University of Utrecht

MICHIGAN STATE UNIVERSITY

mRNA EXPRESSION OF KEY ENZYMES INVOLVED IN THE EICOSANOID BIOSYNTHESIS PATHWAY IN FELINE IDIOPATHIC CYSTITIS

Author: R.D. Breed, Master/Graduate Student Veterinary Medicine, Small Companion Animals
Student no.: 3258653
Period: May-August
Supervisors: Dr. P.J. Venta, Dr. J. M. Kruger, Drs. R.J. Corbee

MERIAL
A SANOFI COMPANY

© R.D. Breed, 2013

Foreword

This study will determine the expression of the mRNA of several eicosanoid enzymes involved in feline idiopathic cystitis, which is sponsored by Merial and commissioned by the Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing in collaboration with the MSU Center for Feline Health and Well-Being.

First of all I want to thank my supervisor of this project, Dr. Pat Venta. He has helped with all the steps in the procedure and gave me many important references to consider. He was always there to help out if something was not clear.

My thanks go to Dr. John Kruger as well, for giving me the opportunity of working on this research project.

Furthermore I want to thank Vilma Yuzbasiyan-Gurkan for her enthusiasm and care in this whole project and during my stay at MSU.

I am thanking Carlie Trabulsy for her support and sociability during the hours in lab (and outside).

I want to thank all the staff members of the canine genetics laboratory, who were all helpful and created a safe and stable environment to work in.

Then I want to give thanks to my supervisor in the Netherlands, Ronald Corbee, for the help in the arrangements at the University of Utrecht.

Finally I want to thank my family and friends for their support during this project.

© R.D. Breed, 2013

Abstract

mRNA EXPRESSION OF KEY ENZYMES INVOLVED IN THE EICOSANOID BIOSYNTHESIS PATHWAY IN FELINE IDIOPATHIC CYSTITIS

Breed RD[1], Venta PJ[2], Kruger JM[2]. [1] University of Utrecht, Utrecht, Netherlands; Merial Summer School Research Program Student, [2] Small Animal Clinical Sciences, Michigan State University, East Lansing, MI

Feline idiopathic cystitis (FIC) is a common inflammatory condition of the bladder that affects young to middle aged cats with unknown cause. Because there may be different pathological mechanisms that could cause FIC, there is a critical need to identify the key pathogenic factors in FIC. Eicosanoids are potent lipid mediators involved in the initiation and resolution of inflammation and in the maintenance of the normal health of the urothelium. Recent studies suggest that FIC may be associated with alterations in bladder eicosanoid metabolism. The altered expression of genes encoding key enzymes involved in the metabolism of eicosanoids could be important in the pathogenesis of FIC. We hypothesized that expression of the mRNAs for these enzymes would differ between affected and healthy cats. In order to test this hypothesis, mRNA was isolated from healthy and FIC cat bladders (obtained by cystotomy and embedded in formalin-fixed paraffin), as well as urolith bladders as a general inflammation control. The mRNAs for COX-1, COX-2, mPGES, cPGES, 15-LOX-2 and PGDH (which all play a role in metabolism of eicosanoids) were quantified by reverse transcription quantitative PCR (RT-qPCR). Four control genes (SDHA, RPS5, RPS19, and POL2A) were developed to allow a relative analysis by the delta delta CT method.

Formalin-fixed paraffin embedded (FFPE) samples of affected cats (n=8), unaffected negative control cats (n=9) and urolith positive control cats (n=8) were obtained and used for the quantification.

No significant differences were found for the expression of the genes in FIC compared to urolith and normal cats, although a trend toward of $p < 0.10$ was found for cPGES and 15-LOX-2.

© R.D. Breed, 2013

The results of these experiments may lead to a better understanding of the contribution of eicosanoids in the pathogenesis of FIC, and may eventually lead to better methods of diagnosis and treatment.

Furthermore primers with high efficiencies were made for the genes, which may be useful for future studies of FIC and other feline inflammatory diseases.

Moreover the use of FFPE tissues for studying mRNA expression in relation with a disease or inflammatory condition is not a common practice. However due to the lack of the availability of fresh frozen tissues, this study took the initiative to use FFPE tissues to study the mRNA expression in relation with FIC. This could make further studies easier to perform due to using stored samples instead of fresh samples.

© R.D. Breed, 2013

Index

© R.D. Breed

Introduction

Feline lower urinary tract diseases (FLUTD) are common clinical problems of cats and are associated with varying combinations of hematuria, dysuria, pollakiuria, strangury, periuria and obstructions (Dru Forrester and Roudebush, 2007; Kruger et al., 2009). When the exact cause of these signs cannot be determined by thorough examination, a cat may be classified as having feline idiopathic cystitis (FIC) (Kruger et al., 2009). In FIC recurring symptoms of lower urinary tract occur and often resolve within 4 to 7 days (Dru Forrester and Roudebush, 2007). FIC is the most common cause of FLUTD and accounts for 54% to 64% of cats with FLUTD in several studies (Defauw et al., 2011). Up to now, there is no consistently effective means of treatment and prevention of FIC, especially the chronic form and is therefore often the reason that affected cats are surrendered or abandoned (Kruger et al., 2009).

FIC is considered to be similar to human interstitial cystitis (IC) and it is therefore used as spontaneous animal model of IC (Treutlein et al., 2012), although the direct relation between the etiopathogenesis is not yet proven (Kruger et al., 2009). Like FIC, human IC is only diagnosed by exclusion of other lower urinary tracts diseases. Despite several studies in both FIC and IC, the etiology of FIC and IC is still unclear. However, they are complex syndromes and it is likely that multiple causative factors are involved in the pathogenesis of these diseases. Because the cause(s) of FIC is still undetermined, a specific therapy is unavailable, while symptomatic therapy is often ineffective (Treutlein et al., 2012).

Since there may be different pathological causes involved in FIC (Kruger et al., 2009), it is important to determine which mechanisms contribute to FIC. In human IC, different theories of the cause have been considered, including infections, autoimmunity, neurogenic inflammation and urothelial defects of the urothelium bladder (Treutlein et al., 2012). In FIC the same theories have

been studied with no single factor emerging as the cause of the disease (Kruger et al., 2009; Snyder et al., 2012).

One common aspect of FIC is the presence of inflammation (edema, vasodilation and inflammatory cell infiltration (Specht et al., 2004; Specht et al., 2003)). Eicosanoids are lipid mediators involved in many pathologic processes, including inflammation, allergies, autoimmunity and carcinogenesis (Buczynski et al., 2009). Furthermore, eicosanoids are known to have important physiologic roles in the integrity of the urothelium, cytoprotection and recovery from injury (Buczynski et al., 2009; Khan et al., 1998). Recent studies suggest that FIC and IC may be associated with alterations in the metabolism of eicosanoids in the urothelium, leading to the hypothesis that eicosanoids may have an important role in mediating these disorders (Khan et al., 2011; Rastogi et al., 2008).

Protein expression of some of the key eicosanoid biosynthetic enzymes [for example PGDH (Khan et al., 2011)], have been studied and therefore the activity of genes encoding these biosynthetic key enzymes are of interest to study in the pathogenesis of FIC. We hypothesized that expression of the mRNAs for selected eicosanoid biosynthetic enzymes will be different between FIC and control bladders. The key enzymes chosen to be studied, are COX-1, COX-2, mPGES, PGES, 15-LOX-2 and PGDH (see Figure 1).

The aim of the study is to gain further insight in the pathogenesis of FIC, enabling the development of more successful diagnostic and therapeutic options for cats afflicted with FIC.

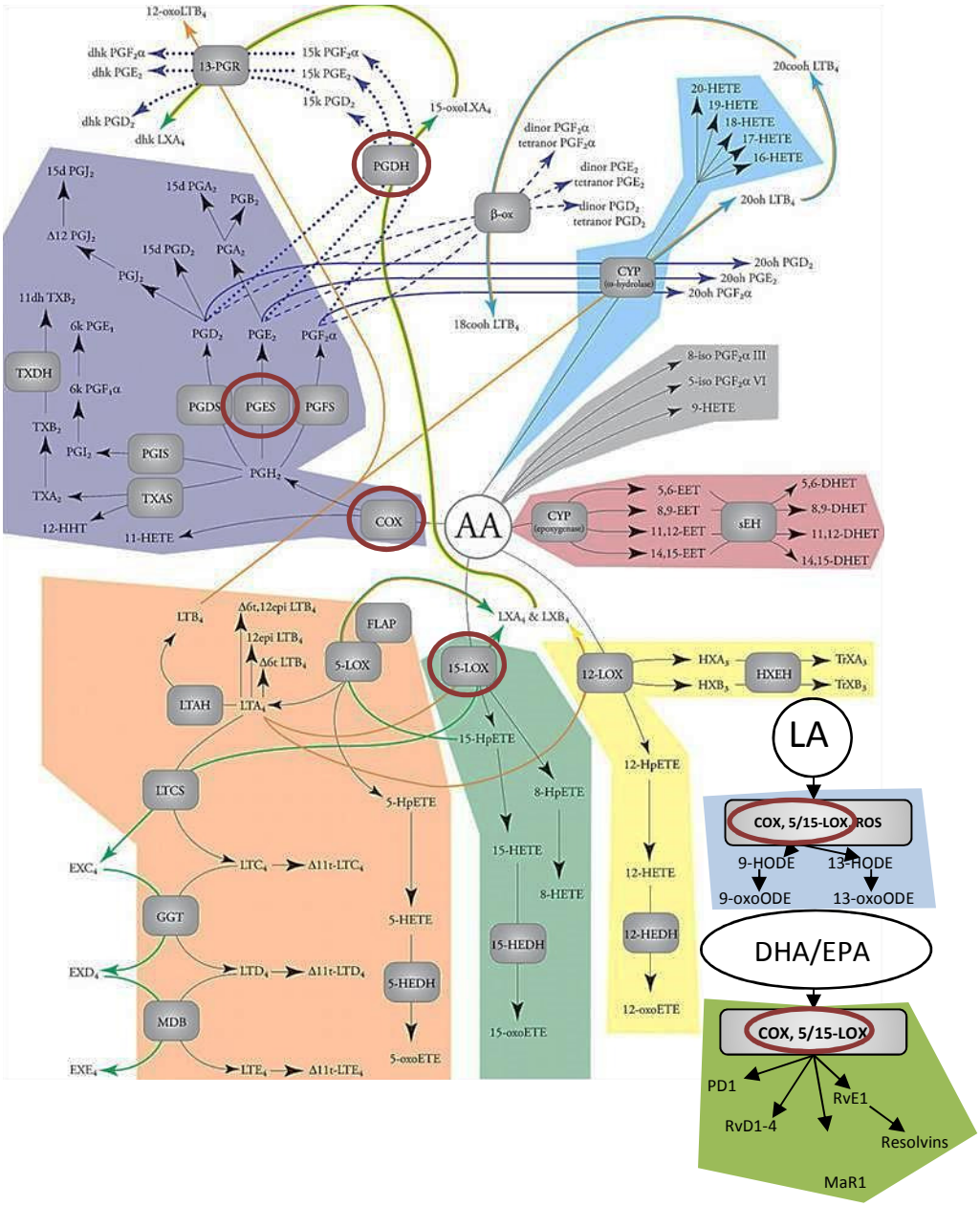


Figure 1 The eicosanoid metabolic pathways, derived from arachadonic acid (AA), eicosapentaenoic acid (EPA), decosahexaenoic acid (DHA) and linoleic acid (LA). The expressions of the enzymes marked in red were evaluated in this study. Figure adapted from Buczynski et al. (2009) by J.M. Kruger (2013).

Materials & Methods

Ethics statement

The materials used in this study were obtained from either animals euthanized for other experiments or collected from tissues that were left over from diagnostic tests. The procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Tissue samples

Samples of the full thickness urinary bladder biopsy specimens were collected by cystotomy and archived as Formaldehyde-Fixed Paraffin Embedded (FFPE) tissues from healthy (n=9) and FIC cat bladders (n=8), as well as urolith bladders (n=8) as a general inflammation positive control. The date range of the samples is shown in Table 1.

Diagnosis

A diagnosis of chronic FIC had been made on duration of the clinical signs and exclusion of other diseases. The cats had no obstructions and a history of two or more clinical signs of FLUTD, for longer than three weeks or with a recurrent frequency (three episodes or more in three months).

All of the cats used had an evaluation with history, physical examination, urinalysis, urine culture for bacteria (prior to empiric antimicrobial therapy), a survey and/or contrast radiographs of the lower urinary tract and an exploratory cystotomy.

Exclusion was made by reports of any other major diseases, if there were previous cystotomies or when the cat had any therapy with steroids or NSAIDs, antihistamines, tricyclics, antidepressants or glycosaminoglycan preparations within 4 weeks prior to the biopsy.

Processing of FFPE tissue

The FFPE issues were collected and immediately fixed in 10% buffered formalin for 12 to 48 hours. Thereafter they were paraffin embedded.

They were cut in the equivalent of a single 20 µm section for RNA extraction.

RNA extraction

The DNA of the fresh tissues was first removed using the steps as described in Table 2. The total RNA of fresh tissues was then isolated using a (small) modified acid guanidinium thiocyanate-phenol-chloroform method, as described by Chomczynski and Sacchi (2006). The volume of isopropanol used, was 0.5 mL instead of 1 mL isopropanol in step 7.

The total RNA of FFPE tissue samples was isolated using the miRNeasy FFPE Kit (Qiagen, Valencia, CA, USA) and using xylene deparaffinization according to the manufacturer's protocol.

RNA was quantified by spectrophotometry using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and integrity has been determined by a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany).

The RNA samples were stored at -20°C until use.

cDNA synthesis

Reverse transcription of the extracted RNA samples was done using a modified method with random hexamers (N6), 5x FS buffer, 10mM dNTPS, dithiothreitol (DTT) and Superscript III (Invitrogen , Carlsbad, CA, USA), as described in Table 3.

Quantitative real-time reverse transcriptase PCR (RT-qPCR) design

The mRNA for COX-1, COX-2, mPGES, cPGES, 15-LOX-2 and PGDH - which all play a role in metabolism of eicosanoids (Buczynski et al., 2009) - was quantified by reverse transcription quantitative PCR (RT-qPCR). Four housekeeping genes (SDHA, RPS5, RPS19, and POLR2A) were selected to normalize results, as described in Derveaux et al. (2010), to allow a relative analysis by the delta-delta CT method (ΔΔCT).

Predicted transcript sequences for the genes were obtained by usage of UCSC Genome Browser (http://genome.ucsc.edu). Using the convert function in the UCSC Genome Browser, the human sequences of the genes could be cross referenced to the cat genome. This was done to take advantage of the better human genome annotation, which helped to prevent accidental primer design to paralogs or processed pseudogenes. The inferred cat gene structure was made out of the correctly aligned segments with all exons included. Exons were then assembled into an inferred "mRNA", which was then translated with Expasy Translate (http://web.expasy.org/translate), to verify the presence of a collinear open reading frame, which was consistent with the RefSeq genes in human and other species.

The tissues are FFPE-derived and therefore it was anticipated, as previously shown, that there is extensive degradation of RNA (Dapson, 2007; Evers et al., 2011). The expression of the various genes was therefore first tested to have high enough expression in fresh frozen tissues (Sanchez-Navarro et al., 2010) and then, a simplified comparison between fresh tissue and FFPE tissue of the same bladder of one healthy cat has been made, to determine that the relative quantity of the expression of the genes is still intact and approximately the same, as done more extensively by other studies (Cronin et al., 2004; Li et al., 2008; Sanchez-Navarro et al., 2010).

Because numerous genes were quantified on a limited amount of total FFPE RNA, a hemi-nested amplification strategy was employed (Stanley and Szewczuk, 2005), that included a first round amplification of pooled "normal" primers, followed by individual gene amplifications using hemi-nested primers.

Some of the normal set primers (COX1, COX2, mPGES, POL2RA) as well as hemi-nested primers (COX1, mPGES and POL2RA) are derived from a preliminary study done by Snyder et al. (2012).

The normal primers were selected by Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/), using the cDNA translated sequences. They were designed to have amplicon sizes of 120-140 bp, to give minimal risk

of getting no amplification for the degraded RNA of the FFPE tissue samples (Antonov et al., 2005) and less shift in Cq values (Li et al., 2008). The hemi-nested primers were selected with amplicon sizes 20 bp less than the original set. Table 4 and Table 5 give an overview of the primers used. Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, CA, USA).

All the RT-qPCRs were performed with StepOnePlus™ (Applied Biosystems, Foster City, CA, USA) and raw data was processed in StepOne™ v. 1.8 (Applied Biosystems, Foster City, CA, USA)). In order to produce accurate results, the efficiencies of all the primer sets (normal and hemi-nested sets) developed for each of these genes have been determined by the production of standard qPCR curves, using 8 µL of six 10-fold serial dilutions of amplified PCR products (obtained with normal primer sets as described in Table 6) with 10 µL SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and 1 µL of forward primer (either normal or hemi-nested) and 1 µL of reverse primer (Pfaffl, 2001). The efficiencies were calculated from the given slopes in Excel 2010 by the formula $E = 10^{(-1/slope)}$ (Pfaffl, 2001), with efficiencies in percentage by $E\% = 100\% \times (10^{(-1/slope)} - 1)$. We maintained the rule that the efficiencies should be within 10% of each other, as suggested by Schmittgen and Livak (2008).

To determine which housekeeping gene was the most stable, an RT-qPCR was set with different normal samples (n=4) and the primers of these genes and further calculations on the Cq values were done with BestKeeper (http://www.gene-quantification.de/bestkeeper.html) and Normfinder (http://www.mdl.dk/publicationsnormfinder.htm).

First round PCR was performed as described in Table 7 with the normal primer set. The second round was a RT-qPCR, in 96 wells format (Applied Biosystems, Foster City, CA, USA). The reaction volume of each well was 10 µL 2x SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA), 8 µL 1µM amplified PCR product, 1 µL 2µM forward hemi-nested primer, 1 µL 2µM reverse primer. To ensure minimization of possible errors, all the samples were performed in duplicate.

Statistical Analysis

Raw Cq data were exported from StepOne™ (Applied Biosystems, Foster City, CA, USA) and used for further calculations in Excel 2010. Cq values that differed more than 1 cycle between duplicates of a gene, were removed due to possible high intra-variation and too low mRNA yields. A simple normalization of the Cq value difference between the housekeeping gene and the target gene could be done by:

$$\Delta Ct = \Delta CP_{target} - \Delta CP_{reference} \qquad \text{Equation 1}$$

Where CP is Cq (mean of duplicates was used), target is the target gene, reference is the used housekeeping gene.

To compare affected groups with unaffected groups, the ΔΔCt formula is used, as:

$$\Delta\Delta Ct = (\Delta CP_{target} - \Delta CP_{reference})_{calibrator} - (\Delta CP_{target} - \Delta CP_{reference})_{sample} \qquad \text{Equation 2}$$

Where calibrator is the unaffected or normal group and sample is the affected group.

But there are differences in efficiencies among the genes and those are not taken into account in the formula, but still could have effects on the Cq values (Pfaffl, 2004). Derived from the formula of Pfaffl, relative ratio calculations could be done including normalization with the housekeeping gene and efficiencies:

$$ratio = \frac{(E_{target})^{\Delta CP_{target\ (control)}}}{(E_{ref})^{\Delta CP_{ref\ (control)}}} / \frac{(E_{target})^{\Delta CP_{target\ (affected)}}}{(E_{ref})^{\Delta CP_{ref\ (affected)}}} \qquad \text{Equation 3}$$

where E=efficiency, control=negative control group and affected=FIC or positive control group.

This ratio is the same as (Yuan et al., 2006):

$$ratio = 2^{-\Delta\Delta Ct} \qquad \text{Equation 4}$$

Because there were different samples with different mean Cq values, the ratio itself cannot be used to compare the different groups. Instead, part of the formula of Equation 3 is used to create a normalization of the difference between target gene and housekeeping gene, with consideration of the efficiencies:

$$-2\log\left(\frac{(E_{target})^{\Delta CP_{target}}}{(E_{ref})^{\Delta CP_{ref}}}\right) \qquad \text{Equation 5}$$

In Equation 5 efficiencies are included and the formula will give normalized Cq value differences between the target gene and the housekeeping gene, instead of a ratio.

The values were calculated in Excel 2010 and the most stable housekeeping gene was used as reference.

With these normalized Cq values, further ANOVA analysis could be made to analyze the acquired data statistically for each target gene. $P < 0.05$ was measured statistically significant and a p-value of < 0.10 was set as trend to significant. If a significance or trend was seen, a group to group comparison was made by an independent t-test and distribution-free Wilcoxon test due the small sample size and possibly not normal distributed samples, which is one of the suggested methods by Yuan et al. (2006). These tests were performed with PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

Results

Efficiencies of primers

All efficiencies of the primer sets were kept in the range of 90% to 100%. Table 8 shows the efficiencies of the normal primer sets that are used in the first round PCR. 15-LOX-2 produced primer dimers (Figure 8) and an efficiency could not be calculated. The efficiencies of the hemi-nested primer sets are displayed in Table 9 and showed a range of 90.1% to 96.9%.

Housekeeping genes

POLR2A seemed to be the best housekeeping gene as indicated by both Bestkeeper and NormFinder Stability Value (see Figure 2). NormFinder advised to use only one housekeeping gene. Therefore further relative quantification calculations were done with POLR2A as reference gene.

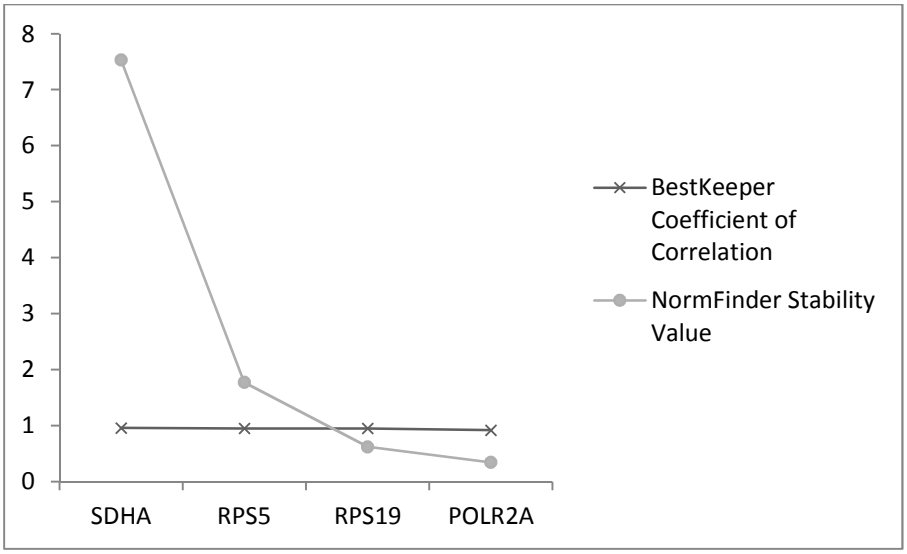


Figure 2 Gene Expression stability values by BestKeeper and NormFinder. Genes with lower NormFinder Stability and BestKeeper Coefficient of Correlation Values are more stable.

Note that a correct hemi-nested primer set for SDHA was not yet established, therefore this gene could be more stable than had been determined at this point.

Comparison of mean Cq for fresh tissue and FFPE tissue

The difference between Cq values of fresh tissue and FFPE tissue for the genes without normalization with POLR2A had a mean of 5.08 (*SD = -1.79*; mPGES not included) cycles. mPGES gave no correct amplification for the FFPE tissue (hence the inadequate shift in Figure 3), thus further inferences with this gene in the relative quantification of this gene are taken with caution.

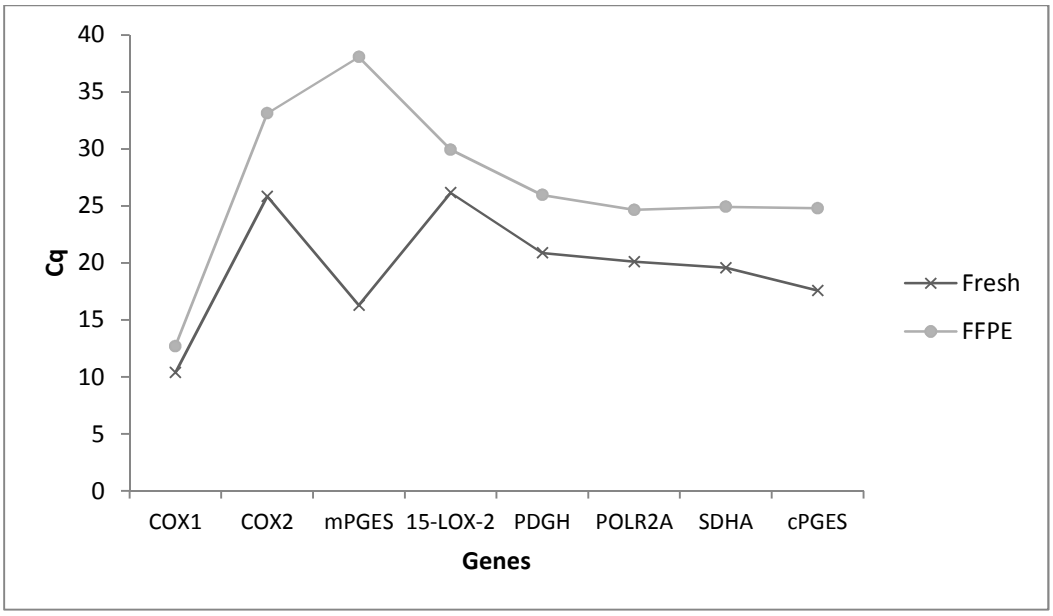


Figure 3 Comparison of mean Cq of the genes in fresh and FFPE tissue. Despite the shift, the relative level of expression is for most of the genes maintained.

With normalization of POLR2A and efficiencies, the difference between FFPE and Fresh tissue was reduced (*M = 1.56, SD = 0.843*; mPGES not included).

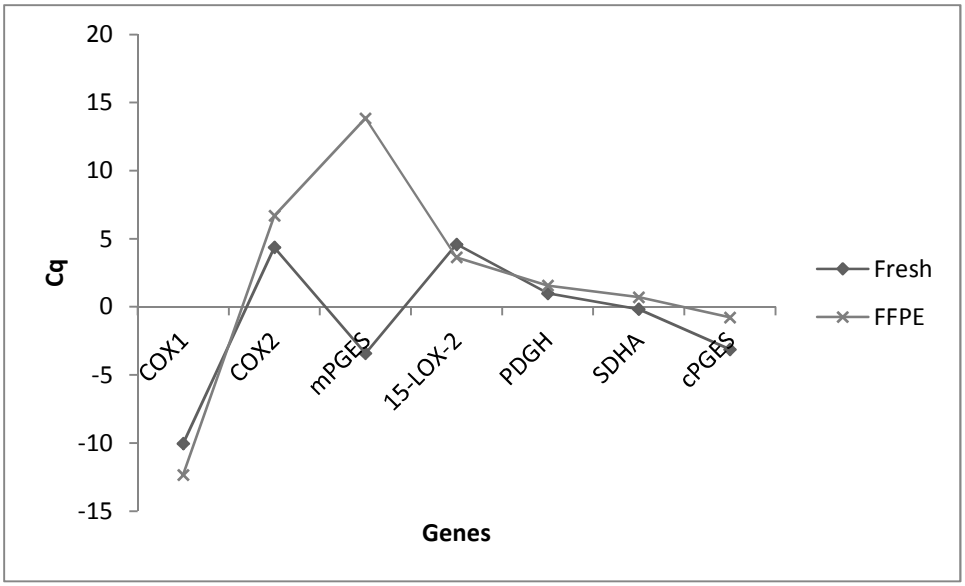


Figure 4 Normalized mean Cq values between target gene and housekeeping gene (POLR2A) in fresh and FFPE tissue.

Furthermore, the genes COX1, COX2 and cPGES are slightly more off in cycles determined by mean and standard deviation than the other genes (see Figure 5).

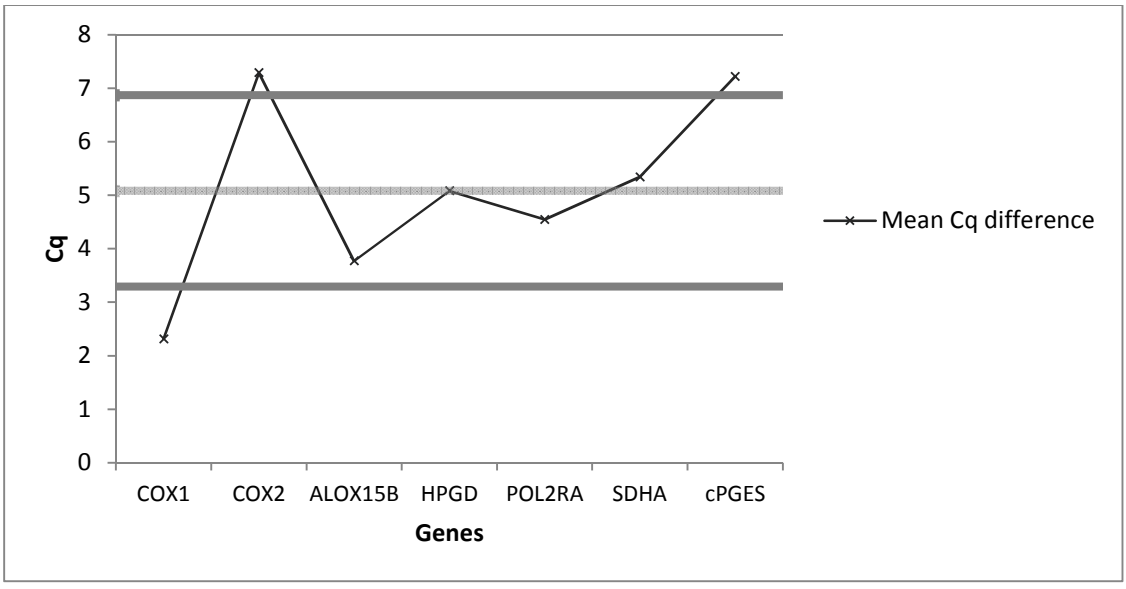


Figure 5 Mean Cq differences of the genes, without normalization. Lines are standard deviation and mean.

Relative quantification of the expression of the genes between normal, FIC and control positive tissue

The RNA amounts of the used FFPE samples are displayed in Table 10 (*M = 1261.56, SD = 941*); the integrity numbers were variable from 1.7 to 2.5 (*M = 2.17, SD = 0.245*).

Three out of eight of the FIC, four out of eight of the urolith and four out of nine of the control samples did not have any significant amplifications during the RT-qPCR and the RNA yields were low. These samples were therefore excluded for further analysis.

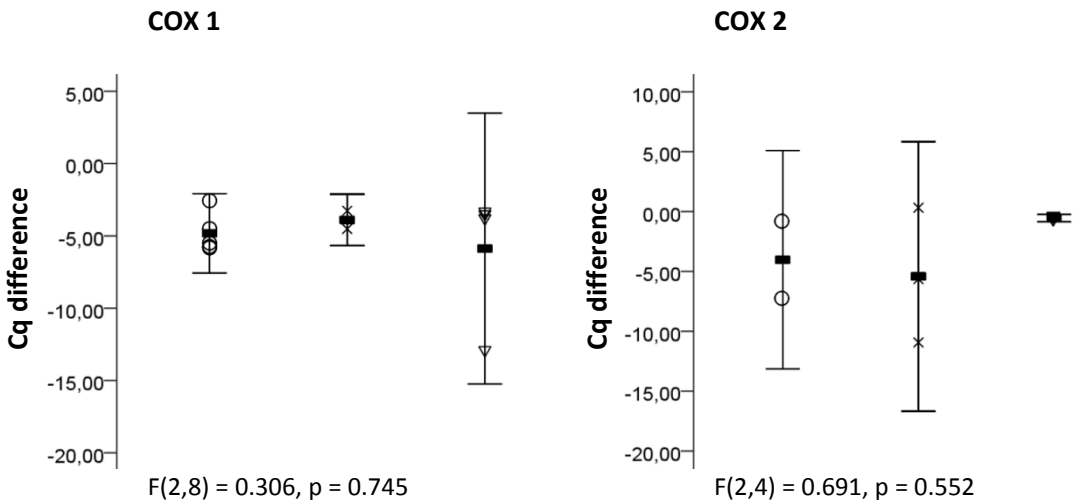
Some of the other samples gave no amplifications of some of the genes and were therefore excluded as well. All inclusions and exclusions are displayed in Table 11.

Analysis with RT-qPCR of the expression of the key enzymes showed no significant differences ($p > 0.05$) between FIC, urolith and normal cats, as can be seen in Figure 6.

COX 1, COX 2 and mPGES did not give sufficient values in all of the groups to do further analysis properly.

The standard deviations for the normalized Cq values of the groups were 3.15, 3.07 and 2.85 for FIC, urolith and normal groups respectively and thus giving a mean of 3.02.

Average intra-assay (duplicate) standard deviation across all genes was 0.51.



© R.D. Breed

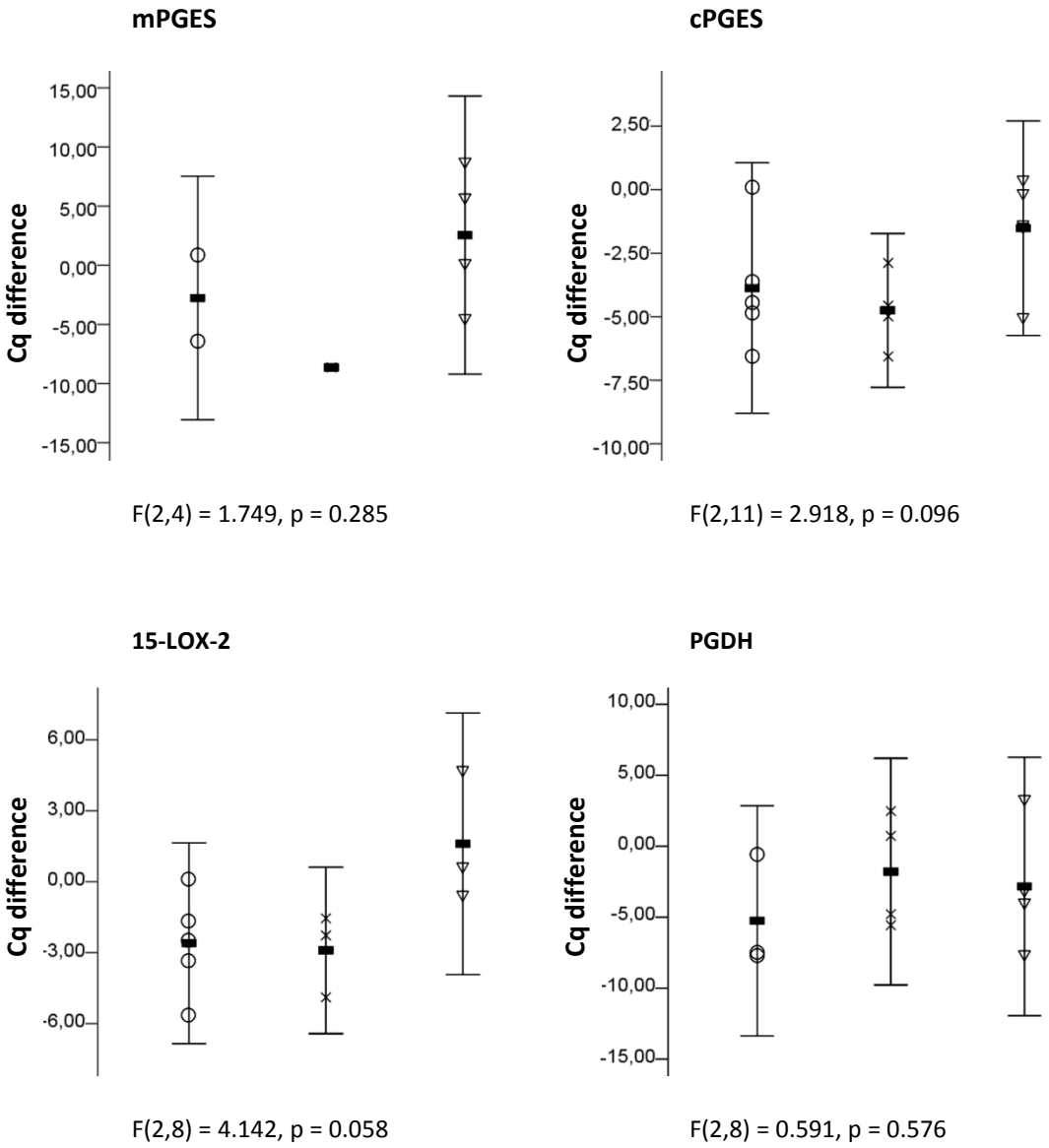


Figure 6 Relative quantification of target genes. Relative quantification of Cq values (ΔCt) of target genes COX 1, COX 2, mPGES, cPGES, 15-LOX-2 and PGDH, between FIC (○), Urolith (×) and normal (▽) cats; including efficiencies in the calculations. Means and 2 x SD are added. Under each plot are the ANOVA test results.

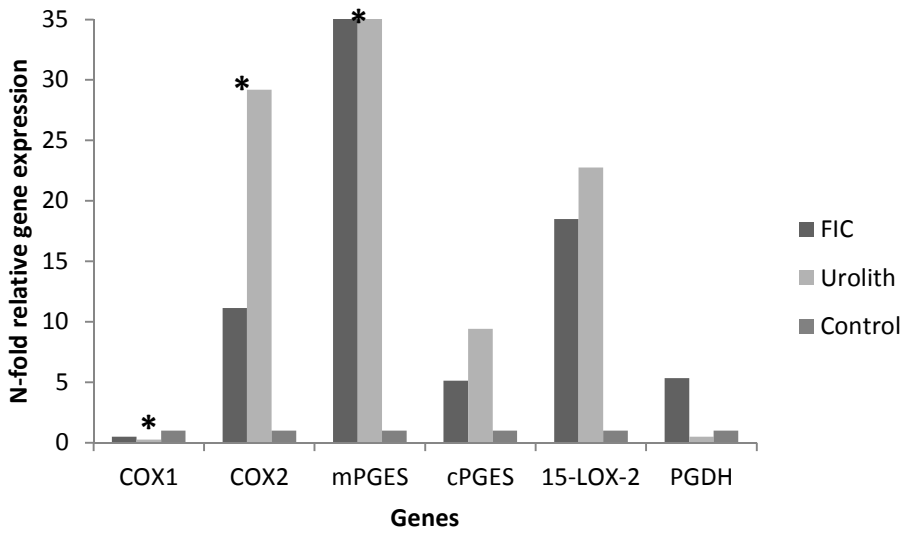


Figure 7 Relative gene expression of target genes. *No sufficient data.

However, for cPGES and 15-LOX-2 there was respectively a trending p-value of 0.096 (F(2,11) = 2.918) and 0.058 (F(2,8) = 4.142).

Independent t-tests for cPGES between FIC (*M = -3.88, SD = 2.47*) and normal cats (*M = -1.52, SD = 2.11*) was t(8) = -1.626, p = 0.143 and between urolith (*M = -4.75, SD = 1.51*) and normal cats was t(7) = -2.568, p = 0.037. Wilcoxon Rank sum test for FIC versus normal and urolith versus normal showed Z = -1.149, p = 0.251 and Z = -1.715, p = 0.086 respectively.

An independent t-test for 15-LOX-2 between FIC (*M = -2.60, SD = 2.12*) and normal cats (*M = 1.61, SD = 2.76*) resulted in t(6) = -2.445, p = 0.050 and between urolith (*M = -2.90, SD = 1.76*) and normal cats t(4) = -2.384, p = 0.076. Wilcoxon Rank sum test was Z = -1.938, p = 0.053 and Z = -1.064, p = 0.050 for FIC versus normal and urolith versus normal respectively.

Discussion

During the design of the primers and determining the efficiencies, several primers (not reported here) displayed two peaks or more in the melt curves indicating that this was not the correct product (Figure 8). Suggestions of primer dimers or hairpins are made, but the cause is not well known despite of the use of online primer software such as Multiple primer analyzer by Thermo Scientific and Oligo Analyzer 3.1 by IDT (Integrated DNA Technologies); searches on further documentation gave no answers to this problem.

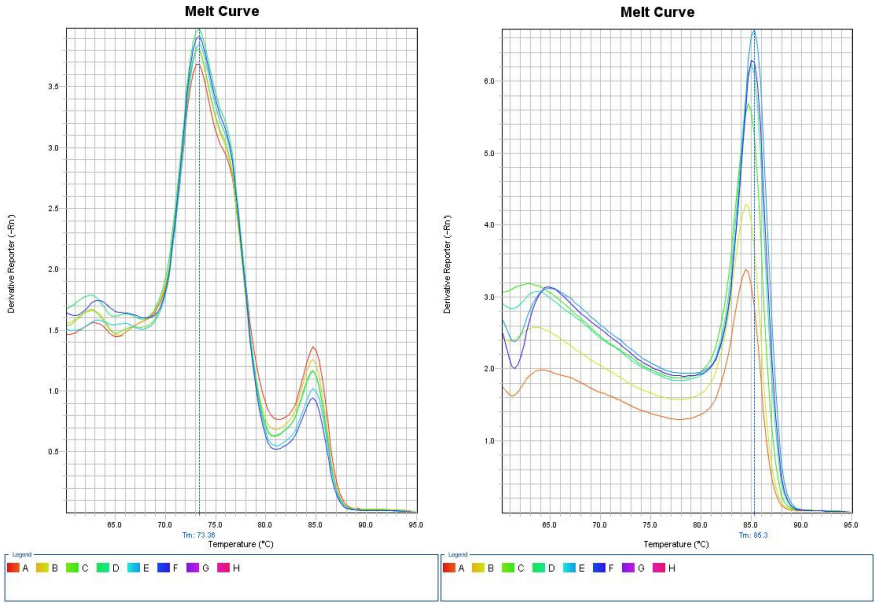


Figure 8 Meltcurves of six 10-fold serial dilutions of normal set and hemi-nested set of 15-LOX-2. The normal set meltcurve gave so-called "primer-dimer" (the smaller peak could possibly be the normal amplification product), while the hemi-nested set gave rather normal curves.

Due to difficulties finding a non-primer dimer set for 5-LOX and FLAP, these genes were not included in this study, although these genes will still be interesting to include in future researches.

As some normal sets gave these difficulties, while the hemi-nested primer set did not gave these problems, the 15-LOX-2 gene was still used in this study, under the assumption that the first round PCR will not have any influence on the relative quantification by RT-qPCR. Still conclusions regarding this gene have to be drawn carefully.

Efficiencies of the working primer sets were all within 90% to 100%. Although it is suggested that a difference of 10% could be used without correction (Schmittgen and Livak, 2008), we still included the efficiencies in our formula for quantification calculations by the Pfaffl method.

Relative quantification of small amplification products of stable genes gave no enormous differences in results for fresh frozen tissue compared to FFPE tissue, except for mPGES. This is in accordance with other studies as Doleshal et al. (2008); Sanchez-Navarro et al. (2010). With normalization, differences came closer by lower values as is more in conjunction with Lebbe et al. (2012); Li et al. (2008). Therefore we suggested that it was correct to use FFPE tissues, although some precautions have to be taken in further procedures and measurements.

Because FIC is not a lethal condition and therefore cystotomies are rarely performed, it was necessary to use archived FFPE tissues to have a sufficient number of biological replicates to allow statistical analyses. However, the use of FFPE gave some problems, because the RNA quantity and quality is much lower than of fresh frozen tissues, due the modification properties of formalin and paraffin (Dapson, 2007; Evers et al., 2011). Furthermore, some genes could not be amplified in all of the samples, possibly due very low expression levels and the possibility of inhibitors of the cDNA synthesis in some of the samples which still had good RNA yields.

Although there needs to be some precautions in making conclusions about 15-LOX-2, it is still remarkable that is approaches near significance, for both FIC and urolith cats compared to normal, unaffected cats. 15-LOX-2 is involved in the synthesis of lipoxins indirectly by the production of 15-HETE; lipoxins have anti-inflammatory effects (Buczynski et al., 2009), although more complex functions are suggested as well. Future research could confirm the alteration of expressions and indicate the relevance of this enzyme in the pathogenesis of FIC.

The ANOVA for cPGES gave a trend toward significance, although an independent t-test and Wilcoxon rank sum test of FIC compared to normal cats gave no significance or trend, while urolith to

normal cats was significant for the independent t-test and gave a trend towards significance for the Wilcoxon rank sum test.

Due the higher standard deviations among the samples (*Mean of the standard deviations of Cq of each gene and group = 3.023*) than normally is found in RT-qPCR studies in non-FFPE samples (De Cremoux et al., 2011), it is suggested that group sample sizes have to be larger in order to find significant differences. For the determined standard deviation value (approx. 3), it is possible to calculate what size the samples have to be to determine differences, using a sample size calculator (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). Depending on what difference has to be expected, the sample size needs to be increased from 16 samples (4 fold difference) to 142 samples (2 fold difference), if $\alpha = 0.05$ and power = 0.80, for a two sided test. Due to time and practical limitations during this study, it was not possible to test additional samples, but these values give suggestions for future studies with FFPE tissues.

Considering that eicosanoids could play a key role in the inflammatory reaction involved in FIC, this may have possible consequences for understanding the pathogenesis of FIC and therefore may lead to some better diagnostics and therapeutic options.

Until now, no treatment is considered to be the exclusive solution for cats with FIC. The main goal is to decrease the severity and recurrence of the symptoms. Current recommendations are stress reduction, moist food instead of dry kibbles, increasing water intake, pain treatment and in severe cases agents such as glycosaminoglycans (GAGs) and amitriptyline, but additional studies are suggested in order to create evidence-based management (Dru Forrester and Roudebush, 2007). The usage of polyunsaturated fatty acid (PUFAs) as nutrient additive is considered to have an effect on the eicosanoid pathway. Fatty acids can have pro-inflammatory or anti-inflammatory effects, depending on their role in the pathway (Saker, 2006). In dogs the use of PUFA's is studied in different inflammatory diseases (Bauer, 2011; Kearns et al., 1999), but the effects of these PUFAs in cats is not as well documented, although recent studies suggest that the use of PUFAs

eicosapentaenoic acid and docosahexaenoic acid have altering effects on the eicosanoid expression with less inflammatory eicosanoids PGE3 and LTB5, compared to eicosanoids of the arachidonic acid pathway (i.e. PGE2 and LTB4) (Corbee et al., 2012a; Corbee et al., 2012b). A randomized controlled clinical trial of diet enrichment with omega-3 PUFAs and antioxidants in cats with acute FIC demonstrated that the frequency of the recurring episodes of lower urinary tract symptoms were significantly reduced (Kruger et al., 2013). These outcomes support the hypothesis that eicosanoids may influence the expression of FIC and this emphasizes the need for future studies to clarify the role of eicosanoids in the pathogenesis of FIC.

Conclusions

No significant differences in expression of the eicosanoid enzymes studied, were found between FIC, urolith and control cats, but for cPGES and 15-LOX-2 there was a trend towards significance ($p < .10$). If the sample size is increased (to increase the power of the study) in further research on the expression of 15-LOX-2 in FIC cats, significant differences in expression in cats with FIC could be observed.

A method has been established to determine mRNA expression in FFPE tissues, although many factors have to be taken into account, especially in the degradation of the RNA and possible high standard deviations among the samples.

With help of sample size calculations based upon the variation observed in the study, possible future studies could use these to determine which samples sizes have to be used for finding significant differences in the mRNA expression of FFPE tissues with RT-qPCR quantifications.

Furthermore, primer sets of good quality with high efficiencies were created for some key eicosanoid enzymes in cats, which can be used in studies of other inflammatory conditions in cats.

© R.D. Breed

Bibliography

Antonov, J., Goldstein, D.R., Oberli, A., Baltzer, A., Pirotta, M., Fleischmann, A., Altermatt, H.J., Jaggi, R., 2005. *Reliable gene expression measurements from degraded RNA by quantitative real-time PCR depend on short amplicons and a proper normalization*. Laboratory Investigation 85, 1040-1050.

Bauer, J.E., 2011. *Therapeutic use of fish oils in companion animals*. Journal of the American Veterinary Medical Association 239, 1441-1451.

Buczynski, M.W., Dumlao, D.S., Dennis, E.A., 2009. *Thematic Review Series: Proteomics. An integrated omics analysis of eicosanoid biology*. Journal of Lipid Research 50, 1015-1038.

Chomczynski, P., Sacchi, N., 2006. *The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on*. Nature Protocols 1, 581-585.

Corbee, R., Barnier, M., van de Lest, C., Hazewinkel, H., 2012a. *The effect of dietary long-chain omega-3 fatty acid supplementation on owner's perception of behaviour and locomotion in cats with naturally occurring osteoarthritis*. Journal of Animal Physiology and Animal Nutrition.

Corbee, R., Booij-Vrieling, H., van de Lest, C., Penning, L., Tryfonidou, M., Riemers, F., Hazewinkel, H., 2012b. *Inflammation and wound healing in cats with chronic gingivitis/stomatitis after extraction of all premolars and molars were not affected by feeding of two diets with different omega-6/omega-3 polyunsaturated fatty acid ratios*. Journal of Animal Physiology and Animal Nutrition 96, 671-680.

Cronin, M., Pho, M., Dutta, D., Stephans, J.C., Shak, S., Kiefer, M.C., Esteban, J.M., Baker, J.B., 2004. *Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay*. The American Journal of Pathology 164, 35-42.

Dapson, R., 2007. *Macromolecular changes caused by formalin fixation and antigen retrieval*. Biotechnic & Histochemistry 82, 133-140.

De Cremoux, P., Valet, F., Gentien, D., Lehmann-Che, J., Scott, V., Tran-Perennou, C., Barbaroux, C., Servant, N., Vacher, S., Sigal-Zafrani, B., 2011. *Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients*. BMC Cancer 11, 215.

Defauw, P.A., Van de Maele, I., Duchateau, L., Polis, I.E., Saunders, J.H., Daminet, S., 2011. *Risk factors and clinical presentation of cats with feline idiopathic cystitis*. Journal of Feline Medicine & Surgery 13, 967-975.

Derveaux, S., Vandesompele, J., Hellemans, J., 2010. *How to do successful gene expression analysis using real-time PCR*. Methods 50, 227-230.

Doleshal, M., Magotra, A.A., Choudhury, B., Cannon, B.D., Labourier, E., Szafranska, A.E., 2008. *Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues*. The Journal of Molecular Diagnostics 10, 203-211.

Dru Forrester, S., Roudebush, P., 2007. *Evidence-based management of feline lower urinary tract disease*. The Veterinary Clinics of North America. Small Animal Practice 37, 533-558.

Evers, D.L., He, J., Kim, Y.H., Mason, J.T., O'Leary, T.J., 2011. *Paraffin embedding contributes to RNA aggregation, reduced RNA yield, and low RNA quality*. The Journal of Molecular Diagnostics 13, 687-694.

Kearns, R.J., Hayek, M.G., Turek, J.J., Meydani, M., Burr, J.R., Greene, R.J., Marshall, C.A., Adams, S.M., Borgert, R.C., Reinhart, G.A., 1999. *Effect of age, breed and dietary omega-6 (n-6): omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs*. Veterinary Immunology and Immunopathology 69, 165-183.

© R.D. Breed

Khan, M., Thompson, C., Mumtaz, F., Jeremy, J., Morgan, R., Mikhailidis, D., 1998. *Role of prostaglandins in the urinary bladder: an update*. Prostaglandins, Leukotrienes and Essential Fatty Acids 59, 415-422.

Khan, O., Kruger, J., Fitzgerald, S., Liebert, M., 2011. Expression of 15-hydroxyprostaglandin dehydrogenase (PGDH) in urothelium of cats with chronic idiopathic cystitis, In Proceedings: Journal of Veterinary Internal Medicine, pp. 724-724.

Kruger, J., Lulich, J., Merrill, J., MacLeay, J., Paetau-Robinson, I., Steck, S., Davidson, S., Osborne, C., 2013. A year-long prospective, randomized, double-masked study of nutrition on feline idiopathic cystitis (FIC), In Proceedings: Annual ACVIM Forum, Seattle WA, p. 504.

Kruger, J.M., Osborne, C.A., Lulich, J.P., 2009. *Changing paradigms of feline idiopathic cystitis*. The Veterinary Clinics of North America. Small Animal Practice 39, 15-40.

Lebbe, C., Guedj, M., Basset-Seguin, N., Podgorniak, M.P., Menashi, S., Janin, A., Mourah, S., 2012. *A reliable method for the selection of exploitable melanoma archival paraffin embedded tissues for transcript biomarker profiling*. PloS ONE 7, e29143.

Li, J., Smyth, P., Cahill, S., Denning, K., Flavin, R., Aherne, S., Pirotta, M., Guenther, S.M., O'Leary, J.J., Sheils, O., 2008. *Improved RNA quality and TaqMan® Pre-amplification method (PreAmp) to enhance expression analysis from formalin fixed paraffin embedded (FFPE) materials*. BMC Biotechnology 8, 10.

Pfaffl, M.W., 2001. *A new mathematical model for relative quantification in real-time RT-PCR*. Nucleic Acids Research 29, e45.

Pfaffl, M.W., 2004. Quantification strategies in real-time PCR, In: AZ of Quantitative PCR. International University Line, pp. 89-113.

Rastogi, P., Rickard, A., Dorokhov, N., Klumpp, D.J., McHowat, J., 2008. *Loss of prostaglandin E2 release from immortalized urothelial cells obtained from interstitial cystitis patient bladders*. American Journal of Physiology-Renal Physiology 294, F1129-F1135.

Saker, K.E., 2006. Evidence-Based Use of Fatty Acids in Feline Disease, In: Proceedings of the 2006 Hill's Global Symposium of Feline Care, pp. 24-30.

Sanchez-Navarro, I., Gamez-Pozo, A., Gonzalez-Baron, M., Pinto-Marin, A., Hardisson, D., Lopez, R., Madero, R., Cejas, P., Mendiola, M., Espinosa, E., Vara, J.A., 2010. *Comparison of gene expression profiling by reverse transcription quantitative PCR between fresh frozen and formalin-fixed, paraffin-embedded breast cancer tissues*. BioTechniques 48, 389-397.

Schmittgen, T.D., Livak, K.J., 2008. *Analyzing real-time PCR data by the comparative C(T) method*. Nature Protocols 3, 1101-1108.

Snyder, C.L., Venta, P.J., Kruger, J.M. 2012. Gene expression analysis of enzymes involved in eicosanoid biosynthesis and catabolism in feline idiopathic cystitis, Michigan State University.

Specht, A., Kruger, J., Fitzgerald, S., 2004. *Histochemical and immunohistochemical features of chronic feline idiopathic cystitis*. Journal of Veterinary Internal Medicine 18, 416.

Specht, A., Kruger, J., Fitzgerald, S., Kiupel, M., 2003. *Light microscopic features of chronic feline idiopathic cystitis*. Journal of Veterinary Internal Medicine 17, 436.

Stanley, K.K., Szewczuk, E., 2005. *Multiplexed tandem PCR: gene profiling from small amounts of RNA using SYBR Green detection*. Nucleic Acids Research 33, e180.

Treutlein, G., Dorsch, R., Euler, K.N., Hauck, S.M., Amann, B., Hartmann, K., Deeg, C.A., 2012. *Novel Potential Interacting Partners of Fibronectin in Spontaneous Animal Model of Interstitial Cystitis*. PloS ONE 7, e51391.

Yuan, J.S., Reed, A., Chen, F., Stewart, C.N., 2006. *Statistical analysis of real-time PCR data*. BMC Bioinformatics 7, 85.

Appendix

Table 1 Date ranges of collected tissue

Tissue group	Collection date
FIC group	8/29/1997 – 12/26/2011
Stone blocks	11/12/1989 – 12/15/2012
Control group	1/4/1995 – 1/17/2013

Table 2 RNA extraction and removal of DNA

Step	Description
1.	Add 4.4 µL extracted RNA with 0.6 µL booster buffer (50% glycerol + 50% H_2O RNase free)
2.	Add 1 µL DNase
3.	Incubate for 20 minutes at room temperature
4.	Incubate for 15 minutes at 80°C
5.	Put on ice

Table 3 Reverse transcription (RNA to cDNA)

Step	Description
1.	Combine following in a 0.5 µL tube:

Component	Amount
RNA	2 µL
Random hexamers (N6)	1.5 µL
10 mM dNTP mix	3 µL
RNase free water	35.5 µL

2. Incubate the tube at 65°C for 5 min, let it cool down at 20°C for 5 min.

3. Place on ice for 2 min.

4. Add the following components:

Component	Amount
5X FS buffer	12 µL
DTT	3 µL
Superscript III	3 µL

5. Incubate as follows:
   60 min at 25°C
   15 min at 70°C

Table 4 Normal primer sets with amplicon size

Protein name	Gene name	Forward primer	Reverse primer	Amplicon size
COX-1	PTGS1	CAAGAGGTTCGGCCTGAG	GGCATCGATGTCTCCATACAG	100
COX-2	PTGS2	GATGAGCGGTTGTTCCAGAC	GAAGTGATAGCCACTCAAGTGTTG	99
15-LOX-2	ALOX15B			
mPGES	PTGES	AGTACTGCCGGAGTGACCAG	AGTAAACGAGGCCCAGGAAC	99
cPGES	PTGES3	TTACGAAAAGGCGAATCTGG	CTGAATCATCCTCCCAGTCTTTC	115
PGDH	HPGD	CGCAGAAGACTCTGTTCATCC	TCACTCCAGCGTTATTGACC	120
SDH	SDHA	AAATGGAGAATGTCGTGGTG	GGCAGATGTGCAGCTGAAGT	126
	RPS5	CATCAAGACCATTGCTGAGTG	CACGTTCCAGCTCATCCTTC	103
	RPS19	ACGTGAACCAGCAGGAGTTC	TTGACAGTGTCCACCCATTC	91
	POLR2A	CAGTGCGCAAATTCACAAAG	CCACGTGGACAGGAACATC	80

Table 5 Hemi-nested primer sets with amplicon size and efficiencies

Protein name	Gene name	Forward primer	Reverse primer	Amplicon size
COX-1	PTGS1	ACCCTACACGTCCTTTCAGGA	GGCATCGATGTCTCCATACAG	82
COX-2	PTGS2	GTTCCAGACGAGCAGGCTAA	GAAGTGATAGCCACTCAAGTGTTG	88
15-LOX-2	ALOX15B			
mPGES	PTGES	ACGTAGATCGCTGCCTCAGA	AGTAAACGAGGCCCAGGAAC	79
cPGES	PTGES3	TCATGGCCAAGGTTAACAAA	CTGAATCATCCTCCCAGTCTTTC	90
PGDH	HPGD	AGTGCGATGTGGCTGACC	TCACTCCAGCGTTATTGACC	99
SDH	SDHA	TTGCGCTGTGCATAGAAGAC	GGCAGATGTGCAGCTGAAGT	104
	RPS5	CCTAGCAGATGAGCTCATCAATG	CACGTTCCAGCTCATCCTTC	80
	RPS19	TCAGAGCTCTGGCAGCCTT	TTGACAGTGTCCACCCATTC	70
	POLR2A	CAAAGAGAGACGTCTTCTTGGAG	CCACGTGGACAGGAACATC	60

Table 6 First round PCR procedure for RT-qPCR efficiencies

Step	Description
1.	Combine following in a 0.5 µL tube:

Component	Amount
Water	16.15 µL
10X PCR buffer	2.5 µL
1 mM dNTPs	2.5 µL
50 mM $MgCl_2$	0.75 µL
Forward primer 2 µM	1 µL
Reverse primer 2 µM	1 µL
Taq polymerase (5 U/ul)	0.1 µL
DNA (10-50 ng/ul)	1 µL

2. Incubate as follows:
   42 cycles:
   1 minute 94 °C
   2 minutes 59 °C
   3 minutes 72 °C

Table 7 First round PCR procedure for RT-qPCR

Step	Description
1.	Combine the following in a 0.5 µL tube to create a cocktail:

Component	Amount
Normal set forward primers 100 µM	2 µL
Normal set reverse primers 100 µM	2 µL
Water	Add so that total will be 100 µL

2. Combine the following in a 0.5 µL tube:

Component	Amount
Water	16.15 µL
10X PCR buffer	2.5 µL
1 mM dNTPs	2.5 µL
50 mM $MgCl_2$	0.75 µL
Cocktail of primers	2 µL
Taq polymerase (5 U/ul)	0.1 µL
DNA (10-50 ng/ul)	1 µL

3. Incubate as follows:
   42 cycles:
   10 seconds 95 °C
   20 seconds 60 °C
   20 seconds 72 °C

© R.D. Breed

Table 8 Efficiencies of normal primer sets.

Gene name	Efficiency%
PTGS1	98
PTGS2	95.4
ALOX15B	N/A*
PTGES	94
PTGES3	94.2
HPGD	96.8
SDHA	96.3
RPS5	94
RPS19	99
POLR2A	100

***No correct amplification and meltcurves reported.**

Table 9 Efficiencies of hemi-nested primer sets

Gene name	Efficiency%
PTGS1	90.1
PTGS2	90.5
ALOX15B	90.1
PTGES	99.6
PTGES3	92.0
HPGD	98.5
SDHA	99.4
RPS5	95.4
RPS19	96.5
POLR2A	96.9

Table 10 RIN integrity values and RNA quantification

Sample no.	Case Number	Group	Total RNA amount (ng)	RIN integrity
1.	SL-06-0000415	FIC	768	
2.	SP-07-0001526	FIC	213	
3.	SP-07-0006413	FIC	615	
4.	SP-08-0000558	FIC	1518	2.3
5.	SP-10-0005722	FIC	663	
6.	Annie Vertex	FIC	2496	2.3
7.	11-0009479	FIC	3471	2.5
8.	10-0007506	FIC	1227	1.7
9.	1928459	Urolith	171	
10.	MC-11A	Urolith	1842	
11.	SP-11-0003773	Urolith	1302	
12.	12-0010363	Urolith	327	
13.	09-0003406	Urolith	2241	2.3
14.	10-0002634	Urolith	1095	2.1
15.	08-0008925	Urolith	831	2.2
16.	1496610	Urolith	396	
17.	CCAS-01	Control	2883	2.3
18.	CCAS-03	Control	2061	
19.	CCAS-04	Control	1884	
20.	CCAS-06	Control	1641	1.8
21.	4	Control	210	
22.	272	Control	624	
23.	88	Control	384	
24.	91	Control	279	
25.	?	Control	2397	2.2

Table 11 Samples that were included and excluded in relative quantifications

Sample no.	Case Number	Included?	Comments
1.	SL-06-0000415	No	No sufficient data of housekeeping gene
2.	SP-07-0001526	No	Too low RNA yield
3.	SP-07-0006413	Yes	COX2, mPGES, PGDH and SDHA too low to measure
4.	SP-08-0000558	Yes	COX2, mPGES too low to measure
5.	SP-10-0005722	Yes	COX2, PGDH too low to measure
6.	Annie Vertex	No	Cq values too low to measure and high variability among measured Cq values.
7.	11-0009479	Yes	
8.	10-0007506	Yes	mPGES too low to measure
9.	1928459	No	Too low RNA yields
10.	MC-11A	No	No sufficient data of housekeeping gene
11.	SP-11-0003773	Yes	COX1, COX2, mPGES, PGDH too low to measure
12.	12-0010363	No	Too low RNA yield
13.	09-0003406	Yes	
14.	10-0002634	Yes	mPGES, 15-LOX-2 too low to measure
15.	08-0008925	Yes	COX1, mPGES too low to measure
16.	1496610	No	Too low RNA yield
17.	CCAS-01	Yes	
18.	CCAS-03	Yes	COX2 too low to measure
19.	CCAS-04	Yes	COX2, 15-LOX-2, PGDH too low to measure

20.	CCAS-06	Yes	COX1, COX2, 15-LOX-2 too low to measure
21.	4	No	Too low RNA yield
22.	272	No	No clear RNA peak at RNA yield measurements
23.	88	No	Too low RNA yield
24.	91	No	Too low RNA yield
25.	?	Yes	mPGES too low to measure