}$). Expression of genes for inflammatory markers that were found to be differentially higher in FCGS-affected cats relative to control cats in other studies[12,13,16,21,24] varied minimally between the FCGS-affected cats and control cats of the present study (**Supplementary Figure S5**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381).

Comparison of host gene expression between cats with responsive FCGS and cats with refractory FCGS

Differences in host gene expression were less substantial between cats with responsive FCGS and cats with refractory FCGS (**Supplementary Figure S6**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381) than between FCGS-affected cats and control cats. Expression of only 1 gene was significantly ($P < 0.001$) greater in cats with responsive FCGS relative to cats with refractory FCGS, whereas expressions of 184 genes were significantly greater in cats with refractory FCGS relative to cats with responsive FCGS. Gene27988 ($P = 2.8 \times 10^{-4}$) expression was very high in 2 cats with responsive FCGS and 1 FCGS-affected cat for which treatment response was unknown. Each of the other genes with significant differential expression between cats with responsive FCGS and cats with refractory FCGS was detected in large amounts in only 1 sample. Genes that had differentially greater expression in cats with refractory FCGS relative to cats with responsive FCGS and had consistent differences in most samples included 2 genes with unknown function (gene20202 [$P = 4.5 \times 10^{-6}$] and gene13606 [$P = 1.5 \times 10^{-5}$]), C-C chemokine receptor-like 2 ($P = 1.5 \times 10^{-5}$), *KCNB2* ($P = 3.1 \times 10^{-5}$), and *LIMK2* ($P = 3.1 \times 10^{-5}$).

Discussion

For over 3 decades, the etiology of FCGS has been considered elusive and challenging. In the present study, unbiased metagenomic and transcriptomic analyses of oral mucosal samples obtained from healthy cats, cats with mild to moderate periodontitis, and cats with FCGS were conducted in the absence of any preconceptions regarding which pathogens might be associated with disease. Although common feline viruses and myriad diverse microbes were identified during the present study, only FCV was positively associated with FCGS ($P = 6.0 \times 10^{-42}$). Feline calicivirus was detected in 21 of 23 FCGS-affected cats but was not detected in any of the 14 healthy cats or 5 cats with periodontitis (control cats). Feline calicivirus was the only organism that was specific to and differentially more abundant in cats with FCGS on the basis of comparisons with both nt and nr. Feline calicivirus was present before treatment and was absent after disease resolution in all cats for which pre- and posttreatment samples were collected except 1. For the cat that was the exception, the FCV detected in the pretreatment sample was genomically distinct from the FCV detected in the posttreatment sample, which suggested the cat cleared the initial infection and was subsequently reinfected with a different strain of the virus.

Of the 11 FCV genomes sequenced in the present study, 2 were identical (and isolated from the same cat at the same sampling time), 2 were 98.5% identical, and the remaining 7 had 78% to 82% sequence identity. Those findings were similar to results of other studies[60,61] in which distinct FCV strains had 78% to 81% sequence identity.

In the present study, a strong positive association was identified between FCV and FCGS. Results of many studies[12,13,15,16,18–20,22,62,a,b] likewise indicate an association between FCV and FCGS. However, investigators of other studies[14,21,24] did not find an association between FCV and FCGS, and the results of those studies discouraged veterinarians from making clinical decisions assuming that such an association exists. In 1 study,[63] experimental inoculation of FCV in 8 specific pathogen–free kittens resulted in acute disease and oral ulceration but failed to induce chronic stomatitis. Results of that study[63] suggest that FCV may be required for initiation of disease but is not sufficient for the development of FCGS and that other factors, such as host age and immune status at the time of FCV infection and host genetics, may contribute to the maintenance of a chronic FCV infection and the development of FCGS.

Anecdotal evidence also suggests an association between FCV and FCGS. In a colony of FIV-infected cats that were routinely monitored for viral infections, cats began to develop FCGS only after an outbreak of FCV was reported in the colony.[22] Because none of the cats in the colony had FCGS before the FCV outbreak, it was believed that FCV contributed to the development of FCGS. There is also anecdotal evidence of the spontaneous resolution of FCGS with clearance of FCV infection. In a case report[62] of a cat with concurrent FCV infection and FCGS that underwent extraction of almost all its teeth, clinical signs of FCGS did not resolve until the FCV infection was cleared 22 months later. Results of the present study also suggested an association between clearance of FCV and resolution of FCGS lesions. Of the 4 FCGS-affected cats for which oral mucosal samples were collected before and after treatment, the 3 that responded to treatment cleared the infection caused by the FCV strain isolated prior to treatment. The cat that did not respond to treatment remained positive for the same FCV strain that was identified prior to treatment.

Other studies[12,13,15,16,18–20,24,a,b] conducted to investigate the relationship between FCGS and FCV used either virus isolation or RT-PCR assays to detect

FCV. Investigators of the studies[11,15,18–20,b] that used virus isolation for detection of FCV generally reported a stronger association between FCV and FCGS than did investigators of the studies[12,13,16,21,24] that used RT-PCR assays for detection of FCV (**Supplementary Table S3**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.5.381). However, other studies[64,65] indicate that virus isolation and RT-PCR assays have similar sensitivities for detection of FCV. The sensitivity of RT-PCR assays varies on the basis of how well the primers bind to the viral genome; therefore, the sensitivity of the technique is vulnerable to variability in the FCV genome. If the RT-PCR primers are unable to bind to the target region of the viral genome, that assay will yield false-negative results. Conversely, virus isolation cultivates and detects live virus and is unlikely to yield false-negative results because of strain variation but may fail to identify virus owing to a small number of infectious virions in a sample, inactivation of the virus during transit, or the presence of virus-neutralizing antibodies in the sample.[66] Both virus isolation and RT-PCR assays are unlikely to yield false-positive results.

The nucleotide mismatches and differences in the estimated annealing temperatures between the FCV genomes assembled in the present study and the RT-PCR primers used in other studies[12,13,16,21,24] suggested that false-negative assay results are likely when any of those primer pairs are used in PCR assays to detect FCV infections in cats. False-negative RT-PCR assay results caused by nucleotide mismatches between the primers and viral genome in tested samples likely account for the discrepancy in reported FCV detection rates between the RT-PCR assay and virus isolation. Studies[12,13,16,21,24] that used RT-PCR primers that matched poorly with target regions of the FCV genome (ie, that contained > 2 nucleotide mismatches in a single primer pair or had 1 nucleotide mismatch within 3 bp of the 3' end) tended to have lower FCV detection rates in FCGS-affected cats than did studies[11,15,18–20,b] that used virus isolation. The exception to that observation is the M_Cali primers, which are used for a nested PCR technique. A nested PCR technique uses an additional set of primers that amplify a target sequence located between the first primer pair during a second round of the assay to increase the sensitivity of the assay.[64] In short, there is a correlation between the fidelity of the RT-PCR primers with variant genomic targets and the frequency of FCV detection in cats, and this correlation might contribute to the apparent contradictory findings regarding the relationship between FCV and FCGS in the veterinary literature.

Results of the present study indicated that PFFV was significantly more abundant in cats with refractory FCGS than in cats with responsive FCGS on the basis of both nt and nr comparisons. Findings of other studies[67,68] suggest there is genomic similarity and possible spillover identity between PFFV and FFV. Feline foamy virus is generally believed to not cause disease in cats.[69–71] However, during an exploratory phase in preparation for the present study, we detected extraordinarily high concentrations of FFV in primary MSC cultures derived from adipose tissue obtained from 2 cats with severe refractory FCGS. Supernatant from those cultures caused aberrant foamy bubbles in previously unaffected MSC cultures, and treatment of the cultures with tenofovir resulted in clearance of FFV and the resumption of robust MSC growth without evidence of the foamy phenotype observed in FFV-infected cultures.[72]

Although no association has been made between FFV or PFFV and any disease, it is possible that a phenotype similar to that observed in the FFV-infected MSC cultures[73] might facilitate persistent coinfection with FCV in FCGS-affected cats. When a host with FCGS becomes infected with PFFV, the additional strain on host tissues could prevent proper in situ MSC growth and differentiation and thereby prevent the gingiva and mucosa from producing healthy tissue, resulting in inflamed granulation tissue that is unlikely to clear FCV or any other pathogen. The association between PFFV and FCGS requires further study. Specifically, the use of tenofovir or anti-retroviral nucleoside analogs to treat cats with refractory FCGS that are coinfected with PFFV warrants consideration.

A secondary goal of the present study was to evaluate associations between cat gene expression and FCGS. Results indicated that differential expression of several members of the GBP family of genes was significantly greater in cats with FCGS than in control cats. Guanylate-binding proteins have an important role in the innate immune system and can be induced by both type I and II interferons to fight bacteria, parasites, and viruses.[73] The *GBP-1* gene was the gene with the greatest differential expression between FCGS-affected cats and control cats. Guanylate-binding protein 1 has activity against RNA viruses, such as vesicular stomatitis virus, encephalomyocarditis virus, and hepatitis C virus.[74–76] The *GBP-5*, *GBP-6*, and Loc109502446 (a gene located within the GBP genomic context, which we putatively identified as a *GBP*) genes were also expressed at greater levels in FGCG-affected cats than in control cats. Guanylate-binding protein 5 promotes inflammasome responses to pathogenic bacteria and impairs infectivity of HIV1 and other retroviruses.[77,78] Guanylate-binding protein 6 and GBP-1 confer cell-autonomous immunity against infection of macrophages by *Listeria* and *Mycobacterium* spp.[79] The fact that genes for this set of known antiviral gene products were specifically upregulated in FCGS-affected cats suggested that a viral infection, such as FCV, might be responsible for the underlying antigenic stimulation that causes FCGS.

In the present study, the use of unbiased mNGS and transcriptomic analysis identified a specific and strong positive association between FCV and FCGS in cats, and the findings provided additional evidence to support the hypothesis that FCV is involved in the

development of FCGS. Evaluation of the full genome sequences for the FCV isolates assembled from the FCGS-affected cats of the present study suggested that the equivocal results within the existing veterinary literature regarding the relationship between FCV and FCGS might have been confounded by the use of RT-PCR primers that target poorly conserved sites in the highly variable FCV genome. We also identified a significant and novel association between PFFV and refractory FCGS, and the clinical significance of that finding remains to be determined. The unbiased nature of mNGS and transcriptomic analysis allowed us to overcome microbe detection challenges posed by FCV genome variability and minimal knowledge of the recently described PFFV genome. Results of the present study suggested that, in cats with FCGS, coinfection with FCV and PFFV may contribute to the persistence of oral lesions following tooth extraction. That finding is consistent with a multifactorial etiology for FCGS and warrants further investigation.

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Footnotes

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f. ZR-Duet DNA/RNA MiniPrep Plus, Zymo Research, Irvine, Calif.
g. NEBNext Ultra-II Library Prep, New England Biolabs, Ipswich, Mass.
h. BioMek NXP, Beckman Coulter, Indianapolis, Ind.
i. Echo 525, Labcyte, San Jose, Calif.
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