DISSERTATION

INVESTIGATION OF TREATMENT WITH MOLNUPIRAVIR AND IMMUNE STIMULANTS FOR USE IN THE MANAGEMENT OF FELINE INFECTIOUS PERITONITIS AND ASSOCIATED COMORBIDITIES

Submitted by

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ABSTRACT

INVESTIGATION OF TREATMENT WITH MOLNUPIRAVIR AND IMMUNE STIMULANTS FOR USE IN THE MANAGEMENT OF FELINE INFECTIOUS PERITONITIS AND ASSOCIATED COMORBIDITIES

Feline coronavirus (FCoV) belongs to the order *Nidovirales*; family *Coronaviridae*; subfamily *Coronavirinae*; genus *Alphacoronavirus*; species *Alphacoronavirus* 1. Feline coronavirus exists in two biotypes; feline enteric coronavirus (FECV), which mainly replicates in the enteric epithelial cells, and feline infectious peritonitis virus (FIPV), which results in lethal infection with efficient systemic replication in monocytes or macrophages. Efficient FCoV replication in activated monocytes and macrophages is a key event in feline infectious peritonitis (FIP) pathogenesis. Fortunately, only a small proportion of FCoV-infected cats go on to develop FIP which typically manifests as a vasculopathy resulting in ('effusive') form (up to 80% of FIP cases have effusions), or granuloma formation resulting in ('non-effusive') mass lesions, or a combination of the two. The effusive form of disease generally is believed to develop in cats with poor cell-mediated immune responses, and the non-effusive form develops in cats with partial cell-mediated immunity. Recently, new antiviral drugs like the nucleoside analog GS-441524 and molnupiravir (MPV) has been used in successful treatment of FIP.

However, more treatment options for cats with FIP, especially those that not responding or relapse, are needed and the goal of this work was to evaluate the use of nucleoside analogue molnupiravir and immune stimulants to determine the best treatment

protocols for cats with FIP. We compared two different immune stimulants for antiviral activity in cats: a TLR 2/6-activating compound polyprenyl immunostimulant; (PI) and a liposome Toll-like receptor 3/9 agonist complexes (LTC) to determine relative abilities to stimulate immune responses in vitro and to study the effects of treatment on immune responses in healthy cats. In these studies, both LTC and PI protocols induced immune enhancing effects, suggesting a possible clinical use for the management of chronic infectious diseases like FIP. We concluded that activating the TLR 3 and 9 pathways (LTC) induced superior broad interferon production in vitro than the activation of the TLR 2 and 6 pathways (PI).

We also determined the pharmacokinetics of molnupiravir in cats with naturally occurring FIP by measuring MPV and EIDD-1931 (β-D-N4-hydroxycytidine; NHC) serum levels in seven cats diagnosed with naturally occurring FIP treated with MPV. The mean NHC concentrations at all time points were at least 4 times the reported in vitro EC_{50} for feline coronavirus strains and twice daily dosing for seven days did not lead to accumulation of drug within the serum. Minimal accumulation of drug was seen in trough concentrations following twice-daily dosing for 7 days. These results supported the use of MPV in the treatment of FIP.

We have conducted a prospective, open-label longitudinal single-center clinical trial with MPV in cats with FIP. A total of 78% cats survived to 6 months and 22% cats died or were euthanized. We also found that the median total bilirubin concentrations were significantly different ($p= 0.0007$) between the survivors vs non-survivors. Relapses occurred in 12% of the cats and all achieved remission during a second course of

treatment. No adverse effects necessitated discontinuation of the treatment. This study showed effectiveness of using 12 week therapy with MPV in treatment of FIP.

While most cats with FIP were euthanized in the past; more cats are now being treated with antiviral drugs. As a result of this, new disease processes that could be associated with FIP are being recognized; for example, immune-mediated hemolytic anemia (IMHA), myocarditis or other cardiac changes, and other comorbidities and the goal of this study was to evaluate the development of IMHA and myocarditis in cats with FIP and its impact on survival and prognosis of these cats.

We have evaluated 45 cats with associative IMHA to FIP in a multicentric study. The median hematocrit for these cats was 18% and anemia was non-regenerative in 36/45 cats with concurrent thrombocytopenia was present in 18/45 cats. All 45 cats were treated with nucleoside analogs and 44 cats with glucocorticoids and at the time of last follow-up 33 cats had survived while 12 had died or were euthanized. Although FIP is likely an uncommon cause of associative IMHA, as more cats with FIP are treated with antiviral therapy, it is important to consider IMHA as a possible cause of anemia in cats with FIP.

We documented the cardiac changes in cats diagnosed with FIP using echocardiography and cardiac troponin I (cTnI). Twenty cats diagnosed with FIP and tested negative for concurrent infections associated with myocarditis underwent an echocardiographic examination. The left ventricular posterior wall from right parasternal long axis and short axis was thickened in 55% (11/20) and 25% (5/20) of cats, respectively. The median cTnI at initial evaluation was 0.37 ng/ml (IQR 0.20-0.83) and recheck of cTnI performed at 12 weeks following antiviral therapy in 6 cats that initially

had elevated cTnI showed normalized cTnI to <0.20 ng/ml. Cats with FIP can present with elevations in cTnI and ventricular wall thickening, which are suggestive of myocarditis and/or myocardial injury.

The results of the studies described the use of antivirals and immune stimulants in FIP and outlined the possible comorbidities clinicians will be facing during the FIP treatment. Further investigation into FIP treatment with antivirals such as nucleoside analogues and protease inhibitors and immune stimulants are needed to increase the success rate in treatment of critically ill cats and prevent relapses in the future. Moreover, studies evaluating comorbidities in cats with FIP such as IMHA, myocarditis, sepsis and gastrointestinal disease are needed to better understand how to manage these cases.

ACKNOWLEDGEMENTS

This work would not have been possible without the support and collaboration of a team of colleagues and friends. I am grateful to everyone who helped me achieve this goal and supported me on my journey in pursuit of saving the lives of many cats from the lethal feline infectious peritonitis virus.

First, I would like to acknowledge the mentorship of Michael R. Lappin. His enthusiasm for science and belief in me as a feline internist have been very important for my career and I look forward to continuing to work together as there is still much more to do in the world of feline infectious diseases.

Thank you to my co-advisor, Steve Dow, whose guidance has been invaluable. He has been a constant source of encouragement and positivity through many ups and downs of my clinical research, and I have learned a lot from him on my academic path.

Thank you to my committee members, Kelly Santangelo and Gregg Dean. Their scientific input and support have given me many experiences throughout this training program. I am inspired by their career accomplishments and look forward to ongoing collaborations with them.

The Lappin lab team: Jen Hawley, Melissa Brewer and Arianne Morris deserves my deepest thanks and appreciation for helping me with my laboratory work. They kept up my spirits in the darker days of my PhD when things did not always look promising. Without them, this work would not have been possible. I would also like to thank McKenna Willis and Kaci Shaw for helping out with the clinical trials patients as well as other studies, without their help, I could not have completed most of my projects.

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I am grateful for the financial support from Merck Animal Health, who funded my last three years at Colorado State University. I was fortunate to have this industry partner that is dedicated to advancing feline health. I also gratefully acknowledge funding support from the Center for Companion Animal Studies. A special thank you to the EveryCat Health Foundation that has been supporting my FIP research. Without their continued funding, it would not have been possible to complete my clinical trials. I appreciate everything they've done for FIP research worldwide.

None of this work would have been possible without the cats and their families who participated in the clinical trials. I am forever grateful that these dedicated people entrusted me with their cats' care and together we fought this lethal disease. The love for their cats resulted in dedication that involved multiple visits to CSU's Veterinary Teaching Hospital, giving medications (on many occasions lot of medications!) to their cats at least twice a day and keeping me updated on their progress. All these cats have contributed tremendously to FIP research and most of them successfully fought this previously lethal disease. Those who sadly lost their fight to this disease will remain in my memory forever.

I have learned more than I could ever have imagined from all of my patients, and I was inspired by their unbelievable strength to fight and their will to live. I am privileged to be part of this miracle in the history of feline medicine.

Outside of CSU and the Veterinary Teaching Hospital, I would like to acknowledge my family, specifically my grandparents, who always supported my passion, my dreams and my career goals. I would not be here today without their unwavering love and support. I would also like to thank my dear friend, Cynthia Cohen, for her encouragement and always believing in me. It has meant so much to me to have someone believing in me even when I did not believe in myself.

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My academic, research, and clinical work would not have been possible without the mentorship and support of Danielle Gunn-Moore. Her love and dedication to feline medicine inspired me as a veterinary student and continues to inspire me daily in my work. Thanks to her encouragement and guidance, I came to CSU to pursue my internal medicine residency and continue FIP research.

DEDICATION

This dissertation is dedicated to the memory of my beloved grandparents, Milan Cerny and Alena Cerna. They always believed in me and supported me in every possible way. In my grandpa's mind, nothing was ever impossible, you simply had to work harder to achieve your goals, I will be forever grateful for everything he taught me and for the person he helped me become.

I miss you both every single day, but you are forever in my heart and mind.

I would also like to dedicate this work to my cats, that I loved and sadly lost to feline infectious peritonitis. Losing them to this cruel disease made me want to dedicate my career to finding a cure for FIP and continues to drive my passion for feline medicine to ensure that no other cats have to suffer and die from this disease.

You all remain forever in my heart.

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Chapter 1: LITERATURE REVIEW

1.1 Overview of feline coronavirus

Feline coronavirus (FCoV) belongs to the order *Nidovirales*; family *Coronaviridae*; subfamily *Coronavirinae*; genus *Alphacoronavirus*; species *Alphacoronavirus* 1, which also includes the enteritis-causing canine coronavirus (CCoV), transmissible gastroenteritis virus and porcine respiratory coronavirus (1, 2). The newly emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is very distinct and different from FCoV, belonging to a different genus: the genus *Betacoronavirus* (3). It is an enveloped, positive, single stranded RNA virus. The genome contains over 29,000 nucleotides and 11 open reading frames (ORFs) that code structural, non-structural, and accessory genes. FCoV is readily inactivated by most disinfectants, steam, and washing at 60 °C (4). A schematic diagram of the FCoV genome is shown in Figure 1.1 and 1.2 The 5′ two-thirds of the positive-sense coronavirus genome consist of two overlapping open reading frames (ORFs), 1a and 1b, that encode non-structural polyprotein (pp) 1 (pp1a and pp1b). The polyproteins are cleaved into individual non-structural proteins (nsps), including RNA-dependent RNA polymerase that plays a role in viral replication. ORF 1a also encodes for viral proteases, including the viral 3C-like protease, which is a target for antiviral therapy. The other third of the genome consists of ORFs encoding structural proteins, a spike (S), a membrane (in the FCoV membrane), a nucleocapsid (the protein wrapped around the FCoV genome), an envelope (also in the FCoV membrane) and non-structural accessory proteins 3a, 3b, 3c, 7a and 7b (5, 6). Non-structural proteins are involved in the replication of the virus and modification of the host

immune response but are not incorporated into the mature virus particle. The RNA-dependent RNA polymerase makes a full-length negative-strand RNA copy of the genome as well as a nested set of smaller subgenomic RNAs with a common 3′ end (7). These negative-strand RNAs serve as templates for new positive-sense genomes and positive-sense subgenomic mRNAs. The 5′ most ORF of each subgenomic mRNA is predominantly used for encoding the proteins, even though the subgenomic mRNAs have more than one coding sequence.

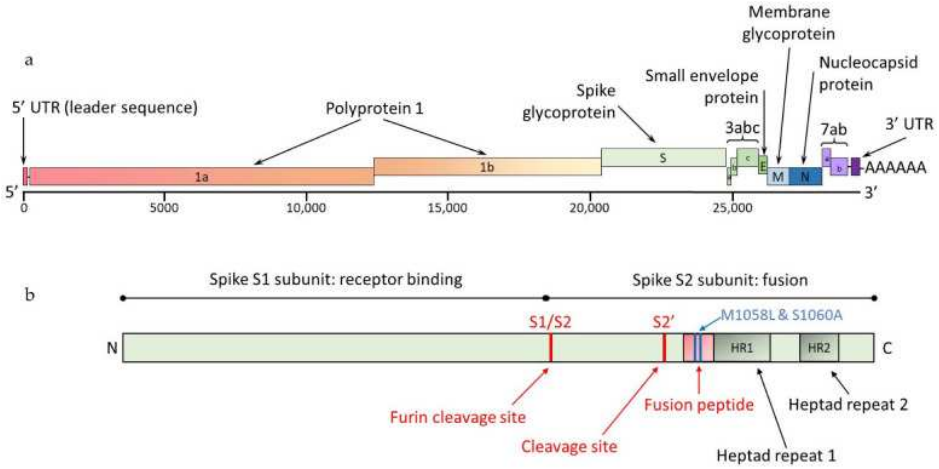


Figure 1.1. Schematic diagrams of type I FCoV: (**a**) Schematic FCoV genome. The FCoV genome of 27–32 kilobases encodes a replicase polyprotein, four structural proteins (spike (S), membrane (M), nucleocapsid (N) and envelope €) and non-structural accessory proteins 3a, 3b and 3c and 7a and 7b. UTR indicates an untranslated region. (**b**) Schematic FCoV spike protein sequence showing the division into the S1 and S2 subunits representing the receptor-binding and fusion domains, respectively, with N- and C-terminals shown. The S1/S2 and S2′ sites represent cleavage sites (in red), and the fusion peptide domain is also shaded in red; the positions of the M1058 and S1060 amino acid residues are also shown (blue lines). Image courtesy of Emi Barker, Langford Vets, University of Bristol, UK (a) and Séverine Tasker, University of Bristol, UK (b). Taken from: Tasker S, Addie DD, Egberink H, Hofmann-Lehmann R, Hosie MJ, Truyen U, Belák S, Boucraut-Baralon C, Frymus T, Lloret A, Marsilio F, Pennisi MG, Thiry E, Möstl K, Hartmann K. Feline Infectious Peritonitis: European Advisory Board on Cat Diseases Guidelines. Viruses. 2023 Aug 31;15(9):1847.

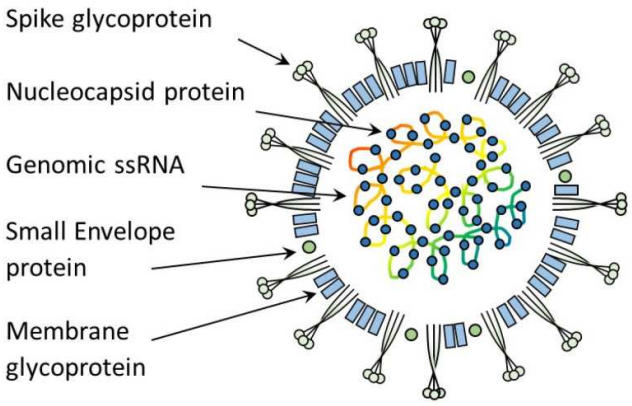


Figure 1.2. Schematic diagram of FCoV structure showing single-stranded (ss) RNA and the structural proteins: spike, envelope, membrane and nucleocapsid proteins. Image courtesy of Emi Barker, Langford Vets, University of Bristol, UK. Taken from: Tasker S, Addie DD, Egberink H, Hofmann-Lehmann R, Hosie MJ, Truyen U, Belák S, Boucraut-Baralon C, Frymus T, Lloret A, Marsilio F, Pennisi MG, Thiry E, Möstl K, Hartmann K. Feline Infectious Peritonitis: European Advisory Board on Cat Diseases Guidelines. Viruses. 2023 Aug 31;15(9):1847.

Feline coronavirus types

FCoV has types I and II and they differ in their ability to grow in vitro as well as genomic properties, and antigenicity (8). Type I FCoV is more prevalent in most parts of the world, with a prevalence of 80–95% (9, 10). Type II FCoV is less common in the cat in vivo, but is easier to be isolated and grown in cell cultures in vitro (2). Both type I and type II FCoV can occur as less-virulent FCoV and as FIP-associated FCoV (11). Type II FCoV strains arise from recombination between type I FCoV and CCoV (Figure 1.3), usually including the spike of CCoV, varying amounts of adjacent 3a, 3b and 3c genes, and envelope genes, but not the nucleocapsid gene, which remains of FCoV origin (6, 12, 13).

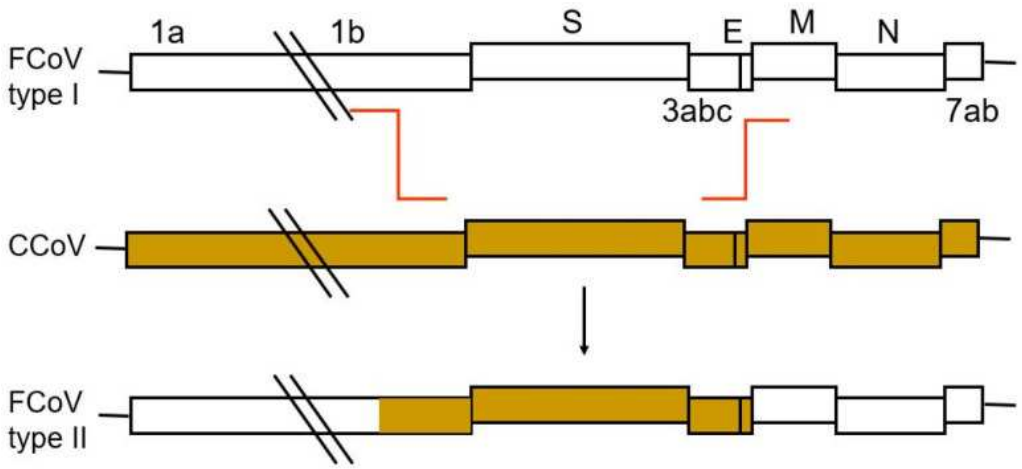


Figure 1.3. Schematic diagram showing how type II FCoV arises from recombination of FCoV type I (shown in white) with CCoV (shown in brown). Image courtesy of Peter Rottier, University of Utrecht, The Netherlands. Taken from: Tasker S, Addie DD, Egberink H, Hofmann-Lehmann R, Hosie MJ, Truyen U, Belák S, Boucraut-Baralon C, Frymus T, Lloret A, Marsilio F, Pennisi MG, Thiry E, Möstl K, Hartmann K. Feline Infectious Peritonitis: European Advisory Board on Cat Diseases Guidelines. Viruses. 2023 Aug 31;15(9):1847

Feline coronavirus exists in two biotypes; feline enteric coronavirus (FECV), which mainly replicates in the enteric epithelial cells, and feline infectious peritonitis virus (FIPV), which results in lethal infection with efficient systemic replication in monocytes or macrophages (14). FIPV mainly replicates systemically; however, FECV has also been shown to replicate systemically in healthy cats and those without FIP (15-17).

FCoV genomes, like all coronaviral genomes, possess a high level of genetic variation due to the high error rate of RNA polymerase leading to different types of mutations, including point mutations, deletions, introduction of stop codons and recombinations (12, 18-20). The ‘internal mutation’ theory is supported by the genetic variation and subsequent selection facilitates the switching of cell tropism from enterocytes to systemic monocytes/macrophages within an FCoV-infected cat that develops FIP (11). Although the genes involved in the FCoV virulence genetic shift are

still unknown, mutations in different genes have been postulated to be associated with the switch of the less-virulent FECV into the virulent FIPV, including the spike gene and accessory genes 3c and 7b (14, 21).

The spike protein comprises two subunits, S1 and S2. The feline host-receptor recognition is mediated by S1 and membrane fusion by S2 (22-24). Following receptor recognition (25), the spike protein is activated by feline host-cell proteases, such as furin (26). Type I FCoV has two cleavage sites, called S1/S2 and S2′; the S1/S2 is cleaved by furin and referred to as the furin cleavage site (26). Type II FCoV contains only the S2′ site (26) and the viral-cell-membrane fusion then occurs via the S2 subunit fusion domain (2). The S2 subunit is composed by the fusion peptide, two heptad repeats (HR1 and HR2), a transmembrane domain and an endodomain (24). Two alternative amino acid differences in the S2 fusion domain of the S protein, namely M1058L (methionine is replaced by leucine at position 1058) and S1060A (serine is replaced by alanine at position 1060) have been reported. These mutations are suggestive of systemic FCoV infection, rather than FIP disease. A novel mutation (M1058F, where F represents phenylalanine) in this region has also been reported in association with FIP (27). The furin cleavage site (S1/S2) at the junction of the receptor binding (S1) and the fusion (S2) subunits of the spike protein, is another genomic region associated with FIP (28). While all less-virulent FCoV had a conserved furin cleavage site, in most FIP-associated FCoV at least one substitution was found (28). Other mutations in the S1/S2 cleavage site have been reported (26, 29-31) and also mutations in the HR1 region of the S gene may be associated with FIP (20, 32).

The ORF 3 gene encodes for a protein for which the exact function is still unknown. Mutations leading to a truncated protein were detected in the 3c genes of FCoV found in tissues of cats with FIP (19, 33-35). The ORF 3 gene is intact in FCoV detected in fecal samples, suggesting that an intact 3c is needed for the infection of the intestinal epithelial cells (21, 33), but is not necessary for replication in monocytes. The non-structural glycoprotein 7b, encoded by ORF 7b has been reported in an association with FCoV virulence in some studies (36), but not in others (19, 37). Moreover, there is no evidence that mutations in the 3a, 3b and 7a genes play a role in the development of FIP (20).

1.2 Epidemiology of feline infectious peritonitis

Feline coronavirus (FCoV) is a ubiquitous virus present in cats worldwide, with up to 50% of cats in single-cat households and up to 90% of cats in multi-cat households being seropositive for FCoV antibodies (38, 39). Although the majority of cats infected with FCoV remain healthy, up to 5-10% of cats may develop FIP (38). Feline infectious peritonitis often affects young cats, most cases are younger than 1 year, from shelters or pedigree cats with certain breeds being more predisposed; however, breed predispositions may vary geographically (40). FCoV is highly contagious and, in households where it is present, the prevalence of FCoV antibodies is often high. This may be due to increased transmission and exposure within places with lot of cats, but also the stress in multi-cat environments.

Some studies are suggesting that domestic shorthair cats are less likely to be FCoV antibody-positive compared to some pedigree breeds (41). In one large study, the FCoV antibody prevalence was 67% in purebred cats and 31% in non-pedigrees (42). In this

study in purebred cats, the seroprevalence increased rapidly in early life, reaching around 80% by three months of age, and remaining at this level until around two years of age. Seroprevalence in pedigree cats is reported to be lower, around 30% in cats aged 14 years or more. In the non-pedigree cats, there is less fluctuation in seroprevalence, with levels remaining at around 30% at all ages. Some breeds (e.g. Persian cats) have been reported to be associated with persistent high FCoV shedding (43). The prevalence of FCoV, determined by the detection of FCoV antibodies and/or FCoV RNA in feces differs in various in studies from different countries.

Transmission of FCoV

FCoV is a contagious virus and transmitted via the oro-fecal route. Feces are the main source of FCoV infection, with the main source of infection being litter trays, cat litter fomites etc. FIP-associated rhinitis has been reported and suggested that the respiratory tract might be a place of entry for the transmission of FCoV (44). Since the virus is found only rarely in the saliva of healthy cats, close contact or sharing feeding bowls are not considered a major route of infection (45). Transplacental transmission has been described from a queen that developed FIP during pregnancy (46), but this is very rare (47); and venereal transmission of FCoV is very unlikely. Transmission of FCoV via blood transfusion has not been reported.

Kittens commonly become infected when young, within a few weeks of age (48). After natural infection, cats begin to shed the virus in the feces in as early as two days (17, 49) and continue to shed for days, weeks, months, and some cats can shed persistently for life (17, 45, 49-51). Shedding typically lasts a few weeks to months, then stops or occurs

intermittently and can recur due to re-infection in an endemic environment as the immunity is short-lived (17, 27, 45, 52-55). A small number of cays (3–9%) never shed FCoV following infection and these cats may be resistant to FCoV infection (45, 53). Although FCoV and CCoV are closely related, contact with dogs does not appear to be a major predisposing factor for coronavirus infection in cats (13).

1.3 Pathogenesis of feline infectious peritonitis

Following ingestion, the virus first enters and replicates within the epithelial cells of the small intestinal villi (56). The FECV biotype has a tropism for intestinal mucosal epithelial cells with a low virulence, resulting in mild or subclinical enteritis (56-59). From two weeks post-infection, the virus is found in the colon (52). In persistently infected carrier cats without clinical signs, the ileum, and especially the colon, are the main sites of persistent viral replication (50, 52, 56). The mesenteric lymph nodes (MLNs), as the most likely first site of FCoV spread from the intestine regardless of subsequent viraemia, have been evaluated for mediators of the innate immune response, and evidence of toll-like receptor involvement has been found in the response to FCoV infection (60). Fortunately, only a small proportion of FCoV-infected cats go on to develop FIP (61). Efficient FCoV replication in activated monocytes and macrophages is a key event in FIP pathogenesis (62). The FIPV biotype has tropism for macrophages as well as plasma cells, monocytes, lymphocytes, and neurocytes. This change in tropism likely occurs die to the mutations in the virus. This biotype causes the clinical syndrome of FIP, and is highly virulent, resulting in significant systemic morbidity and high mortality. The outcome of infection of the monocytes and macrophages is partially dependent on the host cell;

however, virulent strains do replicate more efficiently within permissive monocytes and macrophages (62, 63). The virulence of the virus, the viral load and the cat's immune response determine whether FIP will develop. Thus, both viral genetics and host immunity are likely to play a role in the development of FIP (63-68), FCoV-infected macrophages release cytokines, such as tumor necrosis factor-alpha (TNF-α), which upregulates fAPN (69), causes lymphopenia (70) and inhibits neutrophil apoptosis (71).

The immunity

The development of FIP is associated with the severe suppression of natural killer cells and regulatory T cells, the central players in the innate and adaptive cell-mediated immunity (CMI) (72). An effective early T cell response is believed to critically determine the outcome of infection with FCoV (73). Interferon (IFN) gamma (IFN-γ), which is an important modulator of CMI, has been reported as one of the most important cytokines in FCoV infection. One study suggested that cats resistant to FIP have strong CMI, which can be measured by high serum IFN-γ production (74). If IFN-γ production could prevent the onset of FIP is however not yet clear. Definitive adaptive T cell responses, predictive of disease outcome, were not detected during the early phase of primary infection with FIP-associated FCoV, but recovery antiviral T cell responses were seen later in primary infection for a subset of cats showing slow progression to FIP or resistance to FIP compared to those showing a fast progression of FIP in another study (68). The role of humoral immunity in protecting against FIP is still unclear. Maternally derived antibodies have been suggested to provide protection until about five to six weeks of age (75) until they decline and become undetectable by six to eight weeks of age. However, infection

at two weeks of age has also been detected rarely (48), which questions the protection by maternally derived antibodies.

1.4 Clinical features of feline infectious peritonitis

FIP typically manifests as a vasculopathy resulting in the 'effusive' form (up to 80% of FIP cases have effusions), or granuloma formation resulting in mass lesions ('non-effusive' form), or a combination of the two; indeed, most FIP cases with effusions also have granulomatous lesions visible at post-mortem examination. The effusive form of disease generally is believed to develop in cats with poor cell-mediated immune responses, and the non-effusive form develops in cats with partial cell-mediated immunity. The effusive form of disease is an immune complex vasculitis characterized by leakage of protein-rich fluid into the pleural space, the peritoneal cavity, the pericardial space, and the subcapsular space of the kidneys. Vascular permeability factors, including vascular endothelial growth factor produced by infected monocytes and macrophages might increase the vascular permeability in cats with effusive FIP (76) . In the non-effusive form pyogranulomatous or granulomatous lesions develop in multiple tissues, particularly the eyes, brain, kidneys, omentum, spleen, and liver. Some affected cats have characteristics of both forms of FIP.

Clinical signs seen in both effusive and non-effusive FIP include anorexia, weight loss, lethargy and pyrexia. Pyrexia was far more common in cats with effusive FIP than those with neurological non-effusive FIP in one study (77). Icterus, ocular inflammation, abdominal distention, dyspnea, or CNS abnormalities are also common in cats with FIP. Ocular signs can present as anterior and/or posterior uveitis and neurological signs can

be focal, multifocal or diffuse in nature, often with central vestibular signs, occasionally as a T3–L3 myelopathy) (78). In cats with effusive disease, abdominal distention is common, and pleural effusion can result in dyspnea and a restrictive breathing pattern as well as muffled heart and lung sounds. Male cats sometimes have scrotal swelling. Occasionally masses (pyogranulomas or lymphadenopathy) can be palpated in the abdomen. Lymphadenopathy can also be present in both the effusive and non-effusive forms of FIP. Focal non-effusive FIP can present also as a palpable abdominal mass which can be difficult to initially differentiate from neoplasia, feline gastrointestinal eosinophilic sclerosing fibroplasia and other infections (e.g. mycobacterial infection). Dermatological signs manifesting typically as multiple, non-pruritic papules or nodule have also been reported in FIP (79, 80).

Comorbidities in cats with FIP

As more cats are being treated with new antiviral drugs instead of being euthanized as in the past, new diseases processes that have may be associated with FIP are being recognized. For example, other than pericardial effusion, cardiac lesions associated with FIP were not previously recognized. However, recently a case report of a 9-month-old cat diagnosed with FIP that developed left sided congestive heart failure secondary to hypertrophic cardiomyopathy (HCM) phenotype 4 days after initial diagnosis was published (81). Necropsy performed in this cat confirmed pyogranulomatous lesions affecting multiple organs, including the myocardium. Immunohistochemistry performed on the myocardium revealed feline coronavirus-positive macrophages without evidence of the myofiber disarray that is typically seen in primary HCM (81). A post-mortem diagnosis

of myocarditis associated with feline coronavirus (FCoV) was made. In another study evaluating the efficacy of the nucleoside analog GS-441524 for the treatment of FIP, 2 out of 31 cats being treated died of cardiac related causes (82). One cat had severe biatrial enlargement without wall thickening on echocardiogram; this cat was euthanized but no necropsy was performed (82). The second cat in this study was treated for effusive FIP, but was euthanized 4 weeks after; necropsy confirmed the appearance of congenital hypertrophic cardiomyopathy, although results of histologic evaluation of the heart was not mentioned in the study (82). Interestingly no gross or histologic lesions in the abdomen, chest, eyes brain, or spine associated with FIP were identified in this patient (82). Another case report described fibrinous epicarditis caused by FIP in a three-year-old cat. Inflammatory infiltrate was observed in the epicardium, composed mainly by macrophages, plasmocytes and lymphocytes and immunohistochemistry was performed was positive for feline coronavirus (83). Recently, there was a reported case of a pyogranuloma found in the myocardium in a cat with heart disease; the histopathological finding was that the myocarditis/myocardial necrosis was associated with the presence of *S* gene-mutated FCoV (M1058L biotype) (84).

A study comparing myocardial transcription of cytokines and remodeling enzymes in cats with HCM and cats without cardiac disease, which included 18 cats with FIP, reported the highest myocardial marker transcriptions in cats with multicentric lymphoma, FIP and HCM, followed by diseases likely associated with a systemic inflammatory state. Inflammatory marker transcription predominated in the myocardium of cats with systemic inflammatory disease (85). A study evaluating cytokine production in tissue samples from cats who died of FIP showed that pyrogenic cytokines IL-6 and TNF-a were upregulated

in cardiomyocytes. Although the heart was not directly affected by FIP lesions in any of these cases, the upregulation of these cytokines suggested that cardiomyocytes likely played a role in the systemic inflammation caused by FCoV (60). Upon the infection or injury, the heart muscle triggers an innate immune response, involving cells like macrophages and neutrophils which release cytokines, leading to the inflammatory cascade and recruiting more immune cells to the site of damage. The adaptive immunity also plays a role in development of myocarditis with T cells and B cells contributing to myocarditis through cytokine production. Apart from the limited reported cases above, the potential link between myocarditis and FIP is missing, which is likely in part due to previously limited treatment options. Feline coronavirus shares similarities with another coronavirus, SARS-CoV-2, the etiologic agent for the COVID-19 pandemic. Of note, a higher rate of myocardial injury in patients with SARS-CoV-2 has been documented (86-88). One study in humans identified up to 27.8% cases of COVID-19 having evidence of myocardial injury based on elevated cardiac troponin levels, which has been supported by definitive diagnosis of myocarditis via endomyocardial biopsy and autopsy (86). Humans with SARS-CoV-2 associated myocarditis often have echocardiographic evidence of systolic dysfunction, myocardial edema on cardiac magnetic resonance imaging, and electrocardiographic changes including ST segment changes and ventricular tachycardia (86, 87). Treatment for myocarditis in COVID-19 patients is currently symptomatic, although high-dose steroids and intravenous immunoglobulins have been used (86, 87). COVID-19 patients with suspected or confirmed myocarditis have a high (27%) mortality rate (86, 87). At this time, there is no evidence that myocarditis is directly caused by SARS-CoV-2 in humans; but it is suspected that

myocardial inflammation is secondary to a generalized inflammatory reaction induced by cytokines (86, 87). Myocarditis has now been associated with SARS-CoV-2 in a limited number of dogs and cats in the United Kingdom (89).

Another comorbidity that has been reported in cats with FIP is immune-mediated hemolytic anemia (IMHA), which occurs when the body mounts an immune response against antigens expressed on the surface of erythrocytes (90). In animals with IMHA, the production of antibodies results in the destruction of normal erythrocytes by inappropriate activation of the complement cascade, antibody-dependent cytotoxicity, and/or facilitated phagocytosis in the liver and spleen, resulting in severe anemia. In cats, IMHA can either be non-associative (primary) or be associative (secondary) to an underlying infectious, inflammatory, or neoplastic process (91, 92). Several infections have been documented in cats to cause associative IMHA, including feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), *Leishmania infantum*, *Mycoplasma gatae*, *Mycoplasma haemofelis, Babesia felis* and FIP (91-98). One study also reported 1 FIP and IMHA cat to have co-infection with FeLV (95). While most cats with FIP were euthanized in the past; more cats are now being treated with antiviral drugs. Humans with SARS-CoV-2 infections present with a spectrum of clinical presentations and complications, with associative IMHA being increasingly recognized in patients, either while infected or shortly after infection (99). It has been proposed that SARS-CoV-2 infection could lead to hemolytic anemia directly through cytopathic injury or indirectly through induction of auto-antibodies (100).

1.5 Diagnosis of feline infectious peritonitis

Enteric Coronavirus

There is currently no assay available that can determine whether enteric coronaviruses are associated with diarrhea in cats. Cats with coronavirus RNA in feces should be evaluated for other causes of diarrhea.

Feline Infectious Peritonitis

The diagnosis of FIP can be challenging as routine laboratory testing does not have many specific findings. Complete blood cell count (CBC) usually shows mild, nonregenerative anemia, microcytosis, neutrophilia (with or without toxic changes, 39–57%), lymphopenia (55–77% of cases) and thrombocytopenia or thrombocytosis (77, 101-103).

Serum biochemical findings often include hyperproteinemia, hyperglobulinemia, hypoalbuminemia, increased liver enzyme activities, hyperbilirubinemia, and electrolyte imbalances. Hyperglobulinemia is reported in 89% of cases with hypoalbuminemia or low-normal serum albumin (64.5% of cases) (77). Low albumin:globulin ratio (A:G) can aid in diagnosis of FIP, with A:G ≤0.4 making FIP likely. In one study an albumin/globulin ratio of 0.5 had a positive predictive value of 89%, and an albumin/globulin ratio of 1.0 had a negative predictive value of 91% (104). When hyperglobulinemia is detected, protein electrophoresis can be performed which usually shows polyclonal gammopathy but monoclonal gammopathy has been reported with greatest increases in the gamma (γ)-globulin and alpha $(\alpha)_2$-globulins fractions (105).

Hyperbilirubinemia occurs in 21–63% of FIP cases, and is often seen without marked elevations in alanine aminotransferase (ALT), alkaline phosphatase (ALP) or γ-glutamyl transferase enzyme activity (102). Hyperbilirubinemia can also occur due to IMHA but is less commonly reported with FIP and these cats are also anemic (102). In one study, hyperbilirubinemia was more commonly seen in cats just before their death or euthanasia (103); high bilirubin concentrations have been previously reported in cats as poor prognostic indicator in cats with FIP (106).

Acute phase proteins (APPs) are made in the liver in response to cytokines released from macrophages and monocytes (especially inter-leukins 1 and 6 and tumor necrosis factor α) in many inflammatory and non-inflammatory diseases. Acute phase proteins, mainly αlpha1-acid glycoprotein, are often increased in cats with FIP but can also be increased with other inflammatory diseases (107-110). One small study showed that high AGP concentration (≥1500 µg/mL) can be helpful in diagnosis of FIP and able to distinguish between FIP and non-FIP cases (108).

Serum antibody tests for FCoV are usually ELISAs, indirect immunofluorescence antibody tests or rapid immune-migration tests. A positive FCoV antibody test indicates that the cat has been infected with FCoV and has seroconverted (~ 2–3 weeks from initial infection). Although FIP cats tend to have higher FCoV antibody titers than non-FIP cats, there is much overlap, with no difference between median FCoV antibody titers in healthy and suspected FIP cats (111). Therefore, the titer in an individual animal is of limited use in distinguishing cats with FIP and measuring FCoV antibodies in cats with FIP is not very helpful due to the high number of seropositive cats after FECV infection and about 10% of cats with FIP have been reported to be seronegative (112). Breed-related differences

in FCoV antibody titer have been reported, and may reflect differences in breed response to FCoV infection which may affect diagnosis as well (111, 113).

Diagnostic imaging

Ultrasound often confirms pleural or peritoneal effusion in cases of effusive FIP and abdominal lymphadenopathy and other changed in non-effusive FIP. A recent study showed that cays with FIP have several abnormalities on abdominal ultrasound (114). This study showed that 88% of cats had effusion present, and lymphadenopathy was seen in 80% of cats. Hepatic changes were noted in 80%, the most common being hepatomegaly (58%) and a hypoechoic liver (48%). Intestinal changes were noted in 68% of cats, characterized by asymmetric wall thickening and/or loss of wall layering, with 52% being ileocecocolic junction and/or colonic in location. Splenic changes were present in 36% of cats, including splenomegaly, mottled parenchyma and hypoechoic nodules. Renal changes were present in 32%, encompassing a hypoechoic subcapsular rim and/or cortical nodules. Mesenteric and peritoneal abnormalities were seen in 28% and 16% of cats, respectively. Most cats (92%) had two or more locations of abdominal abnormalities on ultrasound (114).

Changes in the thoracic cavity are less often reported in cats with FIP; however, recent studies are showing increased numbers of cases with abnormalities in the thoracis cavity. One retrospective study with 112 cats showed that 59% had inflammatory histological lesions in the respiratory tract. The major gross patterns were identified as marked fibrin deposition in the thoracic cavity with lung atelectasis; marked fibrin deposition in the thoracic cavity with lung pyogranulomas; and lung pyogranulomas

without thoracic effusion. Histological analysis revealed primary lesions in the visceral pleura and lung parenchyma at a similar frequency, with multifocal to diffuse presentations with most common histological patterns being pleuritis; pleuritis and vasculitis/perivascular injury in the lung parenchyma; pleuritis and pneumonia; perivascular injury in the parenchyma without pleuritis; and pneumonia without pleuritis. This study, however, had higher prevalence of co-infections with retroviruses and feline leukemia virus was detected in 71.2%; therefore, this study might not fully represent retroviral negative cats (115). Another study looking at thoracic radiographs in 33 cats with FIP reported abnormal findings in 91% of the cats (116). Pleural effusion was present in 37% of cats and was either bilateral 85% or unilateral in 15% of cats. The lungs were radiographically abnormal in 71% of cats, with the most common abnormality being an unstructured interstitial pattern (84%), with bronchial (44%) and alveolar (40%) patterns being less common. Sternal lymphadenopathy was reported in 46% of cats and pulmonary nodules were identified in 12% of cats. Moreover, this study also reported an enlarged cardiac silhouette was noted in 17% of cats attributable to myocarditis, pericardial effusion, a high output state, or unrelated cardiomyopathy (116).

Effusion analysis

Fluid analysis and cytology in cases of effusive FIP can be beneficial. The effusions are mostly consistent with modified transudate or exudate and is usually high in protein (≥3.5 g/dL) and of low cellularity (nucleated cells <5000 cells/μL). Mixed inflammatory cell populations of lymphocytes, macrophages, and neutrophils occur most commonly; neutrophils predominate in most cases, but in some cats, macrophages are the primary

cell type seen. Measurement of protein concentrations in effusions and calculation of the albumin/globulin ratio can aid in the diagnosis of effusive FIP.

Histopathology and immunohistochemistry

Surgical biopsies of lymph nodes and/or other organs can be taken for histopathology and IHC to confirm diagnosis of FIP; however, this is not always possible as the cats can be very sick and, in most cases of the non-effusive (and often also effusive) form, a presumptive diagnosis of FIP is usually made based on the combination of clinical and clinicopathologic findings and the exclusion of other causes for the presenting clinical signs. The confirmatory test for diagnosis of FIP is immunohistochemistry (IHC) which detects FCoV antigen in macrophages by immunofluorescence or immunoperoxidase methods. Immunostaining is performed on formalin-fixed tissues using IHC, or on cytological (typically effusion) samples using immuno-cytochemistry (ICC) or immunofluorescence (IF). These techniques exploit the binding of antibodies to host cell-associated FCoV antigens, which are subsequently visualized by enzymatic reactions producing a color change (ICC) or by fluorescence (IF). Positive FCoV antigen immunostaining of tissues can confirm a diagnosis of FIP; however, a negative result does not exclude FIP as a diagnosis as FCoV antigens may be variably distributed within lesions and therefore might not be detected in all histopathological sections prepared from lesions from FIP cases (117, 118).

Immunostaining of effusion samples has shown variable sensitivity (57–100%) as this technique relies on staining FCoV antigen within macrophages in the effusion; if the effusion is often cell-poor and/or the FCoV antigen is masked by FCoV antibodies in the

effusion, a false negative result may be obtained (104, 119, 120). Immunostaining can be used on other samples and FCoV antigen ICC staining has been reported as being successful in detecting FCoV in the CSF of a cat with neurological FIP (121) as well as in aqueous humor samples (122).

Reverse transcriptase polymerase chain reaction

FCoV RNA can also be detected in fluid, tissue aspirates or biopsies by reverse transcriptase polymerase chain reaction (RT-PCR); however, this is not specific for FIP. The specificity and sensitivity of RT-PCR varies based on the test and sample used for diagnostics, with highest specificity being on pleural and peritoneal effusion as FCoV is unlikely to be in effusions from other causes. Diagnosis of non-effusive FIP can be even more challenging due to the absence of effusion. In cases of neurological FIP, cerebrospinal fluid should be taken for analysis and RT-PCR. Cats with ocular involvement can undergo aqueocentesis and RT-PCR performed on aqueous humor. The presence (particularly of high levels) of FCoV RNA in blood, effusion, tissue, CSF and/or aqueous humor samples can be supportive of a diagnosis of FIP. FCoV RT-PCR can also be performed on fecal samples, but this is primarily used to identify cats that are shedding the virus for the management of infection in a multi-cat household. Fecal FCoV RT-PCR is not used to aid in the diagnosis of FIP.

Following the detection of FCoV RNA by RT-PCR, it may be possible to then characterize targeted sections of FCoV genomic sequences present in a sample using molecular techniques such as pyrosequencing, Sanger sequencing or PCR with sequence-specific hydrolysis probes. There are some limitations to sequencing however;

for example, if only low levels of FCoV are present, it can preclude sequence analysis. Characterization of FCoV genomic sequences would be most useful for FIP-specific mutations. Currently, though,there is not a single mutation that seems to be solely responsible for FIP.

One study described amino acid differences in the fusion peptide encoded by the FCoV *S* gene as being markers of FIP suggesting the possibility that detection of the underlying *S* gene mutations could be used to definitively diagnose FIP (123). Similar findings have been reported in another study with amino acid differences in the furin cleavage motif, also encoded by the *S* gene, that have been correlated with FIP disease (28). However, another study compared these *S* gene markers in tissues of FIP cats and feces of healthy non-FIP cats and found that the *S* gene mutations present in most of the FIP tissues were also present in most of the tissues of non-FIP cats that had systemic FCoV infection (124). It has been suggested that the fusion peptide sequence mutations could reflect the cellular tropism of the FCoV (e.g. systemic monocyte/ macrophage-associated FCoV or intestinal epithelium-associated FCoV) rather than being specific for FIP, knowing that non-FIP cats can have systemic FCoV infection (124). Another study confirmed these findings and reported that, if the identification of *S* gene mutated FCoV was included as an additional confirmatory step to the detection of FCoV by RT-PCR, it only slightly increased specificity for the diagnosis of FIP in tissue samples (from 93% to 95%) but the sensitivity was moderately decreased (from 90% to 81%) as non-mutated FCoV was sometimes identified and mutation analysis was not possible in all tissue samples (for example due to low FCoV copy numbers or the presence of type 2 FCoV) (125). Other studies have been looking at the *S* gene mutations in cats with FIP. One

study reported that 71% of FCoV-positive FIP effusion samples had *S* gene mutations, while one did not have a mutation and 24% could not be sequenced due to the low levels of FCoV present (126). In another study, most (89%) FCoV-positive FIP effusion samples had *S* gene mutations, while 8% did not have mutations and one could not be sequenced (127).

1.6 Treatment of feline infectious peritonitis

In the past, most cats with systemic clinical signs of feline infectious peritonitis (FIP) died or were euthanized within days to months of diagnosis and the disease used to carry a very grave prognosis. In recent years, considerable advancements have been made into the treatment of FIP using new antiviral drugs such as nucleoside analogues, including: remdesivir (RDV) and its metabolite GS-441524; and molnupiravir (MPV) and its metabolite EIDD-1931 (NHC). Viral protease inhibitors (nirmatrelvir and GC376) have also shown a promise in the treatment of FIP (82, 128-142). In many countries, including USA, veterinarians can prescribe some or all the following compounded formulations of RDV, GS-441524, MPV and NHC. In many countries, off-label prescription of registered human drugs such as RDV, MPV and Paxlovid are additional legal avenues open to veterinarians. Cats may still require initial supportive and adjunct therapy specific to their FIP presentation and disease severity, however, in the majority of FIP cases, clinicians can anticipate rapid clinical improvement within days of commencing effective antiviral therapy. It is rare in veterinary medicine that we have taken a disease from an untreatable death sentence, to achieving 85% long term survival, with a monotherapy treatment approach.

After binding to the receptor, coronaviruses enter the cell host either via direct fusion at the plasma membrane or endocytosis. Once the nucleocapsid is delivered into the cytoplasm, viral RNA is uncoated for translation of the ORF1a/1b gene into a polyprotein that encodes the 16 non-structural proteins (143). Synthesized proteins proceed to form the replicase-transcriptase complex (RTC). The RTC will initiate the transcription of full-length negative RNA and negative subgenomic (sg)RNAs (143). These will serve as a template for the synthesis of positive full-length RNA and positive sgRNAs. Viral proteins are translated from the positive sgRNAs. After synthesis, they undergo maturation in the Endoplasmic Reticulum and Endoplasmic Reticulum-Golgi apparatus Intermediary Compartment and assemble to form the novel viral particles (143). The viral particles maturation occurs during their transit in the Golgi apparatus and are in fine secreted out of the cell by exocytosis (143). Figure 1.4 shows were different drugs suggested in the treatment of FIP work.

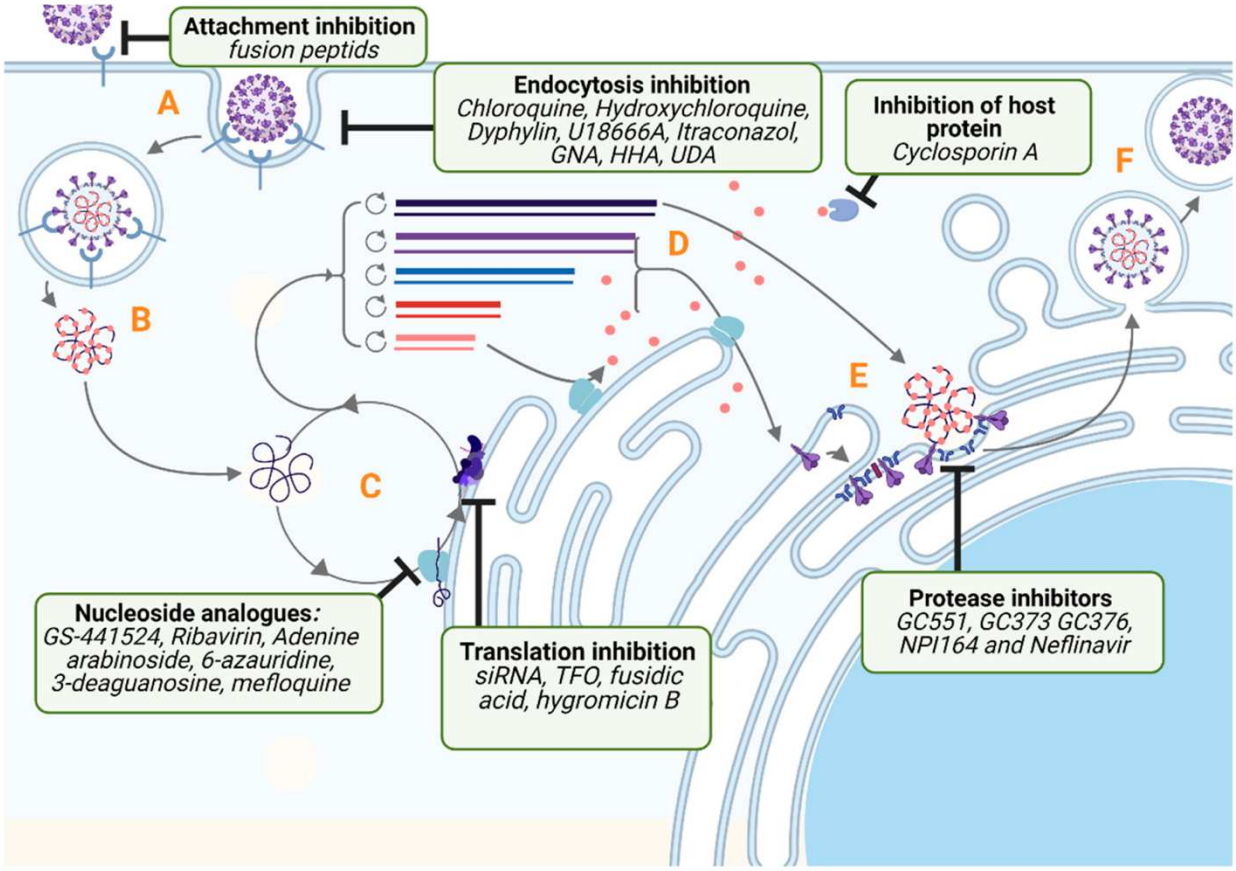


Figure 1.4. FCoV life cycle and mechanisms of actions of current FIPV antivirals. Letters A–F represent major steps of FIPV replication: (**A**) Attachment to cell and endocytosis. (**B**) Liberation of Ribonucleocapsid following fusion of viral and cell membranes and decapsidation. (**C**) Transcription of viral RNAs and creation of Replication/Transcription Complex. (**D**) Translation of the viral structural proteins. (**E**) Assembly of virion in the endoplasmic reticulum or Golgi apparatus. (**F**) Budding of virus in the ERGIC and exocytosis. HHA: Hippeastrum hybrid agglutinin; UDA: Urtica dioica Lectin; GNA: Galanthus nivalis agglutinin; TFO: Triple Helix Forming Oligonucleotides. Image from Delaplace *et al.* Feline Coronavirus Antivirals: A Review. *Pathogens* 2021. 10, 1150.

Nucleoside and nucleotide analogues

Nucleoside and nucleotide analogues are a class of drug with diverse treatment utility, though are primarily employed as antiviral and chemotherapeutic agents (144). A nucleoside (e.g. GS-441524 or NHC), consists of a nucleobase linked to a sugar moiety, whilst a nucleotide (e.g. RDV or MPV), is often a prodrug form that incorporates one or

more phosphate groups into the nucleoside structure (145). *In vivo*, nucleotide analogues are typically metabolized to release their core nucleoside, which is subsequently converted into its active form. This active metabolite disrupts viral replication by mimicking native RNA nucleosides and interfering with viral RNA synthesis. The mechanisms to how RNA synthesis is ultimately disrupted, varies between these drugs, as is detailed below.

Remdesivir & GS-441524

Remdesivir is a nucleotide prodrug that was variably approved around the world for parenteral administration in humans for treatment of SARS-CoV-2 during the COVID-19 pandemic (146, 147). Remdesivir was approved by the U.S. FDA and is available off-label for veterinary use (though sourcing from a human hospital pharmacy can be challenging) and a compounded parenteral formulation is also available in Australia, New Zealand and the UK. Remdesivir undergoes several hydrolysis steps to form the primary metabolite, GS-441524, and then the pharmacologically active triphosphate GS-443902 (148-152). The GS-443902 acts as an adenosine analogue and disrupts viral replication by incorporating into viral RNA via RNA-dependent RNA polymerase, causing delayed chain termination (148, 149, 151, 153). In cats, the *in vivo* conversion from RDV to GS-441524 likely facilitated by blood esterases (rather than hepatic metabolism) (154). Regardless of whether a clinician administers RDV (the prodrug) or GS-441524 (the core nucleoside), the same active triphosphate ultimately disrupts viral replication.

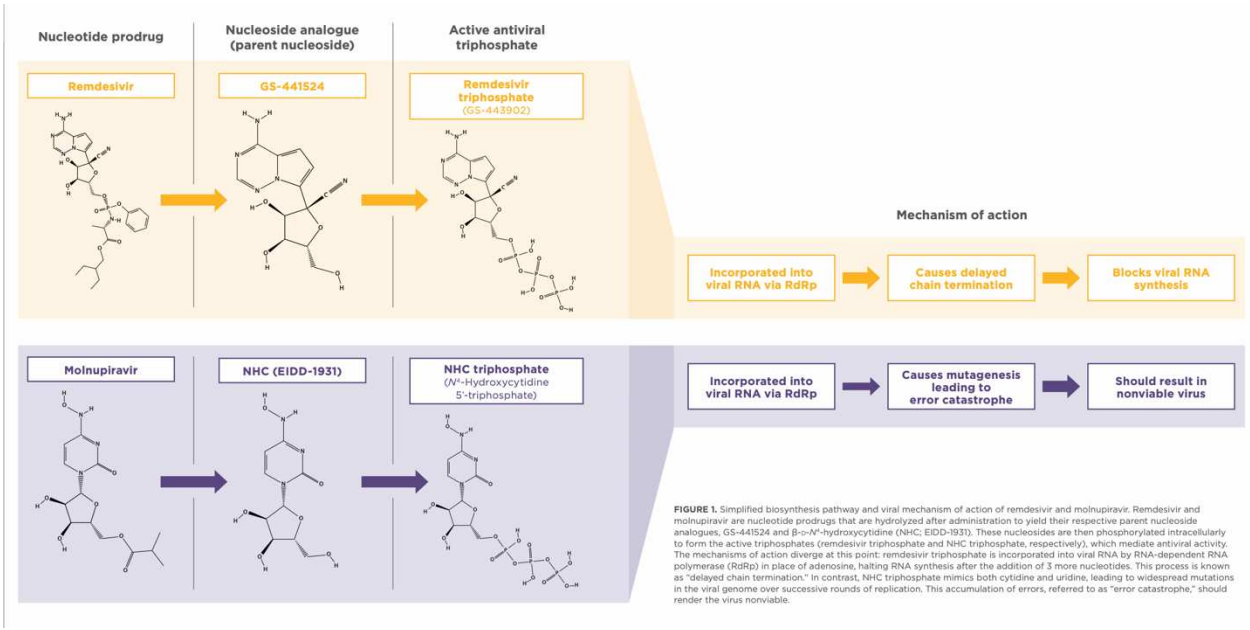


Figure 1.5. Simplified biosynthesis pathway of RDV in humans. Remdesivir diffuses across the cell membrane and is then hydrolysed into first the alanine intermediate metabolite (GS-704277), then subsequently the primary metabolite, GS-441524, followed by tri-phosphorylation into the pharmacologically active triphosphate (GS-443902). This active triphosphate is incorporated into viral RNA via RNA-dependent RNA polymerase (RdRp), disrupting viral replication. Image from Practical pharmacology, Pharmacologic Approaches to Feline Infectious Peritonitis by Dr. Sally Coggins and Dr. Petra Cerna. July/August 2025 at Today's Veterinary Practice.

The pharmacokinetics of GS-441524 have been described in specific pathogen-free (SPF) cats after subcutaneous (SC, n = 2) or intravenous (IV, n = 2) administration of 5 mg/kg (130). The intracellular concentration of the active triphosphate in peripheral blood mononuclear cells remained 8–20 times above the EC_{50} for 72 hours, supporting the efficacy of once-daily dosing of 5 mg/kg GS-441524 by SC or IV routes (130). The *in vivo* pharmacokinetics of GS-441524 following administration of RDV IV have also been described in three clinically normal SPF cats and cats with FIP. These studies established that orally administered MPV at 10 mg/kg, GS-441524 and RDV at 25 mg/kg, and intravenously administered RDV at 7 mg/kg achieves plasma levels greater than the established corresponding EC_{50} values, which are sustained over 24 h for GS-441514

and RDV and that the mean peak plasma concentration of GS-441524 (C_{max}) after a single 15 mg/kg iv dose of RDV was 2632 ng/mL (SD 862); time to reach C_{max} (T_{max}) was 1 hour (SD 0); and elimination half-life ($t_{1/2}$) was 5.14 hours (SD 0.81) (155, 156). Although direct pharmacokinetic studies of oral GS-441524 in cats remain limited, the outcome data strongly supports the efficacy of current oral dosing protocols. Across the existing literature, treatment with GS-441524 and/or RDV as demonstrated remission and long-term survival rates ranging from 77-96% with both SC and oral (PO) therapy (82, 133-138) (Table 1.1).

Table 1.1. Recommended treatment protocols for GS-441524 and RDV by presenting form of FIP.

Form of FIP	GD-441524 PO^	Remdesivir IV*/SC
Effusion(s) and without ocular or neurological signs	15 mg/kg q24hr or split q12hr†	10-15 mg/kg q24hr
No effusion and without ocular or neurological signs	15 mg/kg q24hr or split q12hr†	12-15 mg/kg q24hr
Ocular signs (+/-effusion)	15-20 mg/kg q24hr or split q12hr†	15 mg/kg q24hr
Neurological signs (+/-effusion)	10 mg/kg q12hr	20 mg/kg q24hr

*****Slow IV (intravenously) – Give as CRI over 30mins-2h, can be diluted in saline.
^PO (orally) – Give fasted with water bolus or tbsp wet food, full meal 30mins later
†Divided dose may improve plasma concentrations when using oral GS-441524 and is suggested as preferred by some. SID dosing is still highly effective considered appropriate by the authors, particularly where compliance may be an issue.

Molnupiravir and EIDD-1931

Molnupiravir (MPV; EIDD-2801), an isopropylester prodrug of the nucleoside analogue β-D-*N*4-hydroxycytidine (NHC or EIDD-1931), interferes with the replication of various viruses including SARS-CoV-2 (157, 158). Due to its ability to tautomerize, it can

substitute for either cytidine or uridine and subsequently pair with either guanosine or adenosine in the RNA template. MPV acts as a mutagenizing agent that causes an 'error catastrophe' during viral replication, which causes an increase in mutation frequencies above a tolerable threshold (i.e., a mutation rate that surpasses the level needed to maintain viral viability), resulting in production of replication incompetent genomes in subsequent rounds of RNA synthesis, which ultimately leads to the viral death. A study showing pharmacokinetic data from MPV showed promise for its use and safety in cats (155). After oral administration of 10 mg/kg of MPV, MPV was detected in the serum of all three cats at low levels in the first 12 h post-administration. Its NHC metabolite was detected at much higher levels between 12 to 24 h post-administration; marked variability in detected NHC levels was present between individual cats with the peak detection level for MPV and NHC occurring between 1.5-9h and NHC being detected at a 10-100-fold greater level than MPV. NHC was more consistently and quantifiably detected over the 24-h period, aside for one cat, for which NHC was no longer detected (based on limit of detection) after 12 h post-administration. This study reported no evidence of acute organ toxicity in any of the cats based on the pre- and post-treatment complete blood count (CBC) and serum biochemistry panels; however, all three of the MPV-treated cats demonstrated variable signs of nausea, including hypersalivation and/or vomiting.

One study looking at 18 cats with FIP being treated with MPV showed that MPV might be an effective and safe treatment for domestic cats with FIP at a dose of 10-20 mg/kg twice daily (140). In this study, increased serum alanine transaminase (ALT) activity was found in 3 of the 18 cats, all at days 7-9, and all cats recovered without any medications or need to stop the treatment (140). There is also uncontrolled data from

client surveys that suggests efficacy of this product as rescue treatment following failure of the GS-441524 therapy as well as a first line treatment option for cats with FIP (139). An open-label longitudinal, non-inferiority trial with 10 cats with effusive FIP being treated with MPV (10-15 mg/kg PO q12h) for 84 days showed that MPV is an effective antiviral treatment for effusive FIP (142). A prospective clinical trial with 62 cats with FIP treated with MPV showed that 77% success in treatment with 11% of cats relapsing but all of these responded to a higher dose of MPV (159). A study using NHC orally twice daily at median dose 18.75mg/kg (range 12.0-23.8) showed complete response in 9 cats (6 cats with effusive and 3 cats with non-effusive); however this study reported several side effects such as neutropenia (3 cats), increased ALT (transient in 3 cats, permanent in 1), broken whiskers (1), hyporexia (6). One of these cats relapsed and responded to increased dose (25mg/kg BID) (160). A recent study comparing treatment with GS-441524 and MPV showed similar effect and safety in cats with FIP (141).

By targeting mitochondrial RNA, MPV/EIDD-1931 is intrinsically mutagenic for human RNA and DNA (161). Therefore, gloves should be worn when handling these drugs. It is recommended not to handle these drugs when pregnant. MPV is not prescribed to humans under 18 years of age and the safety for prescription in kittens is yet to be assessed. Despite preliminary comparative survival data suggesting MPV and NHC are unlikely to be significantly worse when compared to RDV and GS-441524, owing to the increased adverse effects and safety issues with the former, the author recommends RDV and GS-441524 should remain the first-line treatment for cats with newly diagnosed FIP where possible (Table 1.2). However, as some cats do not respond

to treatment with GS therapy or relapse, there is a need for investigation other antiviral therapies as well as combination therapies.

Table 1.2. Recommended doses for MPV and its metabolite NHC

Form of FIP	Molnupiravir (EIDD-2801) PO dosage	EIDD-1931 (NHC) PO dosage
Effusion(s) and without ocular or neurological signs	10-15 mg/kg q12hr	15 mg/kg q12hr*
No effusion and without ocular or neurological signs	15 mg/kg q12hr	15 mg/kg q12hr*
Ocular signs (+/-effusion)	15 mg/kg q12hr	15 mg/kg q12hr*
Neurological signs (+/-effusion)	15-20 mg/kg q12hr	20 mg/kg q 12hr*

*these doses are recommended currently recommended for relapses, for first line therapy lower doses may be considered, more studies are needed to provide better guidance on dosing of NHC. The molecular weights of MPV and NHC are 329.31 g/mol 259.22 g/mol respectively, therefore, if there is complete hydrolysis from MPV into NHC, it is expected to get 0.78% the dose of MPV administered (e.g. 10mg/kg MPV would equal 7.8mg/kg NHC).

Protease inhibitors

Following coronaviral viral entry into host cells, there is release of viral RNA, translation, production and subsequent processing into two key enzymes that are vital for viral replication, 3CLpro and papain-like protease (162-165). These enzymes are highly conserved across coronavirus species; therefore, drugs that inhibit them offer promise as broad-spectrum coronavirus antivirals (162). The two main protease inhibitors currently of interest for FIP treatment are GC376 (Anivive) and nirmatrelvir (present in Paxlovid® by Pfizer).

GC376

GC-376 is a 3CLpro protease inhibitor that stops the cleavage and activation of functional viral proteins required for replication and transcription in host cells. *In vitro* assessment of GC367 demonstrated potent antiviral effects against FIPV in various cell cultures (166). The *in vivo* efficacy was then evaluated using murine model of coronavirus infection (165). In one study, GC-376 showed reversal in experimentally induced FIP in 6 out of 8 cats (166), but survival in client-owned cats with naturally acquired FIP was lower (35%) when compared to that achieved with nucleoside analogues (128). A new study looking at combination therapy of GC-376 and GS-441524 treated 46 cats with FIP (36 effusive and 10 non-effusive) for 4 weeks using different dosage regimes reported that 97.8% of cats responded well to the 4-week therapy, though with 2 cats required treatment extension to 7-12 weeks (167). This study demonstrated that GS-441524 combined with GC376 can be safely and effectively used to treat FIP across a shortened treatment period. The absence of a GS-441524 monotherapy control group, however, makes it unclear whether comparable outcomes could have been achieved with GS-441524 alone, particularly given the reported success of 6-week GS-441524 monotherapy (133). Further studies are needed to evaluate the effectiveness of combination therapy.

Nirmatrelvir

Paxlovid®, Pfizer's antiviral combination for SARS-CoV-2, was variably approved around the world. It is available to veterinarians via off-label prescription in some countries, including the US and Australia. Two different tablets are contained within Paxlovid®, the first being nirmatrelvir (another 3CLpro protease inhibitor, but with a very short half-life), the second is ritonavir (a pharmacokinetic booster, which inhibits cytochrome P450 to delay nirmatrelvir metabolism). Nirmatrelvir inhibited *in vitro* replication of FIPV I and II, with increased efficacy when used in combination with other antivirals (155). Paxlovid is being actively investigated for FIP treatment, though anecdotally, it has been used in combination with a nucleoside analogue for a handful of highly refractory cases, as a last resort. Anecdotally, (Dr. Coggins) is aware of 13 cases in which this has been implemented with remission subsequently achieved in all cases (this remains unpublished and anecdotal data). Its use at the time of publication therefore remains preliminary and experimental in nature. If faced, however, with a non-responding cat with confirmed FIP, a dose of nirmatrelvir 75mg/cat with ritonavir 25mg/cat BID PO could be considered in combination with continue nucleoside analogue treatment. As ritonavir is a cytochrome P450 inhibitor, it may delay the metabolism of other medications processed by this pathway.

Judicious use of antivirals by the veterinary community will be important to mitigate risk of antiviral resistance developing (antivirals are antimicrobials too). When looking to the human literature, despite massive usage within an 18mth period of the COVID-19 pandemic, very low levels of remdesivir resistance were observed worldwide (168). In contrast, analysis of SARS-CoV-2 genomes suggest MPV-associated mutational signatures now exist globally following the introduction of MPV for COVID treatment

(169). In human medicine, the introduction of MPV has created new mutations in COVID-19. Neither of these antivirals should be used in healthy cats to eliminate shedding of FCoV due to the risk of resistance as well as high likelihood of reinfection in cats in multi-cat households.

1.7 Conclusions

The treatment landscape for FIP has undergone a revolutionary transformation, with innovative antiviral therapies now offering hope and survival to a condition once considered a death sentence; continued research and refinement of these therapies promise an even brighter future for affected cats and their caregivers.

References:

1. de Groot R, Baker S, Baric R, Enjuanes L, Gorbalenya A, Holmes K, et al. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses: Family Coronaviridae. Elsevier, Amsterdam; 2012.

2. Jaimes JA, Millet JK, Stout AE, André NM, Whittaker GR. A tale of two viruses: the distinct spike glycoproteins of feline coronaviruses. Viruses. 2020;12(1):83.

3. Haake C, Cook S, Pusterla N, Murphy B. Coronavirus infections in companion animals: virology, epidemiology, clinical and pathologic features. Viruses. 2020;12(9):1023.

4. Addie DD, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hartmann K, et al. Disinfectant choices in veterinary practices, shelters and households: ABCD guidelines on safe and effective disinfection for feline environments. Journal of feline medicine and surgery. 2015;17(7):594-605.

5. Horzinek MC, Lutz H. An update on feline infectious peritonitis. Veterinary Sciences Tomorrow. 2001;1.

6. Terada Y, Matsui N, Noguchi K, Kuwata R, Shimoda H, Soma T, et al. Emergence of pathogenic coronaviruses in cats by homologous recombination between feline and canine coronaviruses. PloS one. 2014;9(9):e106534.

7. Lai MM, Cavanagh D. The molecular biology of coronaviruses. Advances in virus research. 1997;48:1-100.

8. Shiba N, Maeda K, Kato H, Mochizuki M, Iwata H. Differentiation of feline coronavirus type I and II infections by virus neutralization test. Veterinary microbiology. 2007;124(3-4):348-52.

9. Kummrow M, Meli ML, Haessig M, Goenczi E, Poland A, Pedersen NC, et al. Feline coronavirus serotypes 1 and 2: seroprevalence and association with disease in Switzerland. Clinical and Vaccine Immunology. 2005;12(10):1209-15.

10. Benetka V, Kübber-Heiss A, Kolodziejek J, Nowotny N, Hofmann-Parisot M, Möstl K. Prevalence of feline coronavirus types I and II in cats with histopathologically verified feline infectious peritonitis. Veterinary microbiology. 2004;99(1):31-42.

11. Pedersen NC. An update on feline infectious peritonitis: virology and immunopathogenesis. The Veterinary Journal. 2014;201(2):123-32.

12. Herrewegh AA, Smeenk I, Horzinek MC, Rottier PJ, de Groot RJ. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. Journal of virology. 1998;72(5):4508-14.

13. Le Poder S, d'Alexandry A-LP-H, Duarte L, Fournier A, Horhogea C, Pinhas C, et al. Infection of cats with atypical feline coronaviruses harbouring a truncated form of the canine type I non-structural ORF3 gene. Infection, Genetics and Evolution. 2013;20:488-94.

14. Gao Y-Y, Wang Q, Liang X-Y, Zhang S, Bao D, Zhao H, et al. An updated review of feline coronavirus: Mind the two biotypes. Virus research. 2023;326:199059.

15. Fish EJ, Diniz PPV, Juan Y-C, Bossong F, Collisson EW, Drechsler Y, et al. Cross-sectional quantitative RT-PCR study of feline coronavirus viremia and replication in peripheral blood of healthy shelter cats in Southern California. Journal of Feline Medicine and Surgery. 2018;20(4):295-301.

16. Kipar A, Baptiste K, Barth A, Reinacher M. Natural FCoV infection: cats with FIP exhibit significantly higher viral loads than healthy infected cats. Journal of feline medicine and surgery. 2006;8(1):69-72.

17. Meli M, Kipar A, Müller C, Jenal K, Gönczi E, Borel N, et al. High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. Journal of Feline Medicine and Surgery. 2004;6(2):69-81.

18. Paltrinieri S, Giordano A, Stranieri A, Lauzi S. Feline infectious peritonitis (FIP) and coronavirus disease 19 (COVID-19): Are they similar? Transboundary and emerging diseases. 2021;68(4):1786-99.

19. Borschensky C, Reinacher M. Mutations in the 3c and 7b genes of feline coronavirus in spontaneously affected FIP cats. Research in Veterinary Science. 2014;97(2):333-40.

20. Bank-Wolf BR, Stallkamp I, Wiese S, Moritz A, Tekes G, Thiel H-J. Mutations of 3c and spike protein genes correlate with the occurrence of feline infectious peritonitis. Veterinary microbiology. 2014;173(3-4):177-88.

21. Pedersen NC, Liu H, Scarlett J, Leutenegger CM, Golovko L, Kennedy H, et al. Feline infectious peritonitis: role of the feline coronavirus 3c gene in intestinal tropism and pathogenicity based upon isolates from resident and adopted shelter cats. Virus research. 2012;165(1):17-28.

22. Zehr JD, Kosakovsky Pond SL, Millet JK, Olarte-Castillo XA, Lucaci AG, Shank SD, et al. Natural selection differences detected in key protein domains between non-

pathogenic and pathogenic feline coronavirus phenotypes. Virus Evolution. 2023;9(1):vead019.

23. Li F. Structure, function, and evolution of coronavirus spike proteins. Annual review of virology. 2016;3(1):237-61.

24. Bosch BJ, Van der Zee R, De Haan CA, Rottier PJ. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. Journal of virology. 2003;77(16):8801-11.

25. Belouzard S, Millet JK, Licitra BN, Whittaker GR. Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses. 2012;4(6):1011-33.

26. Millet JK, Whittaker GR. Host cell proteases: critical determinants of coronavirus tropism and pathogenesis. Virus research. 2015;202:120-34.

27. Meli ML, Spiri AM, Zwicklbauer K, Krentz D, Felten S, Bergmann M, et al. Fecal feline coronavirus RNA shedding and spike gene mutations in cats with feline infectious peritonitis treated with GS-441524. Viruses. 2022;14(5):1069.

28. Licitra BN, Millet JK, Regan AD, Hamilton BS, Rinaldi VD, Duhamel GE, et al. Mutation in spike protein cleavage site and pathogenesis of feline coronavirus. Emerging infectious diseases. 2013;19(7):1066.

29. Healey EA, Andre NM, Miller AD, Whittaker GR, Berliner EA. Outbreak of feline infectious peritonitis (FIP) in shelter-housed cats: molecular analysis of the feline coronavirus S1/S2 cleavage site consistent with a ‘circulating virulent–avirulent theory’of FIP pathogenesis. Journal of Feline Medicine and Surgery Open Reports. 2022;8(1):20551169221074226.

30. André NM, Cossic B, Davies E, Miller AD, Whittaker GR. Distinct mutation in the feline coronavirus spike protein cleavage activation site in a cat with feline infectious peritonitis-associated meningoencephalomyelitis. Journal of Feline Medicine and Surgery Open Reports. 2019;5(1):2055116919856103.

31. Ouyang H, Liu J, Yin Y, Cao S, Yan R, Ren Y, et al. Epidemiology and comparative analyses of the S gene on feline coronavirus in central China. Pathogens. 2022;11(4):460.

32. Lewis CS, Porter E, Matthews D, Kipar A, Tasker S, Helps CR, et al. Genotyping coronaviruses associated with feline infectious peritonitis. Journal of General Virology. 2015;96(6):1358-68.

33. Chang H-W, de Groot RJ, Egberink HF, Rottier PJM. Feline infectious peritonitis: insights into feline coronavirus pathobiogenesis and epidemiology based on genetic analysis of the viral 3c gene. Journal of General Virology. 2010;91(2):415-20.

34. Hsieh L-E, Huang W-P, Tang D-J, Wang Y-T, Chen C-T, Chueh L-L. 3C protein of feline coronavirus inhibits viral replication independently of the autophagy pathway. Research in Veterinary Science. 2013;95(3):1241-7.

35. Pedersen NC, Liu H, Dodd KA, Pesavento PA. Significance of Coronavirus Mutants in Feces and Diseased Tissues of Cats Suffering from Feline Infectious Peritonitis. Viruses. 2009;1(2):166-84.

36. Vennema H, Rossen JWA, Wesseling J, Horzinek MC, Rottier PJM. Genomic organization and expression of the 3′ end of the canine and feline enteric coronaviruses. Virology. 1992;191(1):134-40.

37. Myrrha LW, Silva FMF, Vidigal PMP, Resende M, Bressan GC, Fietto JLR, et al. Feline coronavirus isolates from a part of Brazil: insights into molecular epidemiology and phylogeny inferred from the 7b gene. Journal of Veterinary Medical Science. 2019;81(10):1455-60.

38. Addie DD, Toth S, Murray GD, Jarrett O. Risk of feline infectious peritonitis in cats naturally infected with feline coronavirus. American journal of veterinary research. 1995;56(4):429-34.

39. Pedersen NC. Serologic studies of naturally occurring feline infectious peritonitis. American Journal of Veterinary Research. 1976;37(12):1449-54.

40. Pedersen NC. A review of feline infectious peritonitis virus infection: 1963–2008. Journal of feline medicine and surgery. 2009;11(4):225-58.

41. Spada E, Carrera Nulla A, Perego R, Baggiani L, Proverbio D. Evaluation of Association between Blood Phenotypes A, B and AB and Feline Coronavirus Infection in Cats. Pathogens. 2022;11(8):917.

42. Taharaguchi S, Soma T, Hara M. Prevalence of Feline Coronavirus Antibodies in Japanese Domestic Cats during the Past Decade. Journal of Veterinary Medical Science. 2012;74(10):1355-8.

43. Felten S, Klein-Richers U, Unterer S, Bergmann M, Zablotski Y, Hofmann-Lehmann R, et al. Patterns of Feline Coronavirus Shedding and Associated Factors in Cats from Breeding Catteries. Viruses. 2023;15(6):1279.

44. André NM, Miller AD, Whittaker GR. Feline infectious peritonitis virus-associated rhinitis in a cat. Journal of Feline Medicine and Surgery Open Reports. 2020;6(1):2055116920930582.

45. Addie D, Jarrett O. Use of a reverse-transcriptase polymerase chain reaction for monitoring the shedding of feline coronavirus by healthy cats. Veterinary Record. 2001;148(21):649-53.

46. Pastoret PP, Henroteaux M. Epigenetic transmission of feline infectious peritonitis. Comparative Immunology, Microbiology and Infectious Diseases. 1978;1(1):67-70.

47. Addie DD, Jarrett O. Control of feline coronavirus infection in kittens. Vet Rec. 1990;126(7):164.

48. Lutz H, Gut M, Leutenegger C, Schiller I, Wiseman A, Meli M, editors. Kinetics of FCoV infection in kittens born in catteries of high risk for FIP under different rearing conditions. Second International Feline Coronavirus/Feline Infectious Peritonitis Symposium Glasgow, Scotland; 2002.

49. Stoddart ME, Gaskell RM, Harbour DA, Gaskell CJ. Virus shedding and immune responses in cats inoculated with cell culture-adapted feline infectious peritonitis virus. Veterinary Microbiology. 1988;16(2):145-58.

50. Herrewegh AAPM, Mähler M, Hedrich HJ, Haagmans BL, Egberink HF, Horzinek MC, et al. Persistence and Evolution of Feline Coronavirus in a Closed Cat-Breeding Colony. Virology. 1997;234(2):349-63.

51. Pedersen NC, Allen CE, Lyons LA. Pathogenesis of feline enteric coronavirus infection. Journal of Feline Medicine and Surgery. 2008;10(6):529-41.

52. Kipar A, Meli ML, Baptiste KE, Bowker LJ, Lutz H. Sites of feline coronavirus persistence in healthy cats. Journal of General Virology. 2010;91(7):1698-707.

53. Foley JE, Poland A, Carlson J, Pedersen NC. Patterns of feline coronavirus infection and fecal shedding from cats in multiple-cat environments. J Am Vet Med Assoc. 1997;210(9):1307-12.

54. Bubenikova J, Vrabelova J, Stejskalova K, Futas J, Plasil M, Cerna P, et al. Candidate Gene Markers Associated with Fecal Shedding of the Feline Enteric Coronavirus (FECV). Pathogens. 2020;9(11):958.

55. Vogel L, Van der Lubben M, te Lintelo EG, Bekker CP, Geerts T, Schuijff LS, et al. Pathogenic characteristics of persistent feline enteric coronavirus infection in cats. Vet Res. 2010;41(5):71.

56. Pedersen NC, Boyle JF, Floyd K, Fudge A, Barker J. An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. Am J Vet Res. 1981;42(3):368-77.

57. Sabshin SJ, Levy JK, Tupler T, Tucker SJ, Greiner EC, Leutenegger CM. Enteropathogens identified in cats entering a Florida animal shelter with normal feces or diarrhea. J Am Vet Med Assoc. 2012;241(3):331-7.

58. Hayashi T, Watabe Y, Nakayama H, Fujiwara K. Enteritis due to feline infectious peritonitis virus. Nihon Juigaku Zasshi. 1982;44(1):97-106.

59. Addie DD, Jarrett O. Use of a reverse-transcriptase polymerase chain reaction for monitoring the shedding of feline coronavirus by healthy cats. Veterinary Record. 2001;148(21):649-53.

60. Malbon AJ, Fonfara S, Meli ML, Hahn S, Egberink H, Kipar A. Feline infectious peritonitis as a systemic inflammatory disease: Contribution of liver and heart to the pathogenesis. Viruses. 2019;11(12):1144.

61. Kipar A, May H, Menger S, Weber M, Leukert W, Reinacher M. Morphologic features and development of granulomatous vasculitis in feline infectious peritonitis. Vet Pathol. 2005;42(3):321-30.

62. Malbon AJ, Michalopoulou E, Meli ML, Barker EN, Tasker S, Baptiste K, et al. Colony Stimulating Factors in Early Feline Infectious Peritonitis Virus Infection of Monocytes and in End Stage Feline Infectious Peritonitis; A Combined In Vivo And In Vitro Approach. Pathogens. 2020;9(11):893.

63. Dewerchin HL, Cornelissen E, Nauwynck HJ. Replication of feline coronaviruses in peripheral blood monocytes. Arch Virol. 2005;150(12):2483-500.

64. Rottier PJ, Nakamura K, Schellen P, Volders H, Haijema BJ. Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. Journal of virology. 2005;79(22):14122-30.

65. Malbon AJ, Russo G, Burgener C, Barker EN, Meli ML, Tasker S, et al. The Effect of Natural Feline Coronavirus Infection on the Host Immune Response: A Whole-Transcriptome Analysis of the Mesenteric Lymph Nodes in Cats with and without Feline Infectious Peritonitis. Pathogens. 2020;9(7).

66. Hsieh LE, Chueh LL. Identification and genotyping of feline infectious peritonitis-associated single nucleotide polymorphisms in the feline interferon-γ gene. Vet Res. 2014;45(1):57.

67. Pedersen NC, Liu H, Gandolfi B, Lyons LA. The influence of age and genetics on natural resistance to experimentally induced feline infectious peritonitis. Vet Immunol Immunopathol. 2014;162(1-2):33-40.

68. Mustaffa-Kamal F, Liu H, Pedersen NC, Sparger EE. Characterization of antiviral T cell responses during primary and secondary challenge of laboratory cats with feline infectious peritonitis virus (FIPV). BMC veterinary research. 2019;15:1-15.

69. Takano T, Hohdatsu T, Toda A, Tanabe M, Koyama H. TNF-alpha, produced by feline infectious peritonitis virus (FIPV)-infected macrophages, upregulates expression of type II FIPV receptor feline aminopeptidase N in feline macrophages. Virology. 2007;364(1):64-72.

70. Takano T, Hohdatsu T, Hashida Y, Kaneko Y, Tanabe M, Koyama H. A “possible” involvement of TNF-alpha in apoptosis induction in peripheral blood lymphocytes of cats with feline infectious peritonitis. Veterinary Microbiology. 2007;119(2):121-31.

71. Takano T, Azuma N, Satoh M, Toda A, Hashida Y, Satoh R, et al. Neutrophil survival factors (TNF-alpha, GM-CSF, and G-CSF) produced by macrophages in cats infected with feline infectious peritonitis virus contribute to the pathogenesis of granulomatous lesions. Arch Virol. 2009;154(5):775-81.

72. Vermeulen BL, Devriendt B, Olyslaegers DA, Dedeurwaerder A, Desmarets LM, Favoreel HW, et al. Suppression of NK cells and regulatory T lymphocytes in cats naturally infected with feline infectious peritonitis virus. Veterinary microbiology. 2013;164(1-2):46-59.

73. de Groot-Mijnes JD, van Dun JM, van der Most RG, de Groot RJ. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. Journal of virology. 2005;79(2):1036-44.

74. Giordano A, Paltrinieri S. Interferon-γ in the serum and effusions of cats with feline coronavirus infection. The Veterinary Journal. 2009;180(3):396-8.

75. Addie D, Jarrett O. A study of naturally occurring feline coronavirus infections in kittens. The Veterinary Record. 1992;130(7):133-7.

76. Takano T, Azuma N, Hashida Y, Satoh R, Hohdatsu T. B-cell activation in cats with feline infectious peritonitis (FIP) by FIP-virus-induced B-cell differentiation/survival factors. Archives of virology. 2009;154:27-35.

77. Riemer F, Kuehner KA, Ritz S, Sauter-Louis C, Hartmann K. Clinical and laboratory features of cats with feline infectious peritonitis–a retrospective study of 231 confirmed cases (2000–2010). Journal of feline medicine and surgery. 2016;18(4):348-56.

78. Crawford A, Stoll A, Sanchez-Masian D, Shea A, Michaels J, Fraser A, et al. Clinicopathologic features and magnetic resonance imaging findings in 24 cats with histopathologically confirmed neurologic feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2017;31(5):1477-86.

79. Bauer BS, Kerr ME, Sandmeyer LS, Grahn BH. Positive immunostaining for feline infectious peritonitis (FIP) in a Sphinx cat with cutaneous lesions and bilateral panuveitis. Veterinary Ophthalmology. 2013;16:160-3.

80. Cannon MJ, Silkstone MA, Kipar AM. Cutaneous lesions associated with coronavirus-induced vasculitis in a cat with feline infectious peritonitis and concurrent feline immunodeficiency virus infection. Journal of Feline Medicine and Surgery. 2005;7(4):233-6.

81. Ernandes MA, Cantoni AM, Armando F, Corradi A, Ressel L, Tamborini A. Feline coronavirus-associated myocarditis in a domestic longhair cat. Journal of Feline Medicine and Surgery Open Reports. 2019;5(2):2055116919879256.

82. Pedersen NC, Perron M, Bannasch M, Montgomery E, Murakami E, Liepnieks M, et al. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. Journal of feline medicine and surgery. 2019;21(4):271-81.

83. Araujo G, Matta E, Lallo M, Machado G, Rocha P. Epicarditis in a cat caused by feline infectious peritonitis virus: case report. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2020;72(03):823-6.

84. Guarnieri C, Bertola L, Ferrari L, Quintavalla C, Corradi A, Di Lecce R. Myocarditis in an FIP-diseased cat with FCoV M1058L mutation: Clinical and pathological changes. Animals. 2024;14(11):1673.

85. Fonfara S, Kitz S, Monteith G, Hahn S, Kipar A. Myocardial transcription of inflammatory and remodeling markers in cats with hypertrophic cardiomyopathy and systemic diseases associated with an inflammatory phenotype. Research in Veterinary Science. 2021;136:484-94.

86. Ho JS, Sia C-H, Chan MY, Lin W, Wong RC. Coronavirus-induced myocarditis: a meta-summary of cases. Heart & Lung. 2020;49(6):681-5.

87. Mele D, Flamigni F, Rapezzi C, Ferrari R. Myocarditis in COVID-19 patients: current problems. Internal and emergency medicine. 2021;16(5):1123-9.

88. Irabien-Ortiz Á, Carreras-Mora J, Sionis A, Pàmies J, Montiel J, Tauron M. Fulminant myocarditis due to COVID-19. Revista espanola de cardiologia (English Ed). 2020;73(6):503.

89. Ferasin L, Fritz M, Ferasin H, Becquart P, Corbet S, Ar Gouilh M, et al. Infection with SARS-CoV-2 variant B. 1.1. 7 detected in a group of dogs and cats with suspected myocarditis. Veterinary Record. 2021;189(9):no-no.

90. Mitchell K, Kruth S. Immune-mediated hemolytic anemia and other regenerative anemias. Textbook of veterinary internal medicine. 2010:761-72.

91. Scott D, Schultz R, Post J, Bolton G, Baldwin C. Autoimmune hemolytic anemia in the cat. 1973.

92. Werner LL, Gorman NT. Immune-mediated disorders of cats. The veterinary clinics of North America Small animal practice. 1984;14(5):1039-64.

93. Maede Y, Hata R. Studies on feline haemobartonellosis. II. The mechanism of anemia produced by infection with Haemobartonella felis. 1975.

94. Naik R. Warm autoimmune hemolytic anemia. Hematology/Oncology Clinics. 2015;29(3):445-53.

95. Limlenglert P, Rungsipipat A, Pusoonthornthum R. Application of flow cytometry for early diagnosis and monitoring in cats with immune-mediated hemolytic anemia. The Thai Journal of Veterinary Medicine. 2011;41(2):185-92.

96. Swann J, Szladovits B, Glanemann B. Demographic characteristics, survival and prognostic factors for mortality in cats with primary immune-mediated hemolytic anemia. Journal of veterinary internal medicine. 2016;30(1):147-56.

97. SM N. Immune-mediated hemolytic anemia in cats referring to Veterinary Teaching Hospital of Tehran [2006-2007]. 2009.

98. Piek C, Dekker A, Junius G, Slappendel R, Teske E, editors. Idiopathic immune-mediated hemolytic anemia (IMHA) in cats: differentiation from secondary IMHA and outcome of treatment. 13th European College of Veterinary Internal Medicine Conference, Uppsala, Sweden; 2003.

99. Al Khoufi E, Al-Muhainy B, Algadeeb K, Alsalman M. Autoimmune hemolytic anemia: a late presentation of post-COVID-19 syndrome. Oman Medical Journal. 2023;38(1):e467.

100. Al-Kuraishy HM, Al-Gareeb AI, Kaushik A, Kujawska M, Batiha GE-S. Hemolytic anemia in COVID-19. Annals of hematology. 2022;101(9):1887-95.

101. Sparkes A, Gruffydd-Jones T, Harbour D. Feline infectious peritonitis: a review of clinicopathological changes in 65 cases, and a critical assessment of their diagnostic value. The Veterinary Record. 1991;129(10):209-12.

102. Norris J, Bosward K, White J, Baral R, Catt M, Malik R. Clinicopathological findings associated with feline infectious peritonitis in Sydney, Australia: 42 cases (1990–2002). Australian Veterinary Journal. 2005;83(11):666-73.

103. Tsai H-Y, Chueh L-L, Lin C-N, Su B-L. Clinicopathological findings and disease staging of feline infectious peritonitis: 51 cases from 2003 to 2009 in Taiwan. Journal of Feline Medicine and Surgery. 2011;13(2):74-80.

104. Hartmann K, Binder C, Hirschberger J, Cole D, Reinacher M, Schroo S, et al. Comparison of different tests to diagnose feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2003;17(6):781-90.

105. Taylor SS, Tappin SW, Dodkin SJ, Papasouliotis K, Casamian-Sorrosal D, Tasker S. Serum protein electrophoresis in 155 cats. Journal of Feline Medicine and Surgery. 2010;12(8):643-53.

106. Goto S, Kamiyoshi T, Iwasaki R. Predictive factors associated with short-term mortality in cats with feline infectious peritonitis treated with remdesivir or GS-441524 or both. Journal of Veterinary Internal Medicine. 2025;39(1):e17249.

107. Hazuchova K, Held S, Neiger R. Usefulness of acute phase proteins in differentiating between feline infectious peritonitis and other diseases in cats with body cavity effusions. Journal of feline medicine and surgery. 2017;19(8):809-16.

108. Giori L, Giordano A, Giudice C, Grieco V, Paltrinieri S. Performances of different diagnostic tests for feline infectious peritonitis in challenging clinical cases. Journal of Small Animal Practice. 2011;52(3):152-7.

109. Duthie S, Eckersall P, Addie D, Lawrence C, Jarrett O. Value of α1-acid glycoprotein in the diagnosis of feline infectious peritonitis. Veterinary Record. 1997;141(12):299-303.

110. Paltrinieri S, Giordano A, Tranquillo V, Guazzetti S. Critical assessment of the diagnostic value of feline alpha (1)-acid glycoprotein for feline infectious peritonitis using the likelihood ratios approach (vol 19, pg 266, 2007). JOURNAL OF VETERINARY DIAGNOSTIC INVESTIGATION. 2008;20(3):387-.

111. Bell E, Toribio J, White J, Malik R, Norris J. Seroprevalence study of feline coronavirus in owned and feral cats in Sydney, Australia. Australian veterinary journal. 2006;84(3):74-81.

112. Meli ML, Burr P, Decaro N, Graham E, Jarrett O, Lutz H, et al. Samples with high virus load cause a trend toward lower signal in feline coronavirus antibody tests. Journal of Feline Medicine and Surgery. 2013;15(4):295-9.

113. Bell E, Malik R, Norris J. The relationship between the feline coronavirus antibody titre and the age, breed, gender and health status of Australian cats. Australian veterinary journal. 2006;84(1-2):2-7.

114. Müller TR, Penninck DG, Webster CR, Conrado FO. Abdominal ultrasonographic findings of cats with feline infectious peritonitis: an update. Journal of Feline Medicine and Surgery. 2023;25(12):1098612X231216000.

115. Slaviero M, Cony FG, da Silva RC, De Lorenzo C, de Almeida BA, Bertolini M, et al. Pathological findings and patterns of feline infectious peritonitis in the respiratory tract of cats. Journal of Comparative Pathology. 2024;210:15-24.

116. Repyak K, Atiee G, Cook A, Bryan L, Gremillion C. Thoracic radiographic findings in cats with feline infectious peritonitis. Journal of Feline Medicine and Surgery. 2025;27(2):1098612X241309823.

117. Giordano A, Paltrinieri S, Bertazzolo W, Milesi E, Parodi M. Sensitivity of Tru-cut and fine-needle aspiration biopsies of liver and kidney for diagnosis of feline infectious peritonitis. Veterinary Clinical Pathology. 2005;34(4):368-74.

118. Kipar A, Meli M. Feline infectious peritonitis: still an enigma? Veterinary pathology. 2014;51(2):505-26.

119. Felten S, Matiasek K, Gruendl S, Sangl L, Wess G, Hartmann K. Investigation into the utility of an immunocytochemical assay in body cavity effusions for diagnosis of feline infectious peritonitis. Journal of Feline Medicine and Surgery. 2017;19(4):410-8.

120. Paltrinieri S, Parodi MC, Cammarata G. In vivo diagnosis of feline infectious peritonitis by comparison of protein content, cytology, and direct immunofluorescence test on peritoneal and pleural effusions. Journal of Veterinary Diagnostic Investigation. 1999;11(4):358-61.

121. Ives EJ, Vanhaesebrouck AE, Cian F. Immunocytochemical demonstration of feline infectious peritonitis virus within cerebrospinal fluid macrophages. Journal of Feline Medicine and Surgery. 2013;15(12):1149-53.

122. Felten S, Matiasek K, Gruendl S, Sangl L, Hartmann K. Utility of an immunocytochemical assay using aqueous humor in the diagnosis of feline infectious peritonitis. Veterinary Ophthalmology. 2018;21(1):27-34.

123. Chang H-W, Egberink HF, Halpin R, Spiro DJ, Rottier PJ. Spike protein fusion peptide and feline coronavirus virulence. Emerging infectious diseases. 2012;18(7):1089.

124. Porter E, Tasker S, Day MJ, Harley R, Kipar A, Siddell SG, et al. Amino acid changes in the spike protein of feline coronavirus correlate with systemic spread of virus from the intestine and not with feline infectious peritonitis. Veterinary Research. 2014;45:1-11.

125. Barker EN, Stranieri A, Helps CR, Porter EL, Davidson AD, Day MJ, et al. Limitations of using feline coronavirus spike protein gene mutations to diagnose feline infectious peritonitis. Veterinary Research. 2017;48:1-14.

126. Longstaff L, Porter E, Crossley VJ, Hayhow SE, Helps CR, Tasker S. Feline coronavirus quantitative reverse transcriptase polymerase chain reaction on effusion

samples in cats with and without feline infectious peritonitis. Journal of feline medicine and surgery. 2017;19(2):240-5.

127. Felten S, Weider K, Doenges S, Gruendl S, Matiasek K, Hermanns W, et al. Detection of feline coronavirus spike gene mutations as a tool to diagnose feline infectious peritonitis. Journal of Feline Medicine and Surgery. 2017;19(4):321-35.

128. Pedersen NC, Kim Y, Liu H, Galasiti Kankanamalage AC, Eckstrand C, Groutas WC, et al. Efficacy of a 3C-like protease inhibitor in treating various forms of acquired feline infectious peritonitis. Journal of Feline Medicine and Surgery. 2018;20(4):378-92.

129. Dickinson PJ, Bannasch M, Thomasy SM, Murthy VD, Vernau KM, Liepnieks M, et al. Antiviral treatment using the adenosine nucleoside analogue GS-441524 in cats with clinically diagnosed neurological feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2020;34(4):1587-93.

130. Murphy BG, Perron M, Murakami E, Bauer K, Park Y, Eckstrand C, et al. The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. Veterinary Microbiology. 2018;219:226-33.

131. Krentz D, Zenger K, Alberer M, Felten S, Bergmann M, Dorsch R, et al. Curing Cats with Feline Infectious Peritonitis with an Oral Multi-Component Drug Containing GS-441524. Viruses. 2021;13(11):2228.

132. Krentz D, Zwicklbauer K, Felten S, Bergmann M, Dorsch R, Hofmann-Lehmann R, et al. Clinical Follow-Up and Postmortem Findings in a Cat That Was Cured of Feline Infectious Peritonitis with an Oral Antiviral Drug Containing GS-441524. Viruses. 2022;14(9).

133. Zuzzi-Krebitz A-M, Buchta K, Bergmann M, Krentz D, Zwicklbauer K, Dorsch R, et al. Short Treatment of 42 Days with Oral GS-441524 Results in Equal Efficacy as the Recommended 84-Day Treatment in Cats Suffering from Feline Infectious Peritonitis with Effusion—A Prospective Randomized Controlled Study. Viruses. 2024;16(7):1144.

134. Zwicklbauer K, Krentz D, Bergmann M, Felten S, Dorsch R, Fischer A, et al. Long-term follow-up of cats in complete remission after treatment of feline infectious peritonitis with oral GS-441524. Journal of Feline Medicine and Surgery. 2023;25(8):1098612X231183250.

135. Taylor SS, Coggins S, Barker EN, Gunn-Moore D, Jeevaratnam K, Norris JM, et al. Retrospective study and outcome of 307 cats with feline infectious peritonitis treated with legally sourced veterinary compounded preparations of remdesivir and GS-441524 (2020–2022). Journal of Feline Medicine and Surgery. 2023;25(9):1098612X231194460.

136. Green J, Syme H, Tayler S. Thirty-two cats with effusive or non-effusive feline infectious peritonitis treated with a combination of remdesivir and GS-441524. Journal of Veterinary Internal Medicine. 2023;n/a(n/a).

137. Coggins SJ, Norris JM, Malik R, Govendir M, Hall EJ, Kimble B, et al. Outcomes of treatment of cats with feline infectious peritonitis using parenterally administered remdesivir, with or without transition to orally administered GS-441524. Journal of Veterinary Internal Medicine. 2023;37(5):1772-83.

138. Jones S, Novicoff W, Nadeau J, Evans S. Unlicensed GS-441524-Like Antiviral Therapy Can Be Effective for at-Home Treatment of Feline Infectious Peritonitis. Animals. 2021;11(8):2257.

139. Roy M, Jacque N, Novicoff W, Li E, Negash R, Evans SJM. Unlicensed Molnupiravir is an Effective Rescue Treatment Following Failure of Unlicensed GS-441524-like Therapy for Cats with Suspected Feline Infectious Peritonitis. Pathogens. 2022;11(10):1209.

140. Sase O. Molnupiravir treatment of 18 cats with feline infectious peritonitis: A case series. Journal of Veterinary Internal Medicine. 2023;37(5):1876-80.

141. Sase O, Iwami T, Sasaki T, Sano T. GS-441524 and molnupiravir are similarly effective for the treatment of cats with feline infectious peritonitis. Front Vet Sci. 2024;11:1422408.

142. Reagan KL, Brostoff T, Pires J, Rose A, Castillo D, Murphy BG. Open label clinical trial of orally administered molnupiravir as a first-line treatment for naturally occurring effusive feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2024;38(6):3087-94.

143. Delaplace M, Huet H, Gambino A, Le Poder S. Feline Coronavirus Antivirals: A Review. Pathogens. 2021;10(9):1150.

144. De Clercq E. Antivirals and antiviral strategies. Nature Reviews Microbiology. 2004;2(9):704-20.

145. Yates MKS-R, Katherine L. The evolution of nucleoside analogue antivirals: A review for chemists and non-chemists. Part 1: Early structural modifications to the nucleoside scaffold. Antiviral Research. 2018;154:66-86.

146. Bravo JPK, Dangerfield TL, Taylor DW, Johnson KA. Remdesivir is a delayed translocation inhibitor of SARS-CoV-2 replication. Molecular Cell. 2021;81(7):1548-52.e4.

147. Agostini ML, Andres EL, Sims AC, Graham RL, Sheahan TP, Lu X, et al. Coronavirus Susceptibility to the Antiviral Remdesivir (GS-5734) Is Mediated by the Viral Polymerase and the Proofreading Exoribonuclease. mBio. 2018;9(2):e00221-18.

148. Warren TK, Jordan R, Lo MK, Ray AS, Mackman RL, Soloveva V, et al. Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. Nature. 2016;531(7594):381-5.

149. Avataneo V, de Nicolò A, Cusato J, Antonucci M, Manca A, Palermiti A, et al. Development and validation of a UHPLC-MS/MS method for quantification of the prodrug remdesivir and its metabolite GS-441524: a tool for clinical pharmacokinetics of SARS-CoV-2/COVID-19 and Ebola virus disease. Journal of Antimicrobial Chemotherapy. 2020;75(7):1772-7.

150. Sheahan TP, Sims AC, Graham RL, Menachery VD, Gralinski LE, Case JB, et al. Broad-spectrum antiviral GS-5734 inhibits both epidemic and zoonotic coronaviruses. Science Translational Medicine. 2017;9(396):eaal3653.

151. Humeniuk R, Mathias A, Cao H, Osinusi A, Shen G, Chng E, et al. Safety, Tolerability, and Pharmacokinetics of Remdesivir, An Antiviral for Treatment of COVID-19, in Healthy Subjects. Clinical and Translational Science. 2020;13(5):896-906.

152. Williamson BN, Feldmann F, Schwarz B, Meade-White K, Porter DP, Schulz J, et al. Clinical benefit of remdesivir in rhesus macaques infected with SARS-CoV-2. Nature. 2020;585(7824):273-6.

153. Li Y, Cao L, Li G, Cong F, Li Y, Sun J, et al. Remdesivir Metabolite GS-441524 Effectively Inhibits SARS-CoV-2 Infection in Mouse Models. Journal of Medicinal Chemistry. 2022;65(4):2785-93.

154. Coggins Sally J KB, Malik R,Thompson MF, Norris JM, Govendir M,. Assessing in vitro stability of remdesivir (GS-5734) and conversion to GS-441524 in feline plasma and whole blood. Veterinary Quarterly. 2024;0(0):8.

155. Cook S, Wittenburg L, Yan VC, Theil JH, Castillo D, Reagan KL, et al. An Optimized Bioassay for Screening Combined Anticoronaviral Compounds for Efficacy against Feline Infectious Peritonitis Virus with Pharmacokinetic Analyses of GS-441524, Remdesivir, and Molnupiravir in Cats. Viruses. 2022;14(11):2429.

156. Coggins SJ, Govendir M, Norris JM, Malik R, Hall E, Thompson MF, et al. Pharmacokinetics of GS-441524 following intravenous remdesivir in six cats and preliminary results of therapeutic drug monitoring (22 cats) during treatment of feline infectious peritonitis: 22 cases (2021-2024). Journal of Small Animal Practice. 2025;In Submission (Accepted).

157. Teli D, Balar P, Patel K, Sharma A, Chavda V, Vora L. Molnupiravir: A Versatile Prodrug against SARS-CoV-2 Variants. Metabolites. 2023;13(2).

158. Tian L, Pang Z, Li M, Lou F, An X, Zhu S, et al. Molnupiravir and Its Antiviral Activity Against COVID-19. Front Immunol. 2022;13:855496.

159. Cerna P. 2024 ACVIM Forum Research Abstract Program. Journal of Veterinary Internal Medicine. 2024;38(5):2922.

160. Alex Kennedy JW. 2024 ACVIM Forum Research Abstract Program. Journal of Veterinary Internal Medicine. 2024;38(5):2840-970.

161. Zhou S, Hill CS, Sarkar S, Tse LV, Woodburn BMD, Schinazi RF, et al. β-d-N4-hydroxycytidine Inhibits SARS-CoV-2 Through Lethal Mutagenesis But Is Also Mutagenic To Mammalian Cells. The Journal of Infectious Diseases. 2021;224(3):415-9.

162. Liu Y, Liang C, Xin L, Ren X, Tian L, Ju X, et al. The development of Coronavirus 3C-Like protease (3CLpro) inhibitors from 2010 to 2020. European Journal of Medicinal Chemistry. 2020;206:112711.

163. Thiel V, Ivanov KA, Putics Á, Hertzig T, Schelle B, Bayer S, et al. Mechanisms and enzymes involved in SARS coronavirus genome expression. Journal of General Virology. 2003;84(9):2305-15.

164. Chen Y, Liu Q, Guo D. Emerging coronaviruses: Genome structure, replication, and pathogenesis. Journal of Medical Virology. 2020;92(4):418-23.

165. Kim Y, Shivanna V, Narayanan S, Prior AM, Weerasekara S, Hua DH, et al. Broad-Spectrum Inhibitors against 3C-Like Proteases of Feline Coronaviruses and Feline Caliciviruses. Journal of Virology. 2015;89(9):4942-50.

166. Kim Y, Liu H, Galasiti Kankanamalage AC, Weerasekara S, Hua DH, Groutas WC, et al. Reversal of the Progression of Fatal Coronavirus Infection in Cats by a Broad-Spectrum Coronavirus Protease Inhibitor. PLOS Pathogens. 2016;12(3):e1005531.

167. Lv J, Bai Y, Wang Y, Yang L, Jin Y, Dong J. Effect of GS-441524 in combination with the 3C-like protease inhibitor GC376 on the treatment of naturally transmitted feline infectious peritonitis. Frontiers in Veterinary Science. 2022;9.

168. Focosi D, Maggi F, McConnell S, Casadevall A. Very low levels of remdesivir resistance in SARS-COV-2 genomes after 18 months of massive usage during the COVID19 pandemic: A GISAID exploratory analysis. Antiviral Research. 2022;198:105247.

169. Sanderson T, Hisner R, Donovan-Banfield Ia, Hartman H, Løchen A, Peacock TP, et al. A molnupiravir-associated mutational signature in global SARS-CoV-2 genomes. Nature. 2023;623(7987):594-600.

Chapter 2: COMPARISON OF ANTIVIRAL IMMUNE RESPONSES IN HEALTHY CATS INDUCED BY TWO IMMUNE THERAPEUTICS[1]

2.1 Summary

Effective immunotherapeutic agents for use in cats are needed to aid in the management of intractable viral diseases, including feline infectious peritonitis (FIP) infection. The objectives of this study were to compare two different immune stimulants for antiviral activity in cats: (1) TLR 2/6-activating compound polyprenyl immunostimulant; (PI) and (2) liposome Toll-like receptor 3/9 agonist complexes (LTCs). Comparisons included determining relative abilities to stimulate the induction of type I (IFN-α, IFN-β) and type II (IFN-γ) interferon immune responses in vitro and to study the effects of treatment on immune responses in healthy cats. Cytokine and cellular immune responses to PI and LTC were evaluated using peripheral blood mononuclear cells (PBMCs) from healthy cats incubated with LTC and PI at indicated concentrations using reverse transcriptase polymerase chain reaction assays and ELISA assays. The effects of the immune stimulants on inhibiting FIPV replication were assessed using a feline macrophage cell line (fcwf-4). Cytokine and cellular immune responses to PI and LTC were evaluated in blood samples from healthy cats treated with PI and LTC, using reverse transcriptase polymerase chain reaction (RT-PCR) and ELISA assays. In the in vitro studies, both compounds triggered the upregulated expression of IFN-α, IFN-γ, and IL-1β genes in cat PBMC, whereas treatment with LTC induced significantly greater

[1] This chapter includes the complete published manuscript: Cerna P, Dow S, Wheat W, Chow L, Hawley J, Lappin MR. Comparison of Antiviral Immune Responses in Healthy Cats Induced by Two Immune Therapeutics. Pathogens. 2024 Jul 22;13(7):602.

expression of IFN-α and IFN-γ on Day 1 and IL-1b on Day 3. There was significant protection from FIPV-induced cytopathic effects when fcwf-4 cells were treated with conditioned medium from LTC-activated leukocytes. In the healthy cat study (in vivo), both PI and LTC increased the mRNA signal for IFN-α, IFN-γ, and IL-1β above baseline at multiple time points with statistically greater increases in the LTC group on either Day 1 (IFN-α, IFN-γ) or Day 3 (IL-1β). In addition, RANTES increased over time in cats treated with the LTC. Both LTC and PI protocols induced immune enhancing effects, suggesting a possible clinical use for the management of chronic infectious diseases like FIP. Activating the TLR 3 and 9 pathways (LTC) induced superior broad interferon production in vitro than the activation of the TLR 2 and 6 pathways (PI).

2.2 Introduction

Feline infectious peritonitis (FIP) has been a challenge for veterinarians and has been recognized as a devastating disease among cats for over half a century. Despite marked efforts and many theories, the pathogenesis of FIP is still not fully understood (1, 2). Deficiencies in cell-mediated immunity promote the exuberant production of antibodies to feline coronavirus (FCoV), which results in antibody-dependent cytotoxicity and immune complex deposition (3-5). Cats that resist FIPV are believed to mount a vigorous cell-mediated immune response and overcome the negative effects of FCoV antibodies (6). Cell-mediated immunity is therefore believed to play an important role in controlling or eliminating the mutated virus. Cats in the terminal stages of FIP have severe depletion of the CD4+ and CD8+ T-lymphocytes necessary for mounting cell-mediated immunity (7, 8). Several cytokines have also been reported to play a role in FIP (9-13). With immune

dysregulation being a major component of the pathophysiology of FIP, treatment with an antiviral immunostimulant that triggers strong endogenous interferon production would be a rational approach.

While recent studies have reported the use of drugs like the nucleoside analog GS-441524 for the successful treatment of FIP, their availability in the US is limited, with only remdesivir (Veklury, Gilead USA) and nirmatrelvir and ritonavir (Paxlovid, Pfizer, USA) being FDA approved in humans (14-16). It has been suggested that antiviral drugs can act synergistically with immunomodulatory treatments to improve patient outcome and survival in different viral diseases, such as influenza virus, hepatitis C virus, and human immunodeficiency virus (HIV) (17-19). This synergism could be considered to improve long-term treatment outcomes for cats with FIP as well. Polyprenyl immunostimulant (PI) is believed to upregulate innate immunity via TLR2 and TLR6 and is licensed by the U.S. Department of Agriculture for the reduction in the severity of signs of feline herpesvirus 1 (FHV-1) in cats over 8 weeks of age (20). The product has also been evaluated in two open trials of cats with non-effusive FIP with some positive results (21, 22). The liposome Toll-like receptor 3/9 agonist complex (LTC) immune therapeutic has been studied in different veterinary species, including cats (23). The effects of the LTC on the innate immune system has been well documented in healthy cats (23) and in follow-up studies showing effects against FHV-1 in a feline model (24, 25). Another study showed that a similar compound could be given safely by parenteral inoculation to cats with a positive effect on chronic rhinitis (26). The objectives of this study were to determine the ability of the PI and LTC to stimulate the induction of type I (IFN-α, IFN-β) and type II (IFN-γ) interferon immune responses in vitro, to activate feline leukocytes to secrete

factors capable of suppressing feline infectious peritonitis virus (FIPV) replication in vitro, and to induce evidence for immunomodulation when administered to healthy cats. The primary hypothesis was that both the PI and LTC would activate a variety of innate immune responses in cats that could be measured in vitro or in vivo.

2.3 Materials and methods

Animals

Purpose-bred cats for use in these experiments were originally purchased from Liberty Research (Waverly, New York, NY, USA). The use of the cats to provide peripheral blood mononuclear cells (PBMCs) for in vitro experiments and to assess two in vivo treatment protocols were approved by the Institutional Animal Care and Use Committee (Protocol # 170.063) of a local independent contract research facility. All young adult, mixed sex cats used in the study were group housed with multiple perches and other enrichment devices (ping pong balls; boxes to hide in) with ad libitum access to clean water and dry food. The cats had a complete physical examination performed by a study veterinarian prior to starting the study and then weekly (including body weight) for the duration of the study. All cats had complete blood cell count (CBC), serum biochemistry panel, and urinalysis performed prior to starting the study to assess for normalcy. The cats were assessed for vomiting, diarrhea, appetite, and attitude daily by facility employees for the duration of the study. After the completion of all experiments, the cats were adopted or returned to the colony in the research facility.

In vitro experiments

In vitro experiments with feline PBMC: Feline PBMC (peripheral blood mononuclear cells) were extracted from whole blood of some of the healthy, untreated cats using Ficoll-Paque (Cytiva, Uppsala, Sweden) following manufacturer's protocol. The PBMCs were incubated with LTCs and PI at indicated concentrations (1 µL per well, 0.3 µL per well, and 0.1 µL per well) in 24-well cell culture plates with 2 ×106 cells per well in 1 mL of complete growth media (DMEM, 10%FBS, essential and nonessential amino acids, glutamax, and pen/strep). After 8 h, RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, and RT-PCR was performed using GoTaq® 1-step-PCR-system (Promega, Singapore) on QuantStudio 3 Real-Time PCR system. Primer and probe sequences used in this in vitro work are shown in Table S1. All primer and probe pairs were designed using IDT primer quest tool (IFN-α, IFN-γ, IL-1β, RPL30 housekeeping gene) and were verified by standard curve to have efficiency >95%. To detect the production of IFN-γ and IL-6, supernatants were collected from PBMC cultures with 1 ×106 cells per well in 200 uL of complete media after 24 h. Cells were treated with increasing doses of PI or LTCs as indicated. Supernatants were assayed undiluted using a commercially available kits (Feline IFN-γ DuoSet ELISA and Feline specific IL-6 Duo Set ELISA R&D systems, Minneapolis, MN, USA) according to manufacturer's instructions.

LTC protection from viral infection

FIP virus (FIP79a) was propagated as previously described (27). Optimal viral titer was determined using 50% viability. To assess the ability of LTC to trigger the release of

cytokines capable of suppressing FIPV cytopathic effects on target cells (macrophages). The feline macrophage cell line (fcwf-4, ATCC American Tissue Culture System, Manassas, VA, USA) was grown in cell culture with MEM containing 10%FBS and was used for FIPV infection and cytopathicity studies. After the fcwf-4 cells were confluent, they were incubated with supernatant from LTC-treated feline PBMC cultures (or untreated PBMC supernatant for control); then, FIPV was added at an MOI of 3 and incubated at 37 ◦C and 5% CO2 for another 4 h. After 4 h, the FIPV supernatant was washed away, and the Fcwf cells were incubated for another 72 h in complete growth medium containing FBS. At 72 h post infection, non-adherent dead cells were washed away, and each well was imaged using Olympus CKX41 inverted brightfield microscope. Live cells were counted using ImageJ (28).

In vivo PI and LTC treatment study design

In the in vivo treatment study, 12 young adult cats (6 males; 6 females) were randomly housed in two groups (6 cats in the PI group and 6 cats in the LTC group, each in a 12′×14′room). The PI product was purchased from the manufacturer. The LTC was prepared as described previously (23). Each cat that was randomized to the PI group was administered PI at 3 mg/kg orally once a day for 14 days, as recommended by the manufacturer. Cats that were greater than 5 kg were administered a maximum dose of 15 mg. All cats were monitored for 30 min after each dose of PI for vomiting. For treatment with LTC, a dose of 1 mL was selected for evaluation based on prior experience with LTC administered topically in cats (24, 25) and in dogs as a cancer vaccine adjuvant (29). All cats in the LTC group were administered LTC 1 mL SC twice weekly for 2 weeks.

Temperatures were recorded before LTC or PI administration and at 12 and 24 h after administration. For cats treated with LTC, injection sites were examined for skin reaction and pain prior to each LTC injection and at 12 h and 24 h after each injection.

All cats had complete blood cell count (CBC), serum biochemistry panel, and urinalysis performed at the end of the study to assess for side effects. In vivo PI and LTC treatment study immune assays. Blood was collected from all 12 cats prior to the administration of the PI or LTCs and then on days 1, 3, 7, and 14 for serum separation prior to assay for 19 different cytokines and chemokines (sFas, Flt-3 ligand, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12 (p40), IL-13, IL-18, KC, MCP-1, PDGF-BB, RANTES, SCF, SDF-1, TNF-α) by the use of a commercially available kit (MILLIPLEX MAP Feline Cytokine/Chemokine Magnetic Bead Panel Premixed 19 Plex, FCYTOMAG-20K-PMX—Immunology Multiplex Assay, Millipore Sigma), and assays were performed according to the manufacturer's instructions.

Whole blood in EDTA was subjected to Ficoll density gradient separation to produce PBMCs (see below). The PBMCs were stimulated in vitro and used in quantitative reverse-transcriptase PCR assays (qRT-PCRs) to amplify the mRNA of select cytokine genes (IFN-α, IFN-γ, IFN-β, IL-1β, IL-6, and TNF-α) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a housekeeping gene) using previously published techniques (23, 30).

Statistical methods

In the feline PBMC experiments, comparisons amongst control wells and the 3 concentrations of PI and LTCs were compared using ANOVA, followed by the Tukey

multiple means post-test (Prism9 software; GraphPad, La Jolla, CA, USA). Differences in the extent of cytotoxicity induced by FIPV were compared between treatment groups using a non-parametric t test. In the in vivo PI versus LTC cat treatment study, the results of assays over time were compared between the groups using repeated measures ANOVA and between groups at individual time points by two tailed Student's t test. For t test. For all analyses, statistical significance was set at $p < 0.05$.

2.4 Results

In vitro experiments with feline PBMC: The in vitro studies were set up to assess and compare the immunological activities of two compounds for cats. The first study used vitro cultures of in vitro cultures of PBMC from healthy cats to assess the induction of expression of genes encoding six key antiviral cytokines (IFN-a, IFN-g, IFN-b, TNF-a, IL-1b, and IL-12). When compared to untreated PBMC, the significant expression of IFN-α, IFN-β, or IFN-γ was not induced by PI at any of the three concentrations studied (Figure 2.1). In contrast, the LTC induced significant increases in all three cytokines, with variation amongst different concentrations (Figure 2.1).

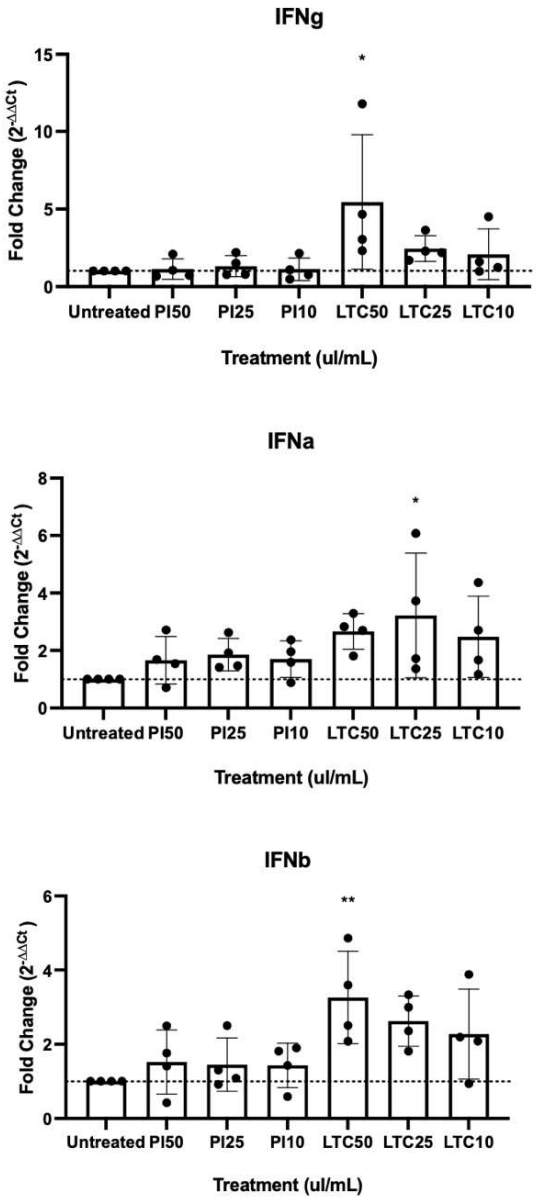


Figure 2.1. In vitro activation of expression of IFN-γ, IFN-α, and IFN-β measured by RT-PCR of specific mRNA after an 8-h incubation of feline PBMCs with three different concentrations of PI (PP), and LTC concentrations are shown in a decreasing scale of 50 µL/mL (1mL media per well), 25 µL/mL, and 10 µL/mL. LTC at concentration 50 µL/mL induced significant expression of interferon IFN-γ ($p = 0.0152$). LTC at concentration 25 µL/mL induced significant expression of IFN-α ($p = 0.0441$), and IFN-β had induced significant expression by LTC at concentration of 50 µL/mL ($p = 0.0060$). Significance * $p < 0.05$ and ** $p < 0.01$.

ELISA assays for feline proinflammatory cytokines IL-6 and IFN-γ were used to determine their release by immune-activated PBMC cultures assessing the impact of LTC and PI treatment on secretion by feline PBMC. The proinflammatory cytokines IL-6 and IFN-γ were measured in vitro from feline PBMCs treated with various concentrations of either PI or LTC. LTC was generally a stronger immune stimulant that PI at high concentrations (Figure 2.2) with higher concentrations of this formulation of LTC mediating the highest release of IL-6.

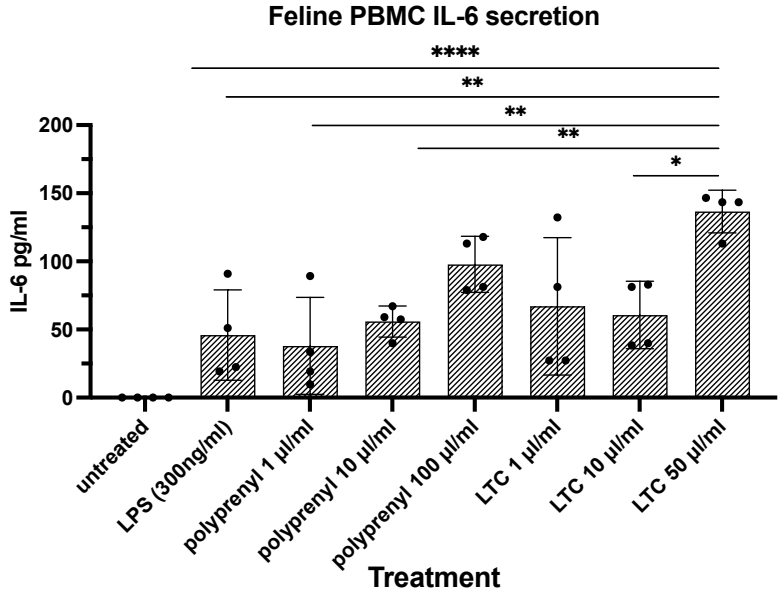


Figure 2.2. IL-6 secretion by feline PBMCs: polyprenyl vs. LTC. Statistical differences were obtained using a one-way ANOVA with Tukey's multiple comparisons test with ****, $p \leq 0.001$; **, $p \leq 0.01$; and *, $p \leq 0.05$.

Relative to untreated PBMC, IFN-γ was significantly increased in PI-treated PBMCs at 20 and 200 µg/mL and in cells treated with all concentrations of LTCs (1, 10, and 50 µg/mL) (Figure 2.3).

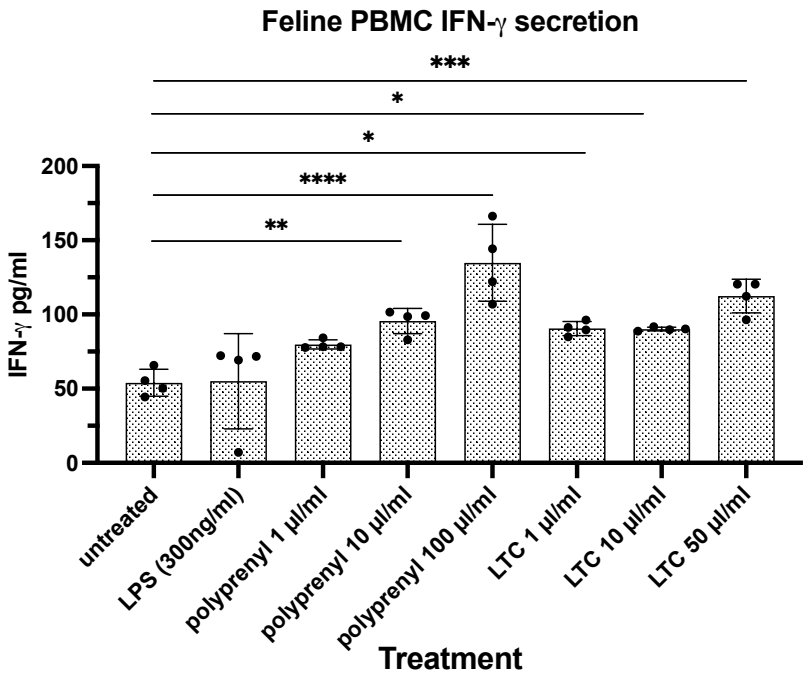


Figure 2.3. IFN-γ secretion by feline PBMCs: polyprenyl vs. LTC. Statistical differences were obtained using a one-way ANOVA with Tukey's multiple comparisons test with ****, $p \leq 0.001$; ***, $p \leq 0.005$; **, $p \leq 0.01$; and *, $p \leq 0.05$.

LTC protection from viral infection

Given that LTC was shown above to activate production and secretion of key antiviral cytokines (whereas PI did not), we next assessed the ability of LTC to stimulate the secretion of antiviral cytokines capable of suppressing FIP replication and cytopathicity. These studies used feline PBMCs as the source of antiviral cytokines, given that therapeutic administration of LTCs would involve the activation of immune cells in lymph nodes and blood. As the readout for antiviral (anti-FIP) activity, the feline tumor macrophage cell line fcwf-4, which has been used previously for FIP replication and treatment studies was utilized (27). There was significant protection from FIPV-induced

cytopathic effects when fcwf-4 cells were treated with conditioned medium from LTC-activated leukocytes (Figure 2.4).

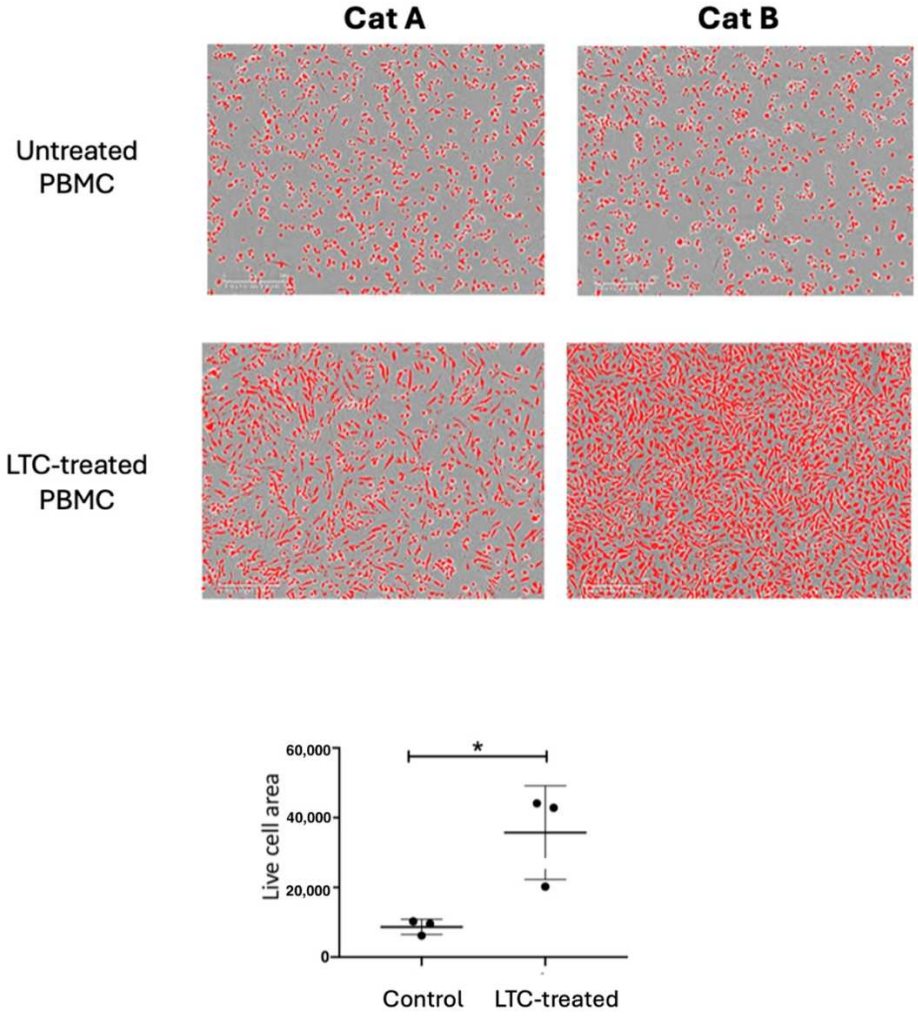


Figure 2.4. LTC activation of feline leukocytes prevents cytopathic effects imposed by FIPV. Untreated
cells (A), or cells treated with LTC overnight (B) were inoculated with virulent FIPVs to assess cytopathic effects and cell survival (live cells appear as red dots in these images). Cell survival data are summarized in C, illustrating significant protection from FIPV-induced killing by factors from LTC-activated leukocytes. Significance defined as * $p \leq 0.05$ using non-parametric Mann–Whitney t-test.

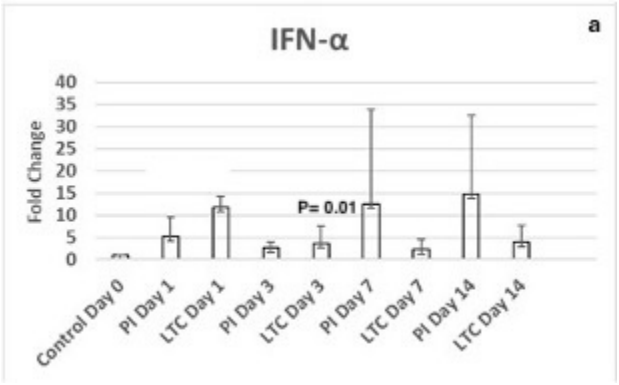
Evaluation of systemic immune responses to treatment with PI or LTCs in healthy cats

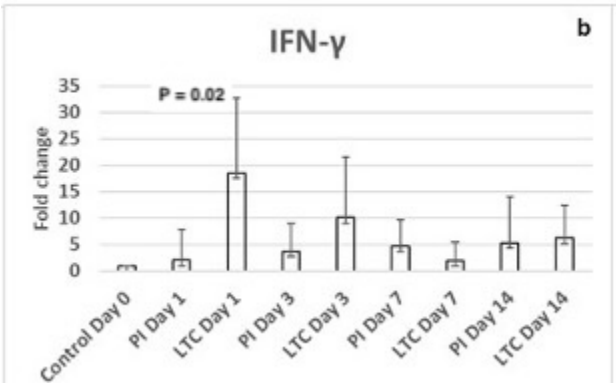
Based on the preceding studies, which demonstrated the ability of LTC to potently activate innate immune responses in cat PBMC, we next determined whether the parenteral administration of LTCs (subcutaneous (SC) injection) would elicit detectable systemic immune responses, using serum cytokine analysis and cytokine RT-PCR for PBMC isolated directly from blood of treated cats. Healthy, purpose-bred cats were treated with PI by oral dosage and/or treated by SC injection with LTC and serum cytokine responses were monitored.

Study animals were also monitored for adverse effects due to PI or LTC administration. Cats treated orally with PI did not exhibit any adverse clinical signs. One cat in the LTC group experienced pain for 24 h at the local site after one injection. No other side effects were noticed in any of the cats in the LTC group or PI group. No changes in CBC, serum biochemistry panel, or urinalysis were noted in the cats in the PI group at the end of the study when compared to prior to the study. Two cats in the LTC group had mild increases (one cat 1.1x and one cat 3.3x the upper reference interval) in alanine aminotransferase and aspartate aminotransferase activities. These values normalized in one cat but remained 2.7× the upper reference interval in the other cat two weeks after the last dose of LTC. For the in vivo evaluation of LTC and PI immune responses, we first assessed concentrations of circulating cytokines, using a feline cytokine multiplex panel of 19 cytokines. For all cats, regardless of day or group, sFas, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-13, IL-18, KC, MCP-1, SCF, and TNF-α were below the limit of detection. The assay was able to detect the measurable concentrations of the following cytokines in either pre- or post-treatment blood samples from animals in this study (Flt-3

ligand, SDF-1, IL-12 (p40), and PDFG-BB). Of the cytokines that could be measured, we observed only significant changes in concentrations of the chemokine RANTES ($p < 0.02$) in cats treated with LTCs and not in cats treated with PI.

Finally, the impact of LTC and PI treatment on the immune response by circulating leukocytes was also evaluated by using targeted RT-PCR for key antiviral cytokines and PBMCs from treated animals, which were measured directly (i.e., without culture). Increases in cytokine gene expression above baseline values (fold change; $2^{-\Delta\Delta CT}$) were noted at all time points for IFN-α, IFN-γ, and IL-1β (but not for IL-6 or TNF-α), with several statistical differences noted between the groups. When compared to PI treatment, there was significantly higher mRNA expression for both IFN-a and IFN-γ at Day 1 post-treatment and for IL-1b at Day 3 in LTC-treated cats (Figure 2.5).





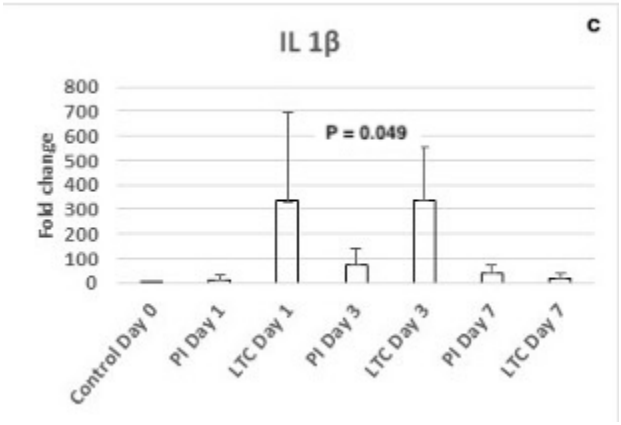


Figure 2.5. Fold change increases in IFN-α (a), IFN-γ (b), and IL-1β (c) mRNA at Days 1, 3, 7, and 14 in cats administered the LTC or PI protocols. When results were significantly different between groups on individual days, the p value is posted above the values.

2.5 Discussion

The primary objective of this study was to compare the immunological and antiviral activities of two innate immune stimulants (PI and LTC) using in vitro studies with cat PBMCs and cat macrophages as well as in healthy cats being treated with PI and LTCs. In the in vitro study, the LTC doses studied induced significant production of IFN-α, IFN-β, and IFN-γ by stimulated feline leukocytes, whereas PI failed to induce these interferons at any dose. These findings are consistent with prior reports from our group, wherein LTC was found to activate local immune responses and the in vitro activation of PBMCs in cats (23).

These findings suggest that activating the TLR 3 and 9 pathways is an effective approach for generating broad interferon production in cats. Moreover, LTC seems to be a stronger immune stimulant for IL-6 release than PI at high concentrations, and a high

concentration of this formulation of LTC does not seem to be as toxic in terms of IL-6 production. It is also possible that the cells released IL-6 before dying. Interleukin-6 plays a central role in host defense due to its wide range of immune and hematopoietic activities and its potent ability to induce the acute phase response, which is an important response in cats with FIP. IL-6 is also known to have antiviral effects (31). There was a significant increase in IFN-γ secretion in PBMC treated with 10.0 µL/mL LTC; however, higher doses of LTC seem to be toxic to cat PBMCs in vitro and lessen IFN-γ secretion.

In addition, treatment of PBMCs with LTCs stimulated the production of cytokines that significantly suppressed the cytopathic effects of FIP in feline macrophage cultures. Thus, these studies revealed the potent innate immune-activating and antiviral activities of LTCs, compared to PI, for therapeutic immune stimulation in cats. This in vitro experiment also showed that there was significant protection from FIPV-induced cytopathic effects when fcwf-4 cells were treated with conditioned medium from LTC-activated leukocytes, suggesting that the cytokines triggered by LTC activation are able to suppress FIPV in permissive macrophages, a key target cell for FIP pathogenesis. Thus, treatment of PBMCs with LTCs stimulated the production of cytokines that significantly suppressed the cytopathic effects of FIP in feline macrophage cultures. Together, these studies revealed the potent innate immune-activating and antiviral activities of LTCs, compared to PI, for therapeutic immune stimulation in cats.

When healthy cats were treated with LTCs and PI, neither protocol induced significant side effects, and the increases in liver enzyme activities induced by the LTC protocol were self-limited. The biological activity of PI and LTC were also assessed in vivo in healthy cats. The primary outcome measure for these studies was cytokine secretion,

as assessed by feline multiplex assay (19 cytokines). When serum samples obtained after PI or LTC administrations were assessed, we found that LTC administration induced the significant upregulation of RANTES secretion. RANTES attracts immune cells from the peripheral blood to sites of inflammation and plays an important role in homing and migration of effector and memory T cells during acute infections. RANTES has been associated with decreased susceptibility to the human immunodeficiency virus (32). Hence, RANTES may increase effective cell-mediated immunity that is often reduced in FIP-prone cats. Other cytokines were either not detected (sFas, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-13, IL-18, KC, MCP-1, SCF, TNF-α) or were not increased by PI or LTC administration.

The failure to detect greater in vivo immune activation using the cytokine assay as a readout may reflect in part the relative insensitivity of circulating cytokine measurement for systemic immune activation. In addition, while the dose of PI administered was the dose recommended by the company marketing PI, the dose of LTC administered was not optimized for SC administration. Our prior studies have found, for example, that the route of LTC administration greatly affects the degree of immune activation that is achieved at the actual dose administered (29). It is also possible that PI administration may induce local immune activation in the gut, while LTC may induce local immune activation in SC tissues and draining lymph nodes, and that neither of these routes would be sufficient to induce changes in concentrations of circulating cytokines.

Both the PI and LTC protocols in healthy cats induced fold increases over baseline for IFN-α, IFN-γ, and IL-1β but not IL-6 or TNF-α. IL-1β had the greatest fold increases and is known to amplify IFN-α-induced antiviral responses (33). Significant differences

between the protocols existed with responses being greater for the LTC protocol on either Day 1 (IFN-α, IFN-γ) or Day 3 (IL-1β) of the study. Whether there is biological significance of the detection of greater or earlier responses for these three cytokines is unknown, and the findings could merely reflect the route of administration with the SQ route inducing changes more quickly.

It has been suggested that antiviral drugs can act synergistically with immunomodulatory treatments to improve patient outcome and survival in different viral diseases, such as influenza virus, hepatitis C virus, and human immunodeficiency virus (HIV) (17-19). Overall, the results of the in vivo and in vitro experiments reported herein support the LTC protocol described to have antiviral properties and should be considered for use in combination with antiviral drugs. This synergism could improve long-term treatment outcomes for cats with FIP as well. In addition, the induction of antiviral innate immune responses could be very beneficial for the management of a variety of feline infectious diseases, not only FIP, but also for FHV-1, feline caliciviruses, and Mycobacteria spp. that still lack effective treatments or have treatments that are expensive, difficult to administer, or are toxic. Considering the results of our in vitro study, it is likely that LTCs can be effective in the treatment of FIP and other infectious diseases, and additional studies are warranted to assess the immune-stimulatory properties of LTCs and to rigorously evaluate their general antiviral properties in vivo, as well as their potential effectiveness as a treatment for FIP.

In contrast to the potential benefits of immune stimulation, it is also possible to potentiate disease. Several cytokines have previously been reported to play a role in FIP. An older study reported that the mRNA levels of IL-2, IL-4, IL-10, IL-12, and IFN-γ were

markedly reduced during the development of FIP in kittens (9). A recent study measured the mRNA levels of IL-1β, IL-6, and TNF-α in liver and heart of cats with FIP and showed that both hepatocytes and cardiomyocytes are sources of inflammatory cytokines in FIP, with hepatic IL-12:IL-10 balance skewed towards IL-12 in cats with FIP (13). In another study, IL-1β, IL-6, IL-12, IL-18, TNF-alpha, macrophage-inhibitory protein (MIP)-1 alpha, and RANTES showed moderate upregulation in cats with neurological FIP and very high upregulation in cats with non-neurological FIP (10). In the same study, the transcription of IFN-γ appeared upregulated in cats with systemic FIP and slightly downregulated in neurological FIP; however, most cytokines in this study had very high variance in non-neurological FIP, but less variability was noted in neurological FIP (10).

Various proinflammatory cytokines and interferon-related genes such as MX1, viperin, CXCL10, CCL8, RANTES, KC, MCP1, IL8, GM-CSF, and IFN-γ were found in FCoV-positive cats (12). Cats with FIP have also been reported to have higher IL-6 expression, and IL-6 is likely involved in the development of immune-complex-mediated vasculitis and in FIP pathogenesis (11). RANTES expression was previously reported to be high in cats with FIP compared to healthy cats (10), suggesting it as a possible target for immunotherapy of cats with FIP. However, dysregulated excessive and persistent synthesis of IL-6 has a pathological effect on acute systemic inflammatory response syndrome and chronic immune-mediated diseases, and therefore, immunotherapy, which might be increasing RANTES and IL-6, might not be as beneficial long term for these cats. There is some clinical information available for the two immune stimulants described here that suggest that a clinical benefit for their use with viral infections is more likely than

the potentiation of disease with FIP and FHV-1 (21, 22, 24, 25, 34), and additional clinical studies in cats with acute or chronic infectious diseases are warranted.

Other than the increased expression of RANTES in the LTC group, neither protocol used in the in vivo experiment induced many changes in the production of the cytokines and chemokines measured in the commercial kit used with healthy cat serum. This commercial kit has shown low values for many analytes in other studies using serum or plasma of cats (35, 36). In one of these studies, over half of the samples were below the lower limit of quantification for both serum and plasma for nine analytes (FAS, GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, CXCL-1, PDGF-BB, and TNF-α), and additional analytes were below the level of detection in plasma samples only (35). These results suggest that this kit may not be optimal for attempting to determine changes in cats with minimal immune simulation or inflammation. However, there could have been other reasons for the undetectable levels of these cytokines such as pre-analytical issues. Other assays, like the PCR assays described here, or bulk RNA sequencing may be more sensitive for use in healthy cat studies(37). There were several limitations to the study. First, the numbers of study animals were small and reflect the pilot nature of these investigations. Timing could have played a role in the measurements as the cytokines were only measured at one time point, and measurements would ideally be taken at various post treatment times to find out the optimal time of secretion before cytotoxicity predominates, which was not performed in this study as these were single time points. Another limitation is that these are in vitro and healthy cat studies that may not fully reflect the effectiveness in cats with FIP, and an in vivo study using immune stimulants such as LTCs and PI should be

performed in cats with FIP as an adjunct treatment to antiviral therapy to further evaluate the effectiveness of these immune stimulants in the treatment of FIP.

2.6 Conclusions

Both protocols studied herein showed evidence for immune modulation that could be of potential benefit to the management of viral infections. While differences were noted between the LTC (TRL3/TRL9) and PI protocols (TLR2/TLR6), care should be taken in concluding that one is better than the other since the routes of administration were different, and there has not been an optimized dose determined for either agent. The studies reported here indicate that the LTC is a potent innate immune agonist in cats, capable of eliciting a high expression of key antiviral genes, including IFN-a, IFN-b, IFN-g, and IL-1b in feline PBMC. In addition, LTCs generated significant antiviral activity against FIP replication in feline macrophages. Therefore, it can be concluded that the LTC was as effective as an innate immune agonist for activating feline antiviral responses both in vitro and in vivo and should therefore be considered a promising new addition to treatment options for FIP and other chronic viral pathogens of cats, and additional studies are warranted to assess the immune-stimulatory properties of LTCs and to rigorously evaluate their antiviral properties in vivo, as well as their potential effectiveness as treatments for FIP.

References

1. Holzworth J. Some important disorders of cats. 1963.

2. Pedersen NC. A review of feline infectious peritonitis virus infection: 1963–2008. Journal of feline medicine and surgery. 2009;11(4):225-58.

3. Weiss R, Scott F. Pathogenesis of feline infectious peritonitis: nature and development of viremia. 1981.

4. Pedersen NC. Virologic and immunologic aspects of feline infectious peritonitis virus infection. Coronaviruses. 1987:529-50.

5. Pedersen N. An overview of feline enteric coronavirus and infectious peritonitis virus infections. 1995.

6. Pedersen NC. An update on feline infectious peritonitis: virology and immunopathogenesis. The Veterinary Journal. 2014;201(2):123-32.

7. de Groot-Mijnes JD, van Dun JM, van der Most RG, de Groot RJ. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. Journal of virology. 2005;79(2):1036-44.

8. Kipar A, Meli M. Feline infectious peritonitis: still an enigma? Veterinary pathology. 2014;51(2):505-26.

9. Gunn-Moore D, Caney S, Gruffydd-Jones T, Helps C, Harbour D. Antibody and cytokine responses in kittens during the development of feline infectious peritonitis (FIP). Veterinary immunology and immunopathology. 1998;65(2-4):221-42.

10. Foley J, Rand C, Leutenegger C. Inflammation and changes in cytokine levels in neurological feline infectious peritonitis. Journal of feline medicine and surgery. 2003;5(6):313-22.

11. Takano T, Azuma N, Hashida Y, Satoh R, Hohdatsu T. B-cell activation in cats with feline infectious peritonitis (FIP) by FIP-virus-induced B-cell differentiation/survival factors. Archives of virology. 2009;154:27-35.

12. Safi N, Haghani A, Ng SW, Selvarajah GT, Mustaffa-Kamal F, Omar AR. Expression profiles of immune mediators in feline Coronavirus-infected cells and clinical samples of feline Coronavirus-positive cats. BMC Veterinary Research. 2017;13:1-13.

13. Malbon AJ, Fonfara S, Meli ML, Hahn S, Egberink H, Kipar A. Feline infectious peritonitis as a systemic inflammatory disease: Contribution of liver and heart to the pathogenesis. Viruses. 2019;11(12):1144.

14. Murphy B, Perron M, Murakami E, Bauer K, Park Y, Eckstrand C, et al. The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. Veterinary microbiology. 2018;219:226-33.

15. Pedersen NC, Kim Y, Liu H, Galasiti Kankanamalage AC, Eckstrand C, Groutas WC, et al. Efficacy of a 3C-like protease inhibitor in treating various forms of acquired feline infectious peritonitis. Journal of feline medicine and surgery. 2018;20(4):378-92.

16. Pedersen NC, Perron M, Bannasch M, Montgomery E, Murakami E, Liepnieks M, et al. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. Journal of feline medicine and surgery. 2019;21(4):271-81.

17. Kahlon J, Kemp M, Yawei N, Carpenter R, Shannon W, McAnalley B. In vitro evaluation of the synergistic antiviral effects of acemannan in combination with azidothymidine and acyclovir. Molecular biotherapy. 1991;3(4):214-23.

18. Lanford RE, Guerra B, Lee H, Averett DR, Pfeiffer B, Chavez D, et al. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly (i)-poly (c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. Journal of virology. 2003;77(2):1092-104.

19. Zheng B-J, Chan K-W, Lin Y-P, Zhao G-Y, Chan C, Zhang H-J, et al. Delayed antiviral plus immunomodulator treatment still reduces mortality in mice infected by high inoculum of influenza A/H5N1 virus. Proceedings of the National Academy of Sciences. 2008;105(23):8091-6.

20. Legendre AM, Kuritz T, Heidel RE, Baylor VM. Polyprenyl immunostimulant in Feline rhinotracheitis: Randomized Placebo-controlled experimental and Field safety studies. Frontiers in Veterinary Science. 2017;4:24.

21. Legendre AM, Kuritz T, Galyon G, Baylor VM, Heidel RE. Polyprenyl immunostimulant treatment of cats with presumptive non-effusive feline infectious peritonitis in a field study. Frontiers in Veterinary Science. 2017;4:7.

22. Legendre AM, Bartges JW. Effect of Polyprenyl Immunostimulant on the survival times of three cats with the dry form of feline infectious peritonitis. Journal of feline medicine and surgery. 2009;11(8):624-6.

23. Wheat W, Chow L, Coy J, Contreras E, Lappin M, Dow S. Activation of upper respiratory tract mucosal innate immune responses in cats by liposomal toll-like receptor ligand complexes delivered topically. Journal of veterinary internal medicine. 2019;33(2):838-45.

24. Contreras ET, Olea-Popelka F, Wheat W, Dow S, Hawley J, Lappin MR. Evaluation of liposome toll-like receptor ligand complexes for non-specific mucosal

immunoprotection from feline herpesvirus-1 infection. Journal of veterinary internal medicine. 2019;33(2):831-7.

25. Lappin M, Wotman K, Chow L, Williams M, Hawley J, Dow S. Nanoparticle ocular immunotherapy for herpesvirus surface eye infections evaluated in cat infection model. Plos one. 2023;18(1):e0279462.

26. Veir JK, Lappin MR, Dow SW. Evaluation of a novel immunotherapy for treatment of chronic rhinitis in cats. Journal of feline medicine and surgery. 2006;8(6):400-11.

27. Bálint Á, Farsang A, Zádori Z, Hornyák Á, Dencső L, Almazán F, et al. Molecular characterization of feline infectious peritonitis virus strain DF-2 and studies of the role of ORF3abc in viral cell tropism. Journal of virology. 2012;86(11):6258-67.

28. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nature methods. 2012;9(7):676-82.

29. Ammons DT, Guth A, Rozental AJ, Kurihara J, Marolf AJ, Chow L, et al. Reprogramming the canine glioma microenvironment with tumor vaccination plus oral losartan and propranolol induces objective responses. Cancer research communications. 2022;2(12):1657-67.

30. Leutenegger CM, Mislin CN, Sigrist B, Ehrengruber MU, Hofmann-Lehmann R, Lutz H. Quantitative real-time PCR for the measurement of feline cytokine mRNA. Veterinary immunology and immunopathology. 1999;71(3-4):291-305.

31. Velazquez-Salinas L, Verdugo-Rodriguez A, Rodriguez LL, Borca MV. The role of interleukin 6 during viral infections. Frontiers in microbiology. 2019;10:445678.

32. Silva MJA, Marinho RL, Dos Santos PAS, Dos Santos CS, Ribeiro LR, Rodrigues YC, et al. The Association between CCL5/RANTES SNPs and Susceptibility to HIV-1 Infection: A Meta-Analysis. Viruses. 2023;15(9):1958.

33. Robichon K, Maiwald T, Schilling M, Schneider A, Willemsen J, Salopiata F, et al. Identification of interleukin1β as an amplifier of interferon alpha-induced antiviral responses. PLoS pathogens. 2020;16(10):e1008461.

34. Černá P, Ayoob A, Baylor C, Champagne E, Hazanow S, Heidel RE, et al. Retrospective survival analysis of cats with feline infectious peritonitis treated with polyprenyl immunostimulant that survived over 365 days. Pathogens. 2022;11(8):881.

35. Gruen ME, Messenger KM, Thomson AE, Griffith EH, Paradise H, Vaden S, et al. A comparison of serum and plasma cytokine values using a multiplexed assay in cats. Veterinary immunology and immunopathology. 2016;182:69-73.

36. Halpin RE, Saunders RS, Thompson BJ, Newgent ASR, Amorim J, Melillo GN, et al. Evaluation of a feline-specific multiplex, bead-based assay for detection of cytokines, chemokines, growth factors, and other immunologically active proteins in serum and plasma samples from cats. American Journal of Veterinary Research. 2016;77(5):495-504.

37. Choi Y, Nam M-W, Lee HK, Choi K-C. Use of cutting-edge RNA-sequencing technology to identify biomarkers and potential therapeutic targets in canine and feline cancers and other diseases. Journal of Veterinary Science. 2023;24(5).

Chapter 3: PHARMACOKINETICS OF MOLNUPIRAVIR IN CATS WITH NATURALLY OCCURING FELINE INFECTIOUS PERITONITIS[2]

3.1 Summary

Antiviral drugs like EIDD-2801 (molnupiravir; MPV) have been successfully used in treatment of feline infectious peritonitis (FIP). Previous study on pharmacokinetics of MPV in healthy cats showed promise for its use and safety. The objective of this study was to determine the pharmacokinetics of molnupiravir in cats with naturally occurring FIP by measuring MPV and EIDD-1931 (β-D-N4-hydroxycytidine; NHC) serum levels. Seven cats diagnosed with naturally occurring FIP were treated with MPV. Blood was collected at 1, 2, 4, 6 and 12h post oral MPV administration and at 12 h post-pill administration 7 days later. Serum concentrations of MPV and NHC were determined using a previously published high performance liquid chromatography tandem-mass spectrometry (HPLC-MS/MS) method. The mean dose of MPV was 15.44 mg/kg (SD± 1.82). The mean peak serum concentration of MPV (C_{max}) after a single PO dose of MPV was 38 ng/mL (SD± 5). The mean peak serum concentration of NHC (C_{max}) after a single PO dose of MPV was 1551ng/mL (SD± 720). Time to reach NHC C_{max} (T_{max}) was 2.6h (SD± 1.4); and NHC elimination half-life was 1.6 h (SD± 1.1). Minimal accumulation of drug was seen in trough concentrations following twice-daily dosing for 7 days. The low MPV levels may be explained by fast conversion to its active metabolite NHC. The mean NHC concentrations at all time points were at least 4 times the reported in vitro EC_{50} for

[2] This chapter includes the complete published manuscript: Černá P., Wittenburg L., Hawley J., Willis M., Siegenthaler B., Lappin M.R. Pharmacokinetics of Molnupiravir in Cats with Naturally Occurring Feline Infectious Peritonitis Pathogens 2025.

feline coronavirus strains and twice daily dosing for seven days did not lead to accumulation of drug within the serum. The results support the use of MPV in the treatment of FIP and, if therapeutic drug monitoring is to be performed, NHC should be measured.

3.2 Introduction

Feline Infectious Peritonitis (FIP) has been a challenge for veterinarians and a devastating disease among cats for over half a century, yet despite marked efforts and many theories, the pathogenesis of FIP is still not fully understood (1). Feline infectious peritonitis is a clinical syndrome that is believed to arise from mutations of feline enteric coronavirus (FECV) that results in changes to viral pathogenicity, enabling FIP viruses (FIPV) to replicate in monocytes and macrophages (1, 2).

Recent studies have reported the use of drugs like the nucleoside analog GS-441524 as a successful treatment of FIP (3-5). However, more treatment options for cats with FIP, especially those that relapse, are needed. Recently, a study showing PK/PD data from the antiviral drug EEID-2801 (FDA Emergency Use Authorization for treatment of SARS-COV-2 in humans) demonstrated promise for its use and safety in cats (6). This recent study of pharmacokinetic properties of molnupiravir (MPV; EIDD-2801) in healthy specific pathogen free cats established that orally administered MPV at 10 mg/kg achieves plasma levels greater than the established corresponding EC_{50} values. In that study, the MPV prodrug was detected in the serum of all three cats at low levels in the first 12 h post-administration while the β-D-N4-hydroxycytidine (NHC; EIDD-1931) metabolite was detected at much higher levels between 12 to 24 h post-

administration. Marked variability in detected NHC serum concentration was present between individual cats. For one cat, the peak serum concentration for MPV and NHC occurred at the 3-h time point with NHC detected at a 10-fold greater level than MPV. For another cat, the peak serum concentration occurred at the 1.5 h time point, with MPV nearly 100-fold below NHC. The peak serum concentration occurred for the 3rd cat at the 9 h time point with NHC detected nearly 100-fold over MPV. NHC was more consistently and quantifiably detected over the 24 h time period, aside for one cat, for which NHC was no longer detected (based on limit of detection) after 12 h post-administration. MPV, however, was not consistently detected at the early time points nor after the 12 h time point. This study reported no evidence of acute organ toxicity in any of the cats based on the pre- and post-treatment complete blood count (CBC) and serum biochemistry panels; however, all three of the MPV-treated cats demonstrated variable signs of nausea, including hypersalivation and/or vomiting, after oral administration of MPV (10 mg/kg).

Another study assessing 18 cats with FIP being treated with MPV showed that molnupiravir might be an effective and safe treatment for domestic cats with FIP at a dose of 10-20 mg/kg twice daily (7). In this study, increased serum alanine transaminase (ALT) activity was found in 3 of the 18 cats, all at days 7-9, and all cats recovered without any medications or need to stop the treatment (7). There is also uncontrolled data from client surveys that suggests efficacy of this product as rescue treatment following failure of the GS-441524 therapy as well as a first line treatment option for cats with FIP (8). A recent study evaluating MPV in cats with FIP showed that it is an effective antiviral treatment for effusive FIP, with 8/10 cats being in remission at 16 weeks while the 2 non-

survivors died in the first 24 hours of treatment (9). Survival of cats treated with oral MPV was non-inferior to historic control cats treated orally with GS-441524 (9).

3.3 Materials and methods

Single dose pharmacokinetics of orally administered MPV

Seven cats with naturally occurring FIP were enrolled in this *in vivo* PK study to determine the feline-specific pharmacokinetics and metabolism of orally administered MPV. This study was approved by the Institutional Animal Care and Use Committee (IACUC #5961). Powdered MPV (NM Pharmtech, City, State, USA) was utilized for the PK study. Orally administered MPV was formulated as excipient-less powder in size 3 gelatin capsules (Monument Pharmacy, Monument, CO, USA).

Physical examination was performed on each cat at study initiation (timepoint zero) and an IV catheter was placed into a cephalic vein, secured in place, and flushed with heparinized saline. Blood was collected from the catheter prior to MPV administration (time point 0) to establish a baseline complete blood count and serum biochemistry analysis panel for the MPV study. These panels were repeated 7 days post-MPV administration to assess for toxicity. After baseline sampling, the cats were administered MPV and then approximately 0.5–1 mL of whole blood was collected from IV catheter at each of the following time points: 1, 2, 4, 6, and 12 h post-administration. At each collection time point, the blood sample was incubated for 5 min at room temperature to allow for clotting and then centrifuged at 12,000 × g for 10 min to separate the serum fraction from the blood cells. One hundred microliters of serum were aliquoted into a 1.5 mL microcentrifuge tube containing 300 µL of acetonitrile to halt compound metabolism

by serum esterases and immediately placed into−80 °C freezer prior to shipping overnight on dry ice for high performance liquid chromatography tandem-mass spectrometry (HPLC/MS-MS) analysis. In addition, oral MPV administration was continued twice daily for seven days and serum trough concentrations of NHC were determined on day 7, 12h after the previous dose of MPV.

Liquid chromatography tandem-mass spectrometry (LCMSMS) quantitation of serum MPV concentrations and pharmacokinetic analysis

Analysis of serum MPV and its main metabolite, NHC (EIDD-1931), was performed by modification of a previously validated and published method for human samples (10). Briefly, stock solution of MPV (EIDD-2801; NM Pharmtech, 99.0% purity) and NHC (EIDD-1931; MedChemExpress, Monmouth Junction, NJ, USA, 99.73% purity) were prepared at 2 mg/mL in methanol and stored at −80 °C. Stock solutions were further diluted in methanol to produce calibration standards for a low concentration curve at 1, 5, 10, 50, and 100 ng/mL of MPV and NHC combined and a high concentration curve at 250, 500, 750, 1000, 2500 and 5000 ng/mL of NHC alone. Calibration curves and quality control samples were generated as described previously (6).

Mass spectrometry and liquid chromatography conditions

Negative ion electrospray ionization mass spectra were obtained on a Sciex 6500+ Q-TRAP triple quadrupole mass spectrometer (AB Sciex LLC, Framingham, MA, USA) with a turbo ionspray source coupled to the Sciex ExcionLC™ UHPLC system with a cooled (15 °C) autosampler. Chromatographic separation was carried out on a

Luna® Phenyl-hexyl 3.0 µm column (50 × 2.0 mm) with a filter frit guard column (both from Phenomenex, Inc., Torrance, CA, USA). A gradient mobile phase was employed consisting of 1 mM ammonium acetate pH 4.3 (mobile phase A) and acetonitrile containing 1 mM ammonium acetate (mobile phase B). Separation was carried out by holding mobile phase B constant at 2% for 1.2 min, increasing linearly to 90% at 2.2 min, holding at 90% until 3.5 min, decreasing linearly back to 2% from at 3.75 min and equilibrating at 2% until 5.0 min. Analytes were identified by monitoring the ion transitions for MPV (m/z 328.1 → 126.0 and 168.1) and for NHC (m/z 257.9 → 125.9 and 107.9). Quantitation was performed by linear regression of analyte peak areas in unknown samples with calibrator samples using $1/x^2$ weighting.

Pharmacokinetic Analysis

Pharmacokinetic parameters for NHC were examined following oral dosing of MPV and were estimated by noncompartmental analysis with the linear trapezoidal-linear interpolation calculation method and uniform weighting using the commercially available software program Phoenix WinNonlin v8.3 (Certara Inc., Princeton, NJ, USA). Estimation of half-life ($T_{1/2}$) was made using a minimum of three time points in the elimination phase. For serum MPV, only maximum serum concentration (Cmax), time to reach maximum serum concentration (Tmax) and area under the serum MPV concentration-time curve from time 0 to 12 hours (AUC_{0-T}) were estimated. Graphs were generated using GraphPad Prism v9.1.2.

3.4 Results

Assay Performance

The calibration curve was linear between 5 and 2,000 ng/mL ($r > 0.997$) with a lower limit of quantitation of 5 ng/mL NHC and 2.5 ng/mL for MPV (signal to noise ratio >10). Accuracy of calibrators was within 15% at all concentrations and accuracy and precision of quality control samples was also within 15% (n=3 per concentration).

Safety and tolerability of oral molnupiravir

The mean oral dose of MPV administered was 15.44 mg/kg (SD± 1.82). No evidence of severe side effects or MPV-associated organ toxicity were noted in any of the cats; however, based on the pre- and post-treatment complete blood count (CBC) and serum biochemistry panels, one cat developed mild increase in ALT activity (142 IU/L; reference interval (RI) 30 – 140). Moreover, one MPV-treated cat showed signs of nausea (hypersalivation) after oral administration of MPV.

Pharmacokinetics of oral MPV and NHC in cats with FIP

The full patient information including the age, sex, breed and FIP form can be seen in Table 3.1. Six out of the seven cats finished 12 weeks of therapy with MPV and are in remission now. One patient died 11 days post diagnosis of FIP, presumably related to FIP but post-mortem examination was not performed. The MPV prodrug was detected in the serum of all seven cats at low concentrations with 5 of 7 cats having serum concentrations above the limit of quantitation at 12 h post-administration (Figure 3.1). There was notable interpatient variability in MPV concentrations at each time point, demonstrated in Figure

3.2. Select PK parameters for MPV are depicted in Table 3.2. Following oral administration of MPV, serum C_{max} was extremely variable with a range of 4 to 236 ng/mL (geometric mean of 37 ± 5 ng/mL). Time to reach maximum serum concentration ranged from 1 hour (4 of 7 cats) to 4 hours (1 cat) with a mean of 1.7 hours. Total MPV exposure from time zero to 12 hours (AUC_{0-T}) ranged from 32 to 685 hr*ng/mL with a geometric mean of 131 ± 3 ng/mL.

Table 3.1. Patient information on age, sex, breed, FIP form and the outcome for individual cats.

Number	Age (years)	Sex	Breed	FIP form	Outcome
107	0.34	MN	DSH	effusive	dead (day 10)
108	3.01	MN	DSH	effusive	in remission
109	1.06	MN	DSH	effusive	in remission
110	1.45	FS	Maine Coon	ocular	in remission
113	0.43	MN	DSH	non-effusive	in remission
114	1.08	MN	DSH	effusive	in remission
116	2.00	FS	Bengal	effusive	in remission

Legend: MN – male neutered, FS – female spayed, DSH – Domestic Shorthair

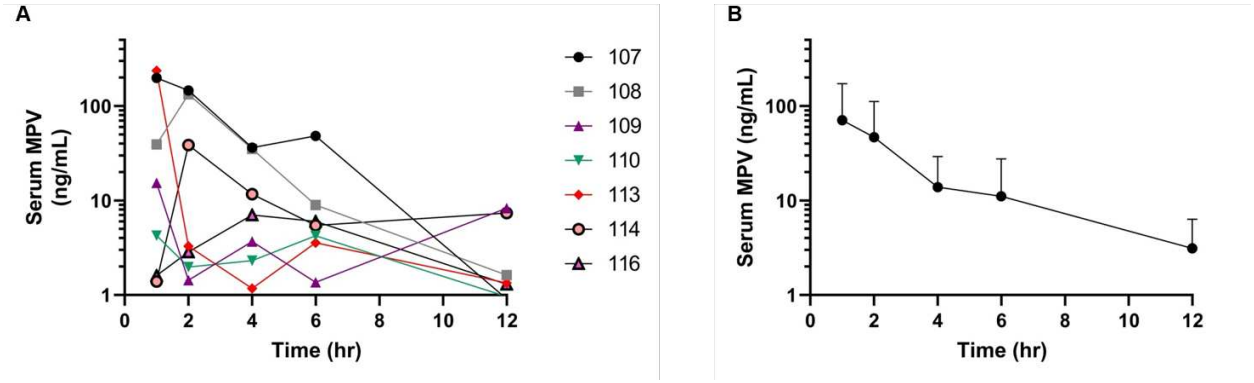


Figure 3.1. Serum concentration-time curves of the prodrug MPV over a 12-h time period in cats. (A) Serum MPV concentrations (ng/mL) of each individual cat over 12 h. (B) Combined serum MPV concentrations in all cats at 1, 2, 4, 6 and 12 hours post MPV administration (black dots are arithmetic mean values, error bars represent standard deviation).

The arithmetic mean serum concentration-time profiles of the active metabolite, NHC, are presented in Figure 3.2. NHC was detectable at the first measured time point (1 hour) and was detectable out to 12 hours in all cats. Estimated PK parameters for NHC are presented in Table 2. C_{max} for NHC ranged from 617 to 2,530 ng/mL (mean of 1,551 ± 720 ng/mL), which is approximately 6 µM. Time to reach maximum serum NHC concentrations was longer than that of MPV, with a mean ± SD T_{max} of 2.6 ± 1.4 hours. Total exposure to NCH was also quite variable and ranged from 4,222 to 12,323 hr*ng/mL (mean ± SD 5919 ± 2873 hr*ng/mL). The harmonic mean serum half-life ($T_{1/2}$) for NHC was 1.6 ± 1.1 hours. Average NHC concentrations at all time points were at least 4 times the reported *in vitro* EC_{50} for feline coronavirus strains: 0.05 µM for Black I (serotype I FIPV) and 0.11 µM for WSU-79-1146 (serotype II FIPV) (6).

Table 3.2. Pharmacokinetic estimates for MPV and NHC following a single oral dose of molnupiravir in seven cats with FIP.

	MPV		NHC	
Parameter	mean ± SD	Median (range)	mean ± SD	Median (range)
C_{max} (ng/mL)	37 ± 5†	39 (232)	1551 ± 720	1630 (1913)
T_{max} (hr)	1.7 ± 1.1	1 (3)	2.6 ± 1.4	2 (3)
AUC_{0-t} (hr*ng/mL)	222 ± 237	127 (653)	5919 ± 2874	4749 (8100)
AUC_{0-Inf} (hr*ng/mL)			6296 ± 3573	4957 (10040)
K_{el} (1/hr)			0.344 ± 0.152‡	0.35 (0.76)
$T_{1/2}$ (hr)			1.6 ± 1.1‡	1.9 (2.7)
Vz/F (L)			24.4 ± 11.3	23.5 (30.6)
Cl/F (L/hr)			9.2 ± 3.9	10.4 (9.8)

Legend: C_{max}: maximum serum concentration; AUC_{0-t}: area under the serum concentration-time curve from time 0 to 12 hours; AUC_{0-Inf}: area under the serum concentration-time curve from time 0 extrapolated to infinity; K_{el}: elimination rate constant; $T_{1/2}$: serum half-life; Vz/F: apparent volume of distribution; Cl/F: apparent clearance. †Geometric mean with geometric standard deviation; ‡Harmonic mean with pseudo-standard deviation.

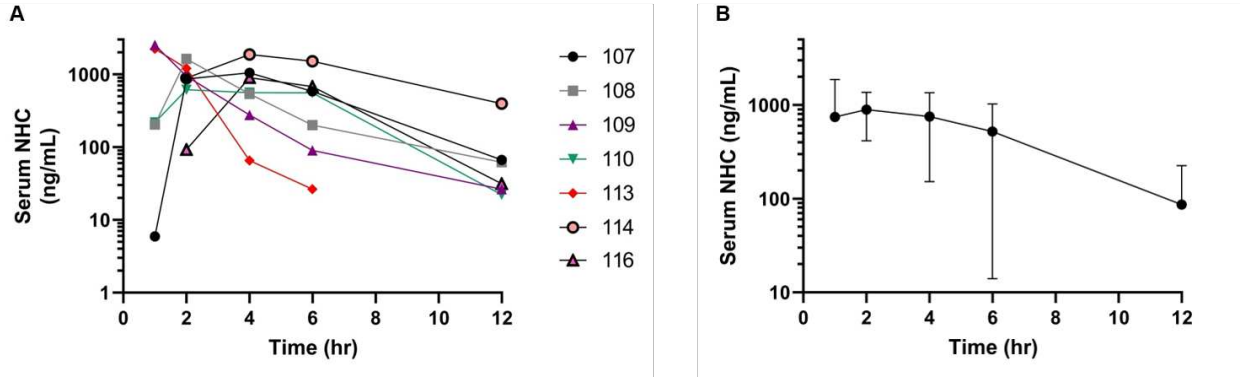


Figure 3.2. Serum concentration-time curves of the active metabolite NHC over a 12-h time period in cats. (**A**) Serum NHC concentrations (ng/mL) of each individual cat over 12 h. (**B**) Combined serum NHC concentrations in all cats at 1, 2, 4, 6 and 12 hours post MPV administration (black dots are arithmetic mean values, error bars represent standard deviation).

Six of the cats had serum NHC concentrations measured again on day 7 at 12 h post dose. In 5/6 cats, the trough serum NHC concentrations were very similar between day 1 and 7; however, in one cat the day 7 concentration was much higher than it was following the day 1 dose (66.8 ng/mL on day 1 vs 223ng/mL) (Figure 3.3). The accumulation ratio for trough serum NHC concentrations ranged from 0.8 to 3.3 (mean 1.5 ± 0.9).

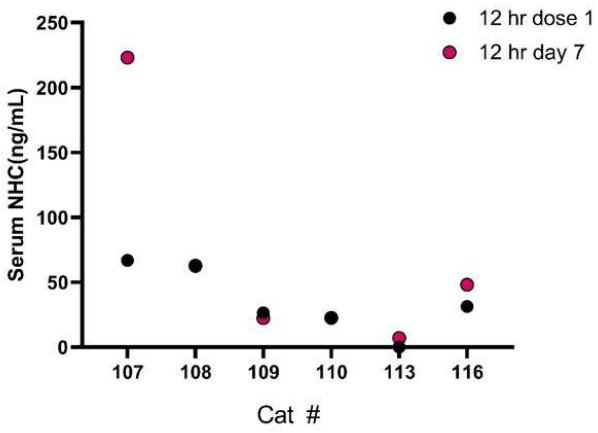


Figure 3.3. Comparison of serum detection of the metabolite NHC at 12-h time point in six cats after one dose and at day 7. Note that cats 108 and 110 have identical 12h sampling at day 1 and 7.

3.5 Discussion

Molnupiravir is an oral prodrug of the broadly active, antiviral ribonucleoside analog *N*-hydroxycytidine (NHC). The primary circulating metabolite NHC is taken up into cells and phosphorylated to NHC-triphosphate (NHC-TP) (11). NHC-TP serves as a competitive substrate for viral RNA-dependent RNA polymerase (RdRp), which results in an accumulation of errors in the viral genome, rendering virus replication incompetent. Molnupiravir has demonstrated activity against SARS-CoV-2 and FCoV. Little to no MPV is observed in plasma due to rapid hydrolysis to NHC, which was found in this study as

well. MPV is rapidly metabolized in cat serum to the active metabolite, NHC (12). In our study, the NHC metabolite was detected at early timepoints and at markedly higher concentrations than the administered prodrug, MPV.

The mean NHC concentrations at all time points were at least 4 times the reported in vitro eC_{50} for feline coronavirus strains; this study also found interindividual variability in MPV metabolism (Figure 3.1 and 3.2). This is similar to the previous study in healthy cats and a definitive cause for this metabolic variability is not clear; however, it is possible that the metabolism could have been affected by the systemic FIP disease (6). It has been suggested that variation in genetic polymorphisms related to metabolic pathways, such as the cytochrome P450 system, might also play a role in this variability (13). Moreover, a minor variability in the timing between centrifugation of individual whole blood samples between cats and the timing of the transfer of feline serum into acetonitrile could also contribute to the variability in measured concentrations of MPV and/or NHC.

An estimated NHC half-life of approximately 3 h was previously reported while our current study estimated an elimination half-life of 1.6 h (6). Moreover, twice daily dosing for seven days did not lead to substantial accumulation of drug within the serum in majority of the cats. The one cat that had higher NHC levels at day 7 was a cat with severely increased total bilirubin levels (1.5 mg/dL; RI (0 - 0.1), which could have led to reduced excretion and thus accumulation of the drug in serum. A similar lack of NHC accumulation following twice-daily dosing of MPV in humans has been reported; however, this same study did detect a greater degree of accumulation of intracellular NHC-TP in PBMCs, which was not measured in this study (14). No evidence of acute toxicity in the MPV-treated cats based on complete blood count and blood chemistry was found apart

from one cat having mild increase in ALT activity and one cat having mild nausea post administration. The associations between administration of MPV and nausea in cats and increased liver enzyme activities has been reported in other studies (6, 7). In addition, the cats were also fasted prior to receiving MPV, which might play a role in the development of nausea. In humans, there was a food effect on the rate of absorption observed after MPV administration; however, the therapeutic exposures during fasted and fed states were comparable (12).

There are several published clinical trials focused on the treatment of cats with naturally occurring FIP (9, 15); however, most focused on treatment of cats without therapeutic drug monitoring or pharmacokinetic studies. There are also no studies looking at the penetration of antiviral compounds into feline tissues. Additional in vivo pharmacokinetic studies, assessment of compound penetration into tissues, and rigorously designed and prospective clinical trials are needed for all drugs used, including MPV. In addition, determination of optimal combination antiviral therapies and consideration of a compound's ability to penetrate cerebrospinal fluid and aqueous humor needs to be determined in order to optimize therapeutic approaches to cats with ocular or neurological FIP and particularly those that experience therapeutic relapse. Measurements of drug levels in serum but also assessments of absorption in tissues, cerebrospinal fluid and aqueous humor are important to establish best dosing protocols of molnupiravir in cats and further optimize and individualize the treatment cats with FIP and provide better therapeutic drug monitoring recommendations for these cats. Further analysis of therapeutic drug monitoring for response to treatment is needed because a recent study showed that assessment of the predictive relationship between median GS-

441524 plasma trough concentration and achieving simple remission failed to demonstrate a significant correlation (16).

3.6 Conclusions

Pharmacokinetic analysis of molnupiravir in cats with naturally occurring FIP was established and showed early metabolization to NHC and interindividual variability in MPV metabolism. The mean NHC concentrations at all time points were at least 4 times the reported in vitro EC50 for feline coronavirus strains and twice daily dosing for seven days did not lead to accumulation of drug within the serum.

References:

1. Pedersen NC. A review of feline infectious peritonitis virus infection: 1963–2008. Journal of feline medicine and surgery. 2009;11(4):225-58.

2. Rottier PJ, Nakamura K, Schellen P, Volders H, Haijema BJ. Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. Journal of virology. 2005;79(22):14122-30.

3. Murphy B, Perron M, Murakami E, Bauer K, Park Y, Eckstrand C, et al. The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. Veterinary microbiology. 2018;219:226-33.

4. Pedersen NC, Perron M, Bannasch M, Montgomery E, Murakami E, Liepnieks M, et al. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. Journal of feline medicine and surgery. 2019;21(4):271-81.

5. Taylor SS, Coggins S, Barker EN, Gunn-Moore D, Jeevaratnam K, Norris JM, et al. Retrospective study and outcome of 307 cats with feline infectious peritonitis treated with legally sourced veterinary compounded preparations of remdesivir and GS-441524 (2020–2022). Journal of feline medicine and surgery. 2023;25(9):1098612X231194460.

6. Cook S, Wittenburg L, Yan VC, Theil JH, Castillo D, Reagan KL, et al. An optimized bioassay for screening combined anticoronaviral compounds for efficacy against feline infectious peritonitis virus with pharmacokinetic analyses of GS-441524, remdesivir, and molnupiravir in cats. Viruses. 2022;14(11):2429.

7. Sase O. Molnupiravir treatment of 18 cats with feline infectious peritonitis: A case series. Journal of Veterinary Internal Medicine. 2023;37(5):1876-80.

8. Roy M, Jacque N, Novicoff W, Li E, Negash R, Evans SJ. Unlicensed molnupiravir is an effective rescue treatment following failure of unlicensed GS-441524-like therapy for cats with suspected feline infectious peritonitis. Pathogens. 2022;11(10):1209.

9. Reagan KL, Brostoff T, Pires J, Rose A, Castillo D, Murphy BG. Open label clinical trial of orally administered molnupiravir as a first-line treatment for naturally occurring effusive feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2024;38(6):3087-94.

10. Amara A, Penchala SD, Else L, Hale C, FitzGerald R, Walker L, et al. The development and validation of a novel LC-MS/MS method for the simultaneous quantification of Molnupiravir and its metabolite ß-d-N4-hydroxycytidine in human plasma and saliva. Journal of pharmaceutical and biomedical analysis. 2021;206:114356.

11. Maas BM, Strizki J, Miller RR, Kumar S, Brown M, Johnson MG, et al. Molnupiravir: Mechanism of action, clinical, and translational science. Clinical and Translational Science. 2024;17(2):e13732.

12. Painter WP, Holman W, Bush JA, Almazedi F, Malik H, Eraut NC, et al. Human safety, tolerability, and pharmacokinetics of molnupiravir, a novel broad-spectrum oral antiviral agent with activity against SARS-CoV-2. Antimicrobial agents and chemotherapy. 2021;65(5):10.1128/aac. 02428-20.

13. Kraemer SD, Testa B. The Biochemistry of Drug Metabolism–An Introduction: Part 7. Intra-Individual Factors Affecting Drug Metabolism. Chemistry & Biodiversity. 2009;6(10):1477-660.

14. Iwamoto M, Duncan KE, Wickremasingha PK, Zhao T, Liberti MV, Lemoine L, et al. Assessment of pharmacokinetics, safety, and tolerability following twice-daily administration of molnupiravir for 10 days in healthy participants. Clinical and Translational Science. 2023;16(10):1947-56.

15. Clark T, Coggins S, Korman R, King J, Malik R. Treatment of feline infectious peritonitis in cats with molnupiravir: clinical observations and outcomes for 54 cases. Australian Veterinary Journal. 2025.

16. Coggins S, Govendir M, Norris J, Malik R, Hall E, Thompson M, et al. Pharmacokinetics of GS-441524 following intravenous remdesivir in six cats and results of therapeutic drug monitoring during treatment of feline infectious peritonitis: 22 cases (2021–2024). Journal of Small Animal Practice. 2025.

Chapter 4: CLINICAL TRIAL OF MOLNUPIRAVIR AS FIRST-LINE TREATMENT OF CATS WITH FELINE INFECTIOUS PERITONITIS

4.1 Summary

Recent studies have reported the use of drugs like the nucleoside analog GS-441524 and molnupiravir as a successful treatment of feline infectious peritonitis (FIP). The goal of this study was to evaluate the efficacy of oral molnupiravir (MPV; EIDD-2801) in cats with naturally occurring FIP with a subset of cats being administered a known immune stimulant orally (LTC). Experiment 1 was to determine of the PMBC transcriptome effects of the immune stimulant in 4 heathy cats. Experiment 2 was a prospective, open-label longitudinal single-center clinical trial. Seventy-three cats with naturally occurring FIP were enrolled and treated with oral MPV (10-21 mg/kg PO q12h) for 84 days. A subset of cats (41 cats with effusive FIP) was randomized to concurrently be administered oral LTC immune stimulant. Cats were evaluated at 0, 4, 8 and 12 weeks followed by 12-week observation period. In experiment 1, the healthy cats showed the most pronounced changes in the transcriptome 2 weeks after starting LTC administration. In the clinical trial, a total of 78% cats survived to 6 months (3 months of therapy and 3 months of observation period) and 22% cats died or were euthanized. The median number of days in which the cats died or were euthanized was 4 (range 1-11). The median total bilirubin concentrations were significantly different (p= 0.0007) between the survivors vs non-survivors. Relapses occurred in 12% of the cats (between 9 to 99 days) and all achieved remission during a second course of treatment. Clinicopathologic features associated with FIP normalized during the study period, but some cats showed decreased cholesterol

levels and lymphocytosis during treatment. No adverse effects necessitated discontinuation of either treatment. No effects of the LTC were apparent in this study. Molnupiravir administered at 10 to 20 mg/kg orally q12h for 12 weeks is well tolerated and an effective treatment (78% success) for all forms of naturally occurring FIP with a relapse rate of 12%. These results support those of others showing that molnupiravir is an effective treatment for cats diagnosed with FIP. Additional studies will be required to determine if any benefits might be derived from the LTC treatment.

4.2 Introduction

Feline infectious peritonitis (FIP) is a disease caused by feline coronaviruses (FCoV) and despite marked efforts and many theories, the pathogenesis of FIP is still not fully understood (1). Left untreated, FIP is almost always fatal, with most cats succumbing within weeks to months of diagnosis (2, 3). Recent studies have reported the use of drugs like the nucleoside analog GS-441524 and molnupiravir (MPV; EIDD-2801) as a successful treatments for FIP (4-9). However, more treatment options for cats with FIP, especially those that relapse, are needed.

Recently, a study showing PK/PD data from the antiviral drug EEID-2801 (FDA Emergency Use Authorization for treatment of SARS-COV-2 in humans) showed promise for its use and safety in cats (10). This recent study of pharmacokinetic properties of MPV in healthy specific pathogen free cats established that orally administered MPV at 10 mg/kg achieves plasma levels greater than the established corresponding EC_{50} values. In that study, the MPV prodrug was detected in the serum of all three cats at low levels in the first 12 h post-administration while the β-D-N4-

hydroxycytidine (NHC; EIDD-1931) metabolite was detected at much higher levels between 12 to 24 h post-administration. This study reported no evidence of acute organ toxicity in any of the cats based on the pre- and post-treatment complete blood count (CBC) and serum biochemistry panels; however, all three of the MPV-treated cats demonstrated variable signs of nausea, including hypersalivation and/or vomiting, after oral administration of MPV (10 mg/kg).

One study looking at 18 cats with FIP being treated with MPV showed that MPV might be an effective and safe treatment for domestic cats with FIP at a dose of 10-20 mg/kg twice daily (7). In that study, increased serum alanine transaminase (ALT) activity was found in 3 of the 18 cats, all at days 7-9, and all cats recovered without any medications or need to stop the treatment (7). There is also uncontrolled data from client surveys that suggests efficacy of this product as rescue treatment following failure of the GS-441524 therapy as well as a first line treatment option for cats with FIP (9).

Another study evaluating MPV in cats with FIP showed that it is an effective antiviral treatment for effusive FIP, with 8/10 cats being in remission at 16 weeks while the 2 non-survivors died in the first 24 hours of treatment (8). Survival of cats treated with oral MPV was non-inferior to historic control cats treated orally with GS-441524 (8). The latest study showed that molnupiravir monotherapy resulted in a cure rate of 72% and molnupiravir utilized as a rescue therapy achieved a cure rate of 100%. Survival analysis revealed no difference in outcomes between cats treated with molnupiravir monotherapy and those treated with remdesivir/GS-441524. Adverse events associated with molnupiravir therapy included neutropenia, and transient elevations in hepatic enzyme activities (11).

Cell-mediated immunity is therefore believed to play an important role in controlling or eliminating the mutated virus. Cats in the terminal stages of FIP have severe depletion of the CD4+ and CD8+ T-lymphocytes necessary for mounting cell-mediated immunity (12, 13). With immune dysregulation being a major component of the pathophysiology of FIP, treatment with an antiviral immunostimulant that triggers strong endogenous interferon production would be a rational approach. It has been suggested that antiviral drugs can act synergistically with immunomodulatory treatments to improve patient outcome and survival in different viral diseases, such as influenza virus, hepatitis C virus and human immunodeficiency virus (HIV) (14-16). This synergism has been proposed to improve long-term treatment outcomes for cats with FIP as well.

Recently, studies evaluating Polyprenyl Immunostimulant (PI) and a new liposome-toll-like receptor agonist (TLR3 and TLR9) complex (LTC) were published, providing some evidence that the compounds are immune stimulants. The commercially available PI is believed to upregulate innate immunity via TLR2 and TLR6 (17), but peer-reviewed data supporting that claim in cats is not available. This product is licensed by the U.S. Department of Agriculture for the reduction in the severity of signs of feline herpesvirus 1 (FHV-1) in cats over 8 weeks and has been evaluated in cats with non-effusive FIP with some positive results (18-21). The LTC immune therapeutic has been extensively studied in multiple different veterinary species, including cats (22, 23). The compound has been patented and is currently in pre-clinical development as a feline immunotherapeutic. The effects of the LTC on the innate immune system has been well documented in healthy cats (22) and, in a follow-up study, we showed effects against FHV-1 in 2 different models (23, 24). In another study, we showed a similar compound could be given safely by

parenteral inoculation to cats with positive effect on chronic rhinitis (25). A new study comparing two different immune stimulants (PI and LTC) for antiviral activity cats by measuring cytokine and cellular immune responses by using peripheral blood mononuclear cells (PBMCs) from healthy cats showed that both compounds triggered the upregulated expression of IFN-α, IFN-γ, and IL-1β genes in cat PBMC (26). Treatment with LTC induced significantly greater expression of IFN-α and IFN-γ suggesting that activating the TLR 3 and 9 pathways induced superior broad interferon production in vitro than the activation of the TLR 2 and 6 pathways. This study also showed significant protection from FIPV-induced cytopathic effects when fcwf-4 cells were treated with conditioned medium from LTC-activated leukocytes (26).

One objective was to analyze the transcriptome of PBMC of healthy cats treated with LTC. It was hypothesized that there would be a significant difference in healthy cats before and after treatment with LTC. Other objectives were to determine the efficacy of the molnupiravir as first-line treatment for naturally occurring FIP in cats and to determine the efficiency of oral LTC as an adjunct treatment of cats with the effusive form of FIP. It was hypothesized that molnupiravir will be effective and safe as first-line treatment for FIP and that LTC will help cats FIP achieve or maintain remission.

4.3 Materials and methods

Experiment 1

The liposome Toll-like receptor 3/9 agonist complex (LTC) immune therapeutic has been studied in different veterinary species, including cats (22). The effects of the LTC on the innate immune system has been well documented in healthy cats (22) and in follow-

up studies showing effects against FHV-1 in a feline model (23, 24). Another study showed that a similar compound could be given safely by parenteral inoculation to cats with a positive effect on chronic rhinitis (25). The LTC has been shown to trigger the upregulated expression of IFN-α, IFN-γ, and IL-1β genes in cat PBMC as well as protection from FIPV-induced cytopathic effects when fcwf-4 cells were treated with conditioned medium from LTC-activated leukocytes (26). The LTC was prepared as described previously (22).

Study design

A prospective study with 4 young healthy specific pathogen-free male neutered cats. Cats were deemed healthy based on physical examination and results of complete blood cell count (CBC) and serum biochemistry panel. Cats were administered 0.1 ml/kg LTC orally once weekly for 3 weeks. Cats were monitored for 1 hour post LTC administration for any sign of side effects. Blood was drawn weekly for transcriptomic analysis and at week 0 and 4 also for CBC and serum biochemistry panels. Prior to blood draws, cats were sedated with 0.3mg/kg butorphanol and 1.5mg/kg alfaxalone intramuscularly without any adverse events. This study was approved by the Institutional Animal Care and Use Committee (Protocol # 170.080).

Sample collection

Whole blood (8 mL) was collected via venipuncture using ETDA tubes (manufacture), peripheral blood mononuclear cells (PBMCs) were separated within 2 hours of collection by density gradient centrifugation (Ficoll-Paque TM plus, GE

Healthcare Bio-Sciences) following manufacture and previous published protocol. Total RNA was extracted from isolated PBMCs using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany), and saved at -80°C until further processing.

RNA sequencing

Total RNA was sent to Novogene Inc. (Sacremento, CA) for processing. Briefly, RNA quality was verified on bioanalyzer (Agilent Technologies, Santa Clara, CA). Average RNA integrity number (RIN) >5, with minimal genomic contamination. mRNA was enriched using oligo (dT) beads, followed by cDNA library generation using TruSeq RNA Library Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed on Illumina Novaseq 6000 machine using 150 bp paired end reads.

RNA sequencing data processing

Demultiplexed Fasq reads from Novogene, Inc. were analyzed using the Alpine High-Performance Computing Resource at the University of Colorado Boulder (27). Fastp v0.23.2 was used to trim adapters and low-quality reads, STAR version 2.7.10b was used for alignment to Felis_catus_9.0 genome assembly and annotated using Ensembl 113. HTseq on Partek Flow version 11.9 (28) was used to compute gene counts, DEseq with paired samples was used for differential expression analysis, significant DEGs (differentially expressed genes) are defined as Log2 fold change >= 2 or <=-2, and p value <=0.05. Pathway analysis was completed using GSEA (Sene set enrichment analysis) v4.3.3. GSEA parameters used include weighted enrichment statistic and Signal2Noise metric for ranking genes. Human genes sets were used including Hallmark,

Reactome, PID (pathway interaction database), wikipathways Biocarta and GOBP (Gene ontology biological pathways) Significance for pathway analysis defined as FDR p-value <=0.25, raw p-value <=0.05. Rstudio 2024.04.0 Chocolate Cosmos' Release was used for graphs.

Experiment 2

Study design

A prospective, open-label longitudinal single-center clinical trial. Orally administered MPV was formulated as excipient-less powder in size 3 gelatin capsules (Monument Pharmacy, Monument, CO, USA). Cats with FIP were enrolled and treated with oral MPV (10-21 mg/kg PO q12h) for 84 days. Cats with effusive FIP were randomized into groups administer the LTC (0.1 ml/kg PO once weekly) vs placebo (0.1 ml/kg PO once weekly). Cats were evaluated at 0, 4, 8 and 12 weeks followed by 12-week observation period. The study followed ethical guidelines and was approved by the Institutional Animal Care and Use Committee (Protocol number 4484, approval date September 8, 2023, and 5961, approval date July 30, 2024). Cats were recruited through the Urgent Care service at the referral hospital as well as by referral from referring veterinarians directly to the clinical trial when patient was suspected to have FIP.

Inclusion criteria

Cats suspected of having FIP were considered for enrollment. To be included, a diagnosis of FIP had to be (a) **confirmed:** positive FCoV RNA using quantitative RT-PCR as previously reported (29) or (b) **presumptive:** have clinical signs (e.g. fever, abdominal

fluid, uveitis, neurological signs) and clinicopathological findings consistent with FIP (e.g. hyperglobulinemia, hypoalbuminemia, low A:G, high protein (>3.5 g/dL effusion), cytology findings assessed by a veterinary clinical pathologist to be consistent with FIP) and other differential diagnoses ruled out (e.g. negative *Toxoplasma gondii* serology, no evidence of neoplasia, bacterial or fungal disease on cytology). To be included in the study, cats were required to negative in blood or serum for feline leukemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibodies within the preceding month. Cats were excluded from the clinical trial if immunosuppressive medications (eg. immunosuppressive dosed of glucocorticoids) or antiviral medications had been administered prior to enrollment. Cats were also excluded if hospitalization for intravenous fluid therapy or blood transfusion for severe anemia was required. Cats with chronic kidney disease IRIS Stage 3 and 4 (30), congestive heart failure, diabetes mellitus and/or uncontrolled hyperthyroidism were also excluded from the study. Cats were characterized based on the form of FIP as effusive (pleural, peritoneal or pericardial) or non-effusive and whether there was neurological or ocular involvement.

Data collected

Patient data collected included: signalment, clinical signs physical examination findings (pyrexia defined as a rectal temperature of >103.1°F (>39.2°C) (31), diagnostic tests (including hematology, serum biochemistry, urinalysis (if performed), infectious disease testing, diagnostic imaging when performed, cytology and/or biochemistry of fluid samples, fine-needle-aspirates (FNA) and testing for FCoV RNA by RT-PCR. Treatment dosage, outcome and any adverse effects of MPV were also recorded.

Statistical analysis

Data were recorded in Excel (Excel, Microsoft, Redmond, Washington, USA). Variables were assessed for normality by visual assessment of histograms and use of the Shapiro–Wilks test. Descriptive statistics were used to report the data. Data were reported as median (range) and interquartile range (IQR). Categorical data of the number of cats alive or dead at the end of the initial treatment period grouped according to whether they had achieved remission or not at the end of the 12-week treatment period. Friedman analysis was used to compare the medians of the clinicopathological parameters in the follow up bloodwork. The chi-square test was used to compare the number of relapses between the LTC vs placebo groups.

4.4 Results

Experiment 1

The LTC treatment in healthy cats

The most pronounced changes in transcriptome after LTC immune stimulation was detected 2 weeks post treatment. Principle component analysis dimensional reduction plot of the immune cell transcriptome showing distribution of each sample is seen in Figure 4.1A. The most significant changes were seen in the PBMCs at week 2 post LTC administration, with a total of 406 significant DEGs (Figure 4.1B). Week 1 samples had a total of 24 significant DEGs (Figure 4.2A), top upregulated and downregulated genes are listed in Figure 4.2B, 4.2C. Top upregulated pathways include those related to protein synthesis, metabolism and a few immune activation, top 10 are shown in Figure 4.2D.

Downregulated gene sets can be categorized as growth factor and signaling related, as well as several antigen-presentation and immune-receptor pathways, top 10 are also included in Figure 4.2D.

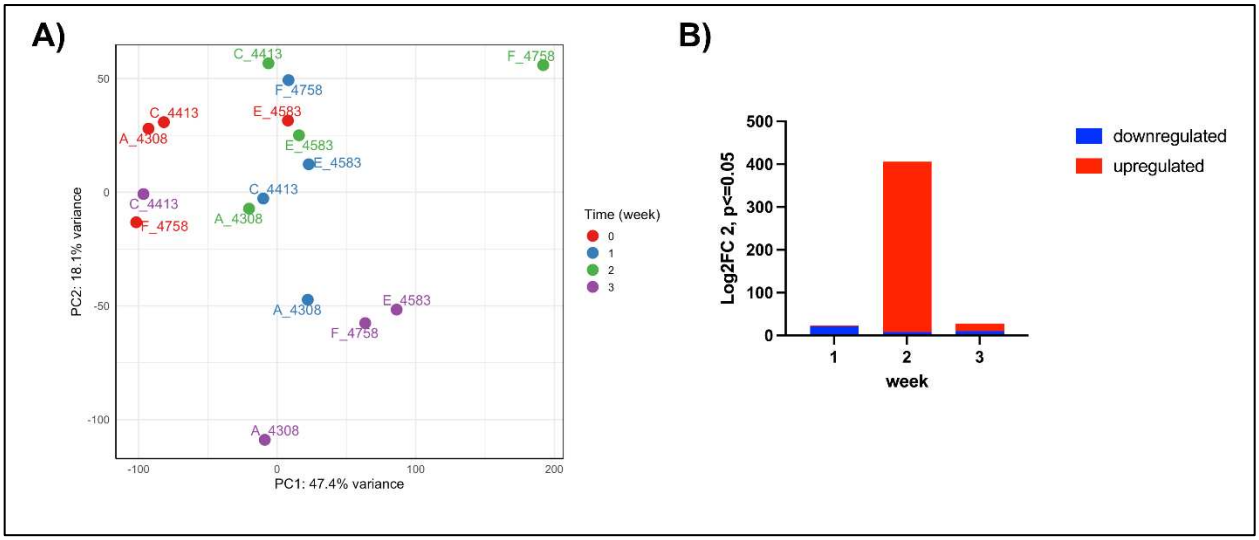
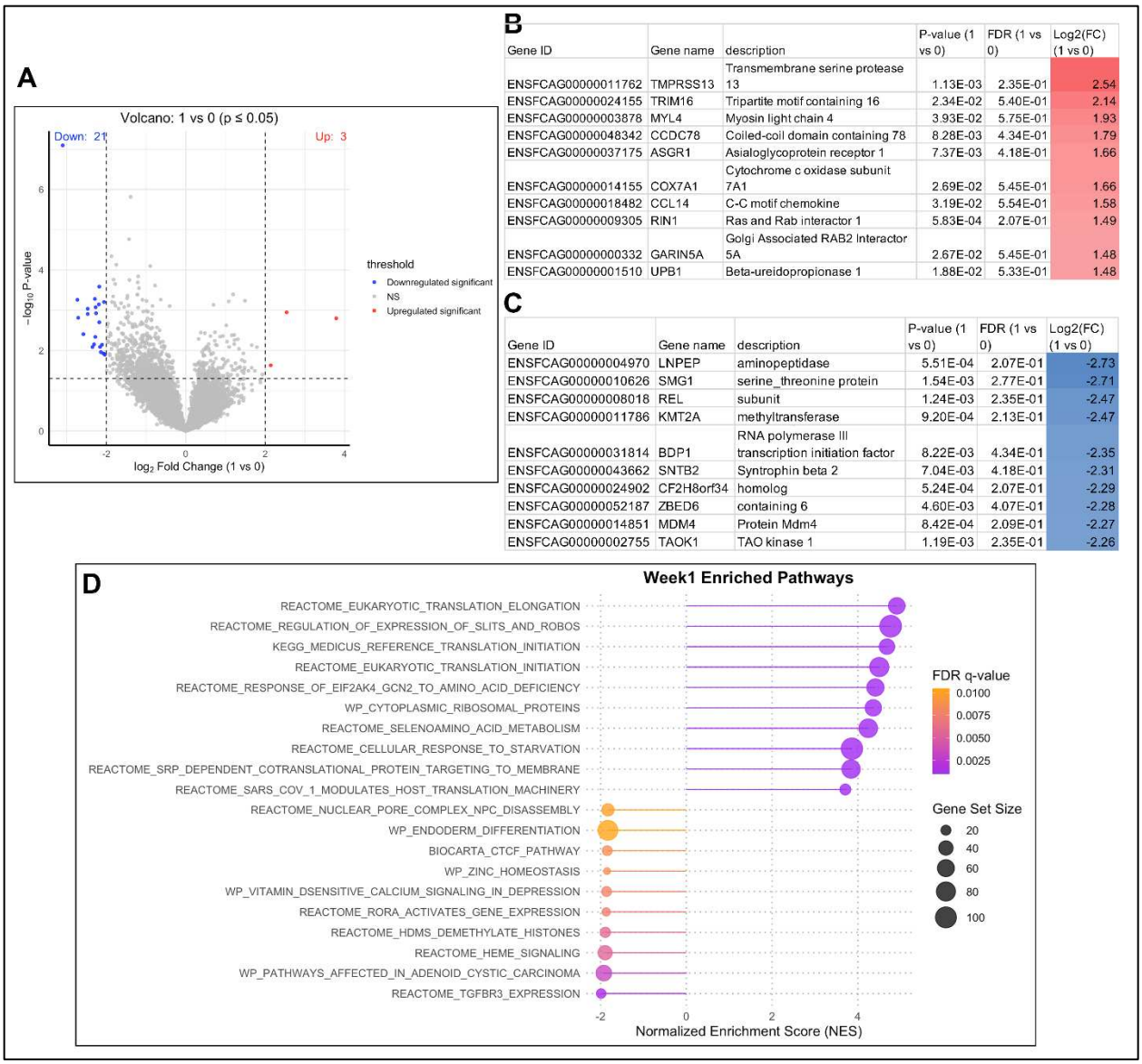


Figure 4.1. Bulk RNA sequencing results from n=4 cats treated with 0.1 mL/kg LTC. A) PCA (principle component analysis) dimensional reduction plot showing distribution of each sample. Colors represent time points in weeks post treatment, cat numbers labels next to each dot. B) Number of significant DEGs (differentially expressed genes) comparing post treatment to pretreatment samples using paired DEseq analysis. Red shows total upregulated significant gene number and blue shows downregulated. Significance defined as raw p value <= 0.05 and Log2 FC (fold change) >= 2 or <= -2.



B

Gene ID	Gene name	description	P-value (1 vs 0)	FDR (1 vs 0)	Log2(FC) (1 vs 0)
ENSFCAG00000011762	TMPRSS13	Transmembrane serine protease 13	1.13E-03	2.35E-01	2.54
ENSFCAG00000024155	TRIM16	Tripartite motif containing 16	2.34E-02	5.40E-01	2.14
ENSFCAG00000003878	MYL4	Myosin light chain 4	3.93E-02	5.75E-01	1.93
ENSFCAG00000048342	CCDC78	Coiled-coil domain containing 78	8.28E-03	4.34E-01	1.79
ENSFCAG00000037175	ASGR1	Asialoglycoprotein receptor 1	7.37E-03	4.18E-01	1.66
ENSFCAG00000014155	COX7A1	Cytochrome c oxidase subunit 7A1	2.69E-02	5.45E-01	1.66
ENSFCAG00000018482	CCL14	C-C motif chemokine	3.19E-02	5.54E-01	1.58
ENSFCAG00000009305	RIN1	Ras and Rab interactor 1	5.83E-04	2.07E-01	1.49
ENSFCAG00000000332	GARIN5A	Golgi Associated RAB2 Interactor 5A	2.67E-02	5.45E-01	1.48
ENSFCAG00000001510	UPB1	Beta-ureidopropionase 1	1.88E-02	5.33E-01	1.48

C

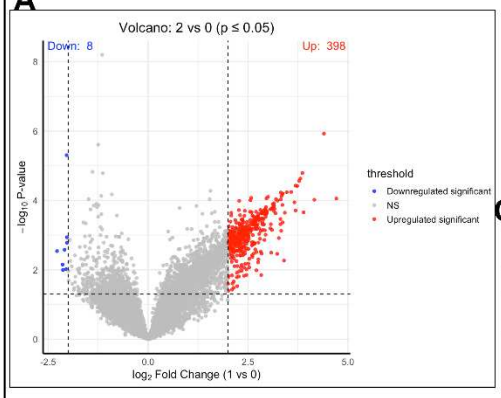
Gene ID	Gene name	description	P-value (1 vs 0)	FDR (1 vs 0)	Log2(FC) (1 vs 0)
ENSFCAG00000004970	LNPEP	aminopeptidase	5.51E-04	2.07E-01	-2.73
ENSFCAG00000010626	SMG1	serine_threonine protein	1.54E-03	2.77E-01	-2.71
ENSFCAG00000008018	REL	subunit	1.24E-03	2.35E-01	-2.47
ENSFCAG00000011786	KMT2A	methyltransferase	9.20E-04	2.13E-01	-2.47
ENSFCAG00000031814	BDP1	RNA polymerase III transcription initiation factor	8.22E-03	4.34E-01	-2.35
ENSFCAG00000043662	SNTB2	Syntrophin beta 2	7.04E-03	4.18E-01	-2.31
ENSFCAG00000024902	CF2H8orf34	homolog	5.24E-04	2.07E-01	-2.29
ENSFCAG00000052187	ZBED6	containing 6	4.60E-03	4.07E-01	-2.28
ENSFCAG00000014851	MDM4	Protein Mdm4	8.42E-04	2.09E-01	-2.27
ENSFCAG00000002755	TAOK1	TAO kinase 1	1.19E-03	2.35E-01	-2.26

Figure 4.2. Changes in transcriptome at week 1 post LTC compared to pretreatment. **A)** Volcano plot of n=4 week 1 compared to week 0 using paired DEseq. Significance defined as raw p-value <=0.05, Log2 FC >=2 or <=-2. Red dots show significantly upregulated genes at week1, blue dots show significantly downregulated genes at week 1. X-axis represents Log2 FC, y-axis negative Log10 p-value. **B)** List of top 10 upregulated protein coding genes with annotation in week 1. FDR= (false discovery rate) adjusted p-value. **C)** List of top 10 downregulated protein coding genes with annotation in week 2. **D)** GSEA (gene set enrichment analysis) results comparing week 1 samples to pretreatment, shown in graph are top 10 highest NES (normalized enrichment score) for pathways upregulated in week 1. Top 10 lowest NES (negative) for pathways downregulated in week 1. Gene set sizes (number of genes mapped to pathway) represented as sized dots, and FDR adjusted p-value shown in color scale. Significance pre-filtered for p-value <=0.05, and FDR <=0.25.

Most pronounced changes in transcriptome after LTC immune stimulation detected 2 weeks post treatment and these samples are the most divergent from the pre-treatment (Figure 4.3A), top upregulated gene tachykinin precursor 4 (TAC4) is commonly found in monocytes, dendritic cells, NK cells, and lymphocytes and may be linked to proinflammatory cytokine production (Figure 4.3B). Downregulated genes include some related to innate antigen processing (LNPEP, SMG1), and pro-inflammatory signaling (REL, CRISPLD2) (Figure 4.3C). Upregulated pathways again include “reactome eukaryotic translation elongation” which is also upregulated at week 1, this along with others suggests increased protein translation (Figure 4.3D). Week 2 also highly upregulates several Innate immunity pathways.

Gene expression at week 3 on average shows less differences compared to the previous week, in part due to 1 out of the 4 samples that have less response to LTC (Figure 4.4A). Top 10 upregulated and downregulated genes in week 3 are shown in Figure 4.4B and 4.4C, although the raw p-value is significant the adjusted p-value (FDR) is high because of the large differences between the biological replicates. Compared to the hundreds of significant pathways in the week 1 and week 2 sample sets, week 3 has less than 90 total pathways which meets the cutoff of FDR <0.25. Top up and downregulated pathways are shown in Figure 4.4D.

A



B

Gene ID	Gene name	description	P-value (2 vs 0)	FDR (2 vs 0)	Log2(FC) (2 vs 0)
ENSFCAG00000002641	TAC4	Tachykinin 4	1.18E-06	5.72E-03	4.40
ENSFCAG00000006183	GUCA2A	Guanylate cyclase activator 2A	9.52E-05	2.99E-02	4.16
ENSFCAG00000046308	HBQ1	Hemoglobin subunit alpha	2.20E-04	2.99E-02	3.89
ENSFCAG00000011223	PCSK1N	Proprotein convertase subtilisin_kexin type 1 inhibitor	1.62E-05	2.27E-02	3.86
ENSFCAG00000043112	POLR2L	DNA-directed RNA polymerases I, II, and III subunit RPABC5	2.34E-05	2.84E-02	3.81
ENSFCAG00000034587	GADD45GIP1	mL64	2.72E-05	2.94E-02	3.78
ENSFCAG00000004711	ELOB	Elongin B	3.82E-05	2.99E-02	3.73
ENSFCAG00000014459	SULT2B1	Sulfotransferase	1.94E-04	2.99E-02	3.69
ENSFCAG00000001343	PMVK	Phosphomevalonate kinase	1.13E-04	2.99E-02	3.66
ENSFCAG00000039538	RPS27	40S ribosomal protein S27	5.77E-05	2.99E-02	3.47

C

Gene ID	Gene name	description	P-value (2 vs 0)	FDR (2 vs 0)	Log2(FC) (2 vs 0)
ENSFCAG00000008018	REL	REL proto-onco, NF-kB subunit	2.88E-03	5.33E-02	-2.28
ENSFCAG00000010832	CRISPLD2	Cysteine rich secretory protein LCCL domain containing 2	7.02E-03	7.40E-02	-2.14
ENSFCAG00000012359	RALGPS2	Ral GEF with PH domain and SH3 binding motif 2	2.62E-03	5.06E-02	-2.09
ENSFCAG00000004970	LNPEP	aminopeptidase	9.60E-03	8.58E-02	-2.05
ENSFCAG00000043407	JCHAIN	and IgM	4.94E-06	1.20E-02	-2.04
ENSFCAG00000010874	SYTL5	Synaptotagmin like 5	1.15E-03	4.04E-02	-2.03
ENSFCAG00000008547	EBF1	EBF transcription factor 1	1.65E-03	4.23E-02	-2.03
ENSFCAG00000010589	CR2	Complement C3d receptor 2	1.07E-02	9.03E-02	-1.99
ENSFCAG00000010626	SMG1	non-specific serine_threonine protein kinase	2.04E-02	1.23E-01	-1.99
ENSFCAG00000014308	CPM	Carboxypeptidase M	1.37E-03	4.05E-02	-1.96

D

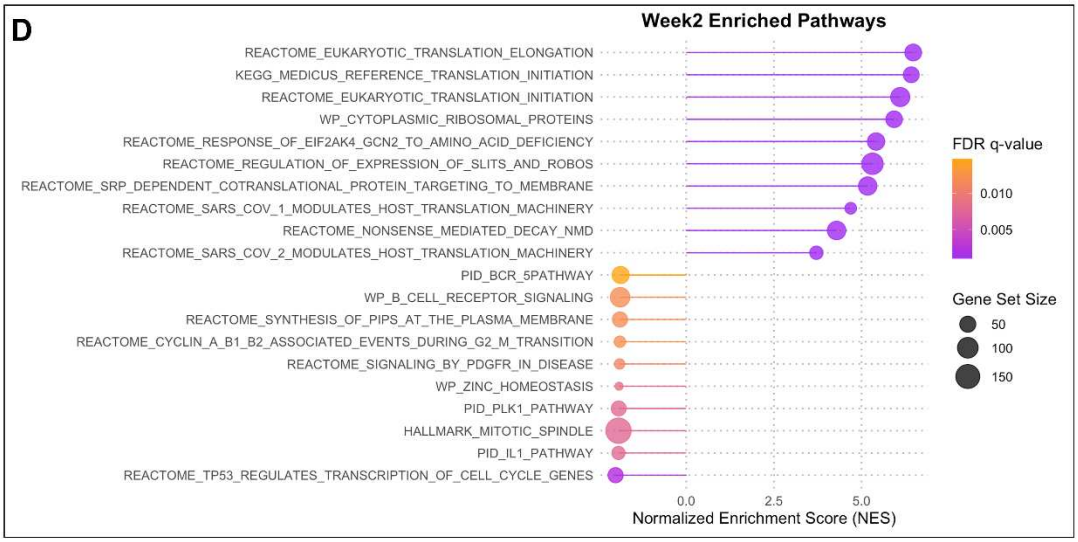
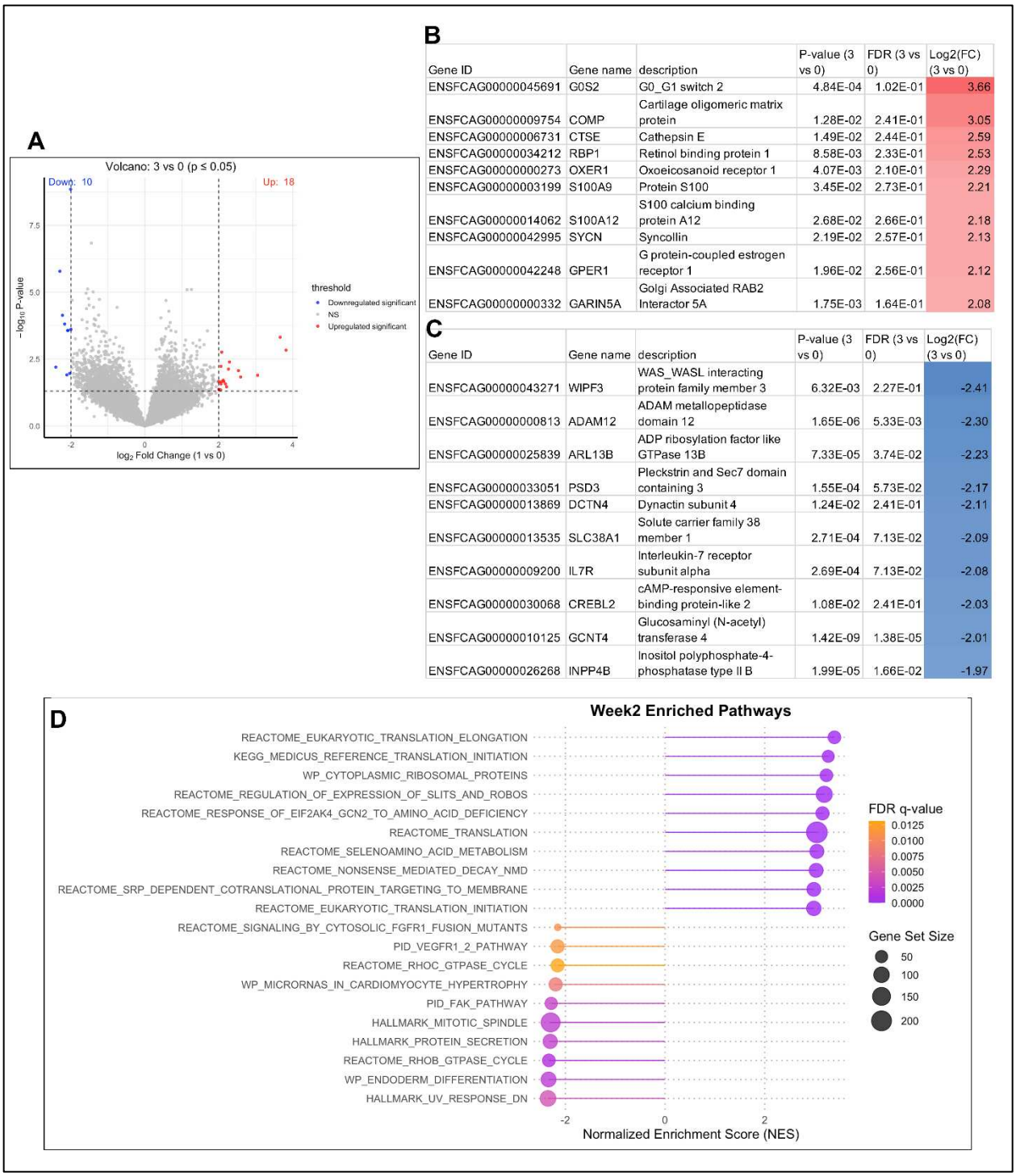


Figure 4.3. Changes in transcriptome at week 2 post LTC compared to pretreatment. A) Volcano plot of n=4 week 2 compared to week 0 using paired DEseq. Red dots show significantly upregulated genes at week2, blue dots show significantly downregulated genes at week 2. **B)** List of top 10 upregulated protein coding genes with annotation in week 2. **C)** List of top 10 downregulated protein coding genes with annotation in week 2. **D)** GSEA results comparing week 2 samples to pretreatment, shown in graph are top 10 highest NES for pathways upregulated in week 2. Top 10 lowest NES (negative) for pathways downregulated in week 2. Significance filters as shown in previous figure.



B

Gene ID	Gene name	description	P-value (3 vs 0)	FDR (3 vs 0)	Log2(FC) (3 vs 0)
ENSFCAG00000045691	G0S2	G0_G1 switch 2	4.84E-04	1.02E-01	3.66
ENSFCAG00000009754	COMP	Cartilage oligomeric matrix protein	1.28E-02	2.41E-01	3.05
ENSFCAG00000006731	CTSE	Cathepsin E	1.49E-02	2.44E-01	2.59
ENSFCAG00000034212	RBP1	Retinol binding protein 1	8.58E-03	2.33E-01	2.53
ENSFCAG00000000273	OXER1	Oxoeicosanoid receptor 1	4.07E-03	2.10E-01	2.29
ENSFCAG00000003199	S100A9	Protein S100	3.45E-02	2.73E-01	2.21
ENSFCAG00000014062	S100A12	S100 calcium binding protein A12	2.68E-02	2.66E-01	2.18
ENSFCAG00000042995	SYCN	Syncollin	2.19E-02	2.57E-01	2.13
ENSFCAG00000042248	GPER1	G protein-coupled estrogen receptor 1	1.96E-02	2.56E-01	2.12
ENSFCAG00000000332	GARIN5A	Golgi Associated RAB2 Interactor 5A	1.75E-03	1.64E-01	2.08

C

Gene ID	Gene name	description	P-value (3 vs 0)	FDR (3 vs 0)	Log2(FC) (3 vs 0)
ENSFCAG00000043271	WIPF3	WAS_WASL interacting protein family member 3	6.32E-03	2.27E-01	-2.41
ENSFCAG00000000813	ADAM12	ADAM metallopeptidase domain 12	1.65E-06	5.33E-03	-2.30
ENSFCAG00000025839	ARL13B	ADP ribosylation factor like GTPase 13B	7.33E-05	3.74E-02	-2.23
ENSFCAG00000033051	PSD3	Pleckstrin and Sec7 domain containing 3	1.55E-04	5.73E-02	-2.17
ENSFCAG00000013869	DCTN4	Dynactin subunit 4	1.24E-02	2.41E-01	-2.11
ENSFCAG00000013535	SLC38A1	Solute carrier family 38 member 1	2.71E-04	7.13E-02	-2.09
ENSFCAG00000009200	IL7R	Interleukin-7 receptor subunit alpha	2.69E-04	7.13E-02	-2.08
ENSFCAG00000030068	CREBL2	cAMP-responsive element-binding protein-like 2	1.08E-02	2.41E-01	-2.03
ENSFCAG00000010125	GCNT4	Glucosaminyl (N-acetyl) transferase 4	1.42E-09	1.38E-05	-2.01
ENSFCAG00000026268	INPP4B	Inositol polyphosphate-4-phosphatase type II B	1.99E-05	1.66E-02	-1.97

Figure 4.4. Changes in transcriptome at week 3 post LTC treatment. **A)** Volcano plot of n=4 week 3 compared to week 0 using paired DEseq. Red dots show significantly upregulated genes at week3, blue dots show significantly downregulated genes at week 3. **B)** List of top 10 upregulated protein coding genes with annotation in week 3. **C)** List of top 10 downregulated protein coding genes with annotation in week 3. **D)** GSEA results comparing week 3 samples to pretreatment, shown in graph are top 10 pathways

upregulated in week 3 and top 10 pathways downregulated in week 3. Significance filters as shown in previous figures.

Experiment 2

Study cohort

Seventy-three cats with a confirmed or presumptive diagnosis of FIP were recruited between September 2023 and November 2024. Of these, majority were Domestic shorthairs (54; 74%) or longhairs (8; 11%) and only 11 (15%) cats were pedigree breeds: Maine Coon (4 cats), Sphynx (1), British shorthair (1), Ragdoll (1), Bengal (1), Siamese (1), Snowshoe (1) and Siberian (1). There were 38 (53%) neutered males, 27 (37%) spayed females, 4 (5%) intact males and 4 (5%) entire females. The majority (54/73; 74%) of cats were aged <1 year at diagnosis, the median age was 0.6 years (IQR 0.5-1.0) and the oldest cat enrolled in the study was 13 years old.

Clinical findings

Most cats (47/73; 64%) were diagnosed with effusive FIP: peritoneal effusion (39; 53%), pleural effusion (7; 10%) and 1 (1%) cat had both pericardial and pleural effusion. Non-effusive FIP was diagnosed in 26 (36%) cats and 10 (14%) cats had ocular involvement, 4 (5%) cats had neurological involvement, and 2 (3%) cats had both ocular and neurological involvement. Out of these cats, 43 (59%) had confirmed FIP by RT-PCR, while 30 (41%) cats had presumptive FIP with 22 (30%) cats PCR negative and in 8 (11%) cats no PCR was performed. The RT-PCR was positive in 30 (41%) cats on peritoneal effusion, in 5 (7%) cats on pleural effusion, in 3 (4%) cats on jejunal LN, 3 (4%) cats on spleen, 1 (1%) cat on aqueous humor and 1 (1%) cat on mass in the ileum. Additionally, in 4 (5%) cats, RT-PCR was positive on more than one sample: jejunal lymph nodes in 2

(3%) cats, colic lymph node in 1 (1%) cat and pancreatic lymph node in 1 (1%) cat. The most common clinical signs and findings are shown in Table 4.1.

Table 4.1. The most common clinical signs and findings at the time of FIP diagnosis.

Clinical abnormality	**Number of cats**	**%**
Lethargy	57	78
Weight loss	45	62
Hyporexia	43	60
Pyrexia	42	58
Upper respiratory tract signs	12	16
Uveitis	12	16
Diarrhea	7	10
Neurological signs	6	8
Polydipsia	5	7
Constipation	3	4
Difficulty gaining weight	3	4
Vomiting	1	1
Hematochezia	1	1

Laboratory results

Table 4.2 shows CBC results at the initial visit for 71 cats; in two cats the EDTA blood clotted, and CBC could not be performed. A total of 51/71 (72%) cats were anemic with median hematocrit of 28.0 (IQR 24.0-32.0). Neutrophilia was seen in 38/71 (54%) cats and one cat presented with mild neutropenia (1.7x10^3/uL). A total of 46/71 (65%) cats had lymphopenia on presentation and 1 cat presented with mild lymphocytosis

(6.7x10^3/uL). Serum biochemistry results for all 73 cats are shown in Table 3. A low serum AG ratio of $\leqslant$0.4 occurred in 25/73 (34%) of cats, with only 10/73 (14%) of cats having a serum AG ratio of $\geqslant$0.6. Hyperglobulinemia occurred in 54/73 (74%) cats and hypoalbuminemia occurred in 66/73 (90%) of cats. A total of 48/73 (66%) cats presented with hyperbilirubinemia.

Table 4.2. Complete blood cell counts results at the time of FIP diagnosis (week 0).

Clinical parameter	Median (IQR)	Reference interval
Hemoglobin	9.6 (8.2-10.6)	9.8 - 15.5 g/dL
Hematocrit	28.0 (24.0-32.0)	32 - 47%
MCV	43.0 (40.8-46.0)	40 - 52 fl
MCHC	34.0 (33.0-34.0)	32 - 36 g/dL
Reticulocytes	30.2 (21.6-44.2)	0 - 50 10^3/uL
Platelets	273 (150-381)	200 - 500 10^3/uL
Nucleated cell count	14.6 (10.0-22.4)	4.0 - 14.0 10^3/uL
Neutrophils	11.9 (7.3-19.3)	2 - 12 10^3/uL
Lymphocytes	1.2 (0.5-2.2)	1.5 - 6 10^3/uL
Monocytes	0.2 (0.1-0.5)	0 - 0.8 10^3/uL
Eosinophils	0.0 (0.0-0.2)	0 - 1.2 10^3/uL
Basophils	0.0 (0.0-0.0)	0 - 0.1 10^3/uL

MCV - mean corpuscular volume, MCHC - mean corpuscular hemoglobin concentration

Table 4.3. Serum biochemistry results at the time of FIP diagnosis (week 0).

Clinical parameter	Median (IQR)	Reference interval
Glucose	99.0 (82.0-115.0)	68 - 140 mg/dL
BUN	19.0 (16.0-23.0)	18 - 35 mg/dL
Creatinine	0.8 (0.6-0.9)	0.8 - 2.4 mg/dL
Phosphorus	6.5 (5.7-7.5)	3 - 6 mg/dL
Calcium	8.8 (8.3-9.5)	9.2 - 11.1 mg/dL
Magnesium	2.2 (2.1-2.4)	2 - 2.7 mg/dL
Total protein	7.6 (6.7-8.9)	6.3 - 8 g/dL
Albumin	2.4 (2.1-2.7)	3.1 - 4.4 g/dL
Globulin	5.2 (4.2-6.1)	2.7 - 4.2 g/dL
A:G	0.4 (0.4-0.6)	0.8 - 1.6
Cholesterol	135.0 (105.0-166.0)	95 - 270 mg/dL
Creatinine kinase	429 (169-1180)	60 - 350 IU/L
Total bilirubin	0.3 (0.1-1.2)	0 - 0.1 mg/dL
ALP	19.0 (11.0-31.0)	10 - 80 IU/L
ALT	45.0 (27.0-76.0)	30 - 140 IU/L
AST	69.0 (39.0-124.0)	15 - 45 IU/L
GGT	0.0 (0.0-0.0)	0 - 0.5 IU/L
Sodium	147.0 (145.0-149.0)	149 - 157 mEQ/L
Potassium	4.4 (4.0-4.8)	3.7 - 5.4 mEQ/L
Chloride	114.2 (111.1-116.4)	115 - 125 mEQ/L

A:G - albumin:globulin ratio, ALP - alkaline phosphatase, ALT - *alanine transaminase,* AST - **aspartate aminotransferase**, GGT - gamma-glutamyl transferase

Urinalysis was performed in 29/73 (40%) of cats and showed median urine specific gravity of 1.053 (IQR 1.041-1.059), median pH of 6.5 (IQR 6.0-7.0) and median urine protein creatinine ratio of 0.36 (IQR 0.25-0.57).

All but one cat were feline immunodeficiency virus and feline leukemia virus negative. One cat was FeLV/FIV negative at the referring veterinarian records prior to inclusion but was then diagnosed with FeLV antigen and confirmed by PCR with FeLV infection after enrolment.

Abdominal ultrasound

Abdominal ultrasound was performed in 23/73 (32%) cats. All cats (23/23; 100%) had more than one abnormality on abdominal ultrasound with median number of abnormalities in one cat being 4 (range 2-6). The most common findings were lymphadenopathy (21/23; 91%), peritoneal effusion (21/23; 91%), mottled or heterogenous spleen (7/23; 31%), intestinal wall thickening (7/23; 31%), splenomegaly (5/23; 22%), medullary rim sign (5/23; 22%), heterogenous kidneys (5/23; 22%), gall bladder thickening or edema (5/23; 22%), renomegaly (4/23; 17%), heterogenous or hypoechoic liver (4/23; 17%), nodular liver (3/23; 13%), renal nodules (2/23; 9%), hepatomegaly (1/23; 4%), hyperechoic mesentery (1/23; 4%), splenic nodules (1/23; 4%), pancreatic nodules (1/23; 4%) and ileal mass (1/23; 4%).

Cytology findings

Fluid analysis and cytology was performed in 46/47 (97%) of the cats with effusive FIP. The median total protein on the effusion was 6.0 (IQR 5.4-7.1). The median nucleated cell count was 3200 (IQR 1900-5700).

Treatment and follow up

Antiviral treatment

All cats were treated with MPV for 12 weeks. A total of 4 (5%) cats had therapy prolonged to 16 weeks due to incomplete resolution of clinicopathological abnormalities or clinical signs at week 12. One cat had to be excluded from the study because of disease progression and owners elected to add GS-441524 to the treatment. The median dosage of MPV was 13.8mg/kg (IQR 12.5-15.4) and the maximum dosage was 21.4mg/kg PO BID. A total of 57/73 (78%) cats survived to 6 months (3 months of therapy and 3 months of observation period) and 16/73 (22%) cats died or were euthanized. The median number of days in which the cats died or were euthanized was 4 (range 1-11). The median total bilirubin concentrations were significantly different (p= 0.0007) between the survivors vs non survivors (Figure 4.5). The median total bilirubin in surviving cats was 0.2 (IQR 0.1-0.8) vs in cats that died the median total bilirubin was 1.9 (IQR 0.5-3.0). Of the 57 cats that lived, 7 (12%) relapsed and required a secondary course of treatment, when signs referable to FIP (pyrexia, lethargy, and inappetence) recurred between 9 and 99 days after discontinuing treatment. All 7 cats commenced a second course of treatment using a median dosage of 22.4 mg/kg (IQR 21.8-24.8) which was higher than their primary course, and all achieved remission during this second course of treatment.

Of note is that none of the cats relapsed with same form of FIP; all cats had non-effusive form with 3/7 cats also having neurological involvement while during the first time, 4/7 cats had effusive form and 3/7 non effusive with 2 of those having ocular involvement. The 2 cats with ocular involvement both presented with neurological signs at the time of the relapse.

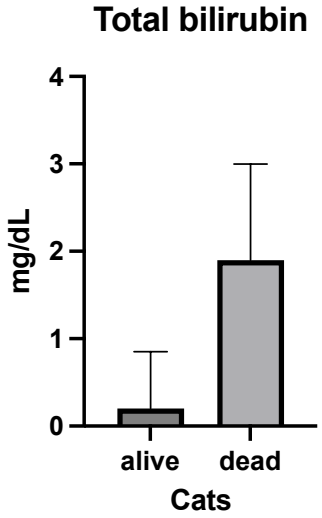


Figure 4.5. Total bilirubin concentrations at diagnosis in cats that survived vs died. The box represents the median and vertical line is the interquartile range.

Side effects from molnupiravir were minimal. Most common side effects were gastrointestinal: nausea (4 cats), hypersalivation (4), constipation (3), diarrhea (2), hyporexia (1) and vomiting (1). Apart from 1 cat, all of the side effects observed resolved within the first week of therapy, but this 1 cat remained intermittently nauseous and had to be treated with ant-nausea medications throughout the treatment. Pruritus was reported in 3 cats during the study. One cat developed folded ears, this was a cat that relapsed and had to be treated again with a high dose of molnupiravir (26.4 mg/kg PO BID). Mild neutropenia developed in 1 cat at week 8 and persisted until week 12 and 1 cat developed mild neutropenia at week 12. At week 4, 8/56 cats developed mild to

moderate increase in *alanine transaminase* (ALT); out of these, 2 cats had high ALT already at diagnosis) and all of these cats had normal ALT by week 8. At week 8, 2 cats had mild increase in ALT (these were different that the cats with high ALT at week 4) and at week 12, ALT was mildly increased in 2 cats (1 new cats and 1 that had ALT increase at week 8). No other side effects were noticed. No adverse effects necessitated drug discontinuation.

Clinicopathologic findings during the first 12 weeks of treatment

Marked and rapid clinical improvements were observed in all 56 cats that completed 12 weeks of treatment. Follow up bloodwork was available for 56 cats that were alive at 4,8 and 12 weeks of treatment and these can be seen in Tables 4.4-4.9. On CBC, there was a significant improvement in hematocrit (p=<0.0001) and neutrophils (p=<0.0001) – Figures 4.6 and 4.7. Mild eosinophilia developed in 3 cats on week 4 of treatment and resolved in all 3 (2 cats by week 8 and 1 cat by week 12). One cat developed mild eosinophilia week 8 of treatment that persisted until week 12 and additional 3 cats developed mild eosinophilia on week 12. On serum biochemistry, there was a significant difference in improvement in A:G (p=<0.0001; Figure 4.8), albumin (p=<0.0001; Figure 4.9) and globulin (p=<0.0001; Figure 4.10) concentrations as well as total bilirubin levels (p=<0.0001; Figure 4.11). There was also an increase in lymphocytes with statistical significance (p=<0.0001; Figure 4.12) and decrease in cholesterol (p=<0.0311; Figure 4.13) while on antiviral treatment.

Table 4.4. Complete blood cell counts results at week 4 of treatment.

Clinical parameter	Median (IQR)	Reference interval
Hemoglobin	11.5 (10.7-12.4)	9.8 - 15.5 g/dL
Hematocrit	34.0 (31.5-37.0)	32 - 47%
MCV	44.0 (42.0-47.0)	40 - 52 fl
MCHC	34.0 (33.0-35.0)	32 - 36 g/dL
Reticulocytes	32.4 (21.1-44.0)	0 - 50 10^3/uL
Platelets	366 (234-529)	200 - 500 10^3/uL
Nucleated cell count	11.1 (8.2-15.3)	4.0 - 14.0 10^3/uL
Neutrophils	7.0 (5.2-9.9)	2 - 12 10^3/uL
Lymphocytes	2.7 (1.7-4.0)	1.5 - 6 10^3/uL
Monocytes	0.2 (0.1-0.3)	0 - 0.8 10^3/uL
Eosinophils	0.4 (0.2-0.8)	0 - 1.2 10^3/uL
Basophils	0.0 (0.0-0.0)	0 - 0.1 10^3/uL

MCV - mean corpuscular volume, MCHC - mean corpuscular hemoglobin concentration

Table 4.5. Serum biochemistry results at week 4 of treatment.

Clinical parameter	Median (IQR)	Reference interval
Glucose	98.0 (87.8-111.3)	68 - 140 mg/dL
BUN	25.5 (21.0-30.0)	18 - 35 mg/dL
Creatinine	0.9 (0.7-1.0)	0.8 - 2.4 mg/dL
Phosphorus	7.3 (6.1-8.3)	3 - 6 mg/dL
Calcium	9.9 (9.5-10.3)	9.2 - 11.1 mg/dL
Magnesium	2.1 (2.1-2.3)	2 - 2.7 mg/dL
Total protein	7.5 (7.1-7.9)	6.3 - 8 g/dL
Albumin	3.2 (3.0-3.6)	3.1 - 4.4 g/dL
Globulin	4.3 (3.6-4.7)	2.7 - 4.2 g/dL
A:G	0.7 (0.6-1.0)	0.8 - 1.6
Cholesterol	108.0 (89.0-123.0)	95 - 270 mg/dL
Creatinine kinase	188 (121-268)	60 - 350 IU/L
Total bilirubin	0.0 (0.0-0.1)	0 - 0.1 mg/dL
ALP	55.0 (30.0-84.3)	10 - 80 IU/L
ALT	50.0 (37.8-76.5)	30 - 140 IU/L
AST	24.0 (20.0-31.3)	15 - 45 IU/L
GGT	0.0 (0.0-0.0)	0 - 0.5 IU/L
Sodium	150.0 (148.0-151.0)	149 - 157 mEQ/L
Potassium	4.6 (4.4-4.8)	3.7 - 5.4 mEQ/L
Chloride	114.5 (113.3-116.1)	115 - 125 mEQ/L

A:G - albumin:globulin ratio, ALP - alkaline phosphatase, ALT - *alanine transaminase,* AST - **aspartate aminotransferase,** GGT - gamma-glutamyl transferase

Table 4.6. Complete blood cell counts results at week 8 of treatment.

Clinical parameter	Median (IQR)	Reference interval
Hemoglobin	2.8 (11.9-13.8)	9.8 - 15.5 g/dL
Hematocrit	37.0 (34.0-39.0)	32 - 47%
MCV	44.0 (41.0-46.0)	40 - 52 fl
MCHC	35.0 (34.0-35.9)	32 - 36 g/dL
Reticulocytes	33.1 (22.1-39.2)	0 - 50 10^3/uL
Platelets	270 (145-405)	200 - 500 10^3/uL
Nucleated cell count	8.4 (5.6-12.8)	4.0 - 14.0 10^3/uL
Neutrophils	5.3 (3.2-7.5)	2 - 12 10^3/uL
Lymphocytes	2.5 (1.8-3.7)	1.5 - 6 10^3/uL
Monocytes	0.2 (0.1-0.3)	0 - 0.8 10^3/uL
Eosinophils	0.4 (0.2-0.8)	0 - 1.2 10^3/uL
Basophils	0.0 (0.0-0.0)	0 - 0.1 10^3/uL

MCV - mean corpuscular volume, MCHC - mean corpuscular hemoglobin concentration

Table 4.7. Serum biochemistry results at week 8 of treatment.

Clinical parameter	Median (IQR)	Reference interval
Glucose	95.5 (86.8-114.3)	68 - 140 mg/dL
BUN	27.0 (23.8-30.0)	18 - 35 mg/dL
Creatinine	1.0 (0.9-1.1)	0.8 - 2.4 mg/dL
Phosphorus	7.1 (5.7-8.0)	3 - 6 mg/dL
Calcium	10.1 (9.6-10.4)	9.2 - 11.1 mg/dL
Magnesium	2.1 (2.1-2.3)	2 - 2.7 mg/dL
Total protein	7.2 (6.8-7.4)	6.3 - 8 g/dL
Albumin	3.4 (3.2-3.7)	3.1 - 4.4 g/dL
Globulin	3.6 (3.3-4.1)	2.7 - 4.2 g/dL
A:G	1.0 (0.8-1.1)	0.8 - 1.6
Cholesterol	106.5 (87.8-131.8)	95 - 270 mg/dL
Creatinine kinase	170 (129-257)	60 - 350 IU/L
Total bilirubin	0.0 (0.0-0.0)	0 - 0.1 mg/dL
ALP	54.5 (35.8-75.3)	10 - 80 IU/L
ALT	48.0 (38.3-63.8)	30 - 140 IU/L
AST	24.0 (18.8-29.3)	15 - 45 IU/L
GGT	0.0 (0.0-0.0)	0 - 0.5 IU/L
Sodium	151.1 (150.0-152.0)	149 - 157 mEQ/L
Potassium	4.4 (4.3-4.8)	3.7 - 5.4 mEQ/L
Chloride	115.7 (114.4-116.9)	115 - 125 mEQ/L

A:G - albumin:globulin ratio, ALP - alkaline phosphatase, ALT - *alanine transaminase,* AST - **aspartate aminotransferase**, GGT - gamma-glutamyl transferase

Table 4.8. Complete blood cell counts results at week 12 of treatment.

Clinical parameter	Median (IQR)	Reference interval
Hemoglobin	13.2 (12.3-13.9)	9.8 - 15.5 g/dL
Hematocrit	37.0 (35.0-40.0)	32 - 47%
MCV	44.0 (41.0-46.0)	40 - 52 fl
MCHC	35.0 (34.0-35.0)	32 - 36 g/dL
Reticulocytes	28.6 (21.0-41.4)	0 - 50 10^3/uL
Platelets	283 (171-388)	200 - 500 10^3/uL
Nucleated cell count	8.5 (6.7-12.5)	4.0 - 14.0 10^3/uL
Neutrophils	5.0 (3.6-7.7)	2 - 12 10^3/uL
Lymphocytes	2.8 (1.7-4.0)	1.5 - 6 10^3/uL
Monocytes	0.2 (0.1-0.3)	0 - 0.8 10^3/uL
Eosinophils	0.4 (0.2-0.8)	0 - 1.2 10^3/uL
Basophils	0.0 (0.0-0.0)	0 - 0.1 10^3/uL

MCV - mean corpuscular volume, MCHC - mean corpuscular hemoglobin concentration

Table 4.9. Serum biochemistry results at week 12 of treatment.

Clinical parameter	Median (IQR)	Reference interval
Glucose	95.0 (88.0-118.8)	68 - 140 mg/dL
BUN	26.0 (23.0-29.0)	18 - 35 mg/dL
Creatinine	1.1 (0.9-1.3)	0.8 - 2.4 mg/dL
Phosphorus	6.8 (5.6-7.9)	3 - 6 mg/dL
Calcium	10.0 (9.6-10.3)	9.2 - 11.1 mg/dL
Magnesium	2.2 (2.1-2.3)	2 - 2.7 mg/dL
Total protein	7.1 (6.8-7.3)	6.3 - 8 g/dL
Albumin	3.5 (3.3-3.7)	3.1 - 4.4 g/dL
Globulin	3.5 (3.2-4.0)	2.7 - 4.2 g/dL
A:G	1.0 (0.9-1.1)	0.8 - 1.6
Cholesterol	110.5 (91.0-124.3)	95 - 270 mg/dL
Creatinine kinase	181 (133-274)	60 - 350 IU/L
Total bilirubin	0.0 (0.0-0.0)	0 - 0.1 mg/dL
ALP	52.0 (35.0-69.5)	10 - 80 IU/L
ALT	47.0 (37.8-62.5)	30 - 140 IU/L
AST	22.0 (18.8-28.0)	15 - 45 IU/L
GGT	0.0 (0.0-0.0)	0 - 0.5 IU/L
Sodium	152.0 (150.0-154.0)	149 - 157 mEQ/L
Potassium	4.5 (4.1-4.8)	3.7 - 5.4 mEQ/L
Chloride	117.1 (114.6-118.8)	115 - 125 mEQ/L

A:G - albumin:globulin ratio, ALP - alkaline phosphatase, ALT - *alanine transaminase,* AST - **aspartate aminotransferase**, GGT - gamma-glutamyl transferase

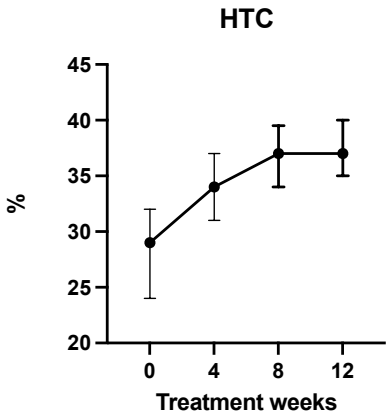


Figure 4.6. Hematocrit % over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

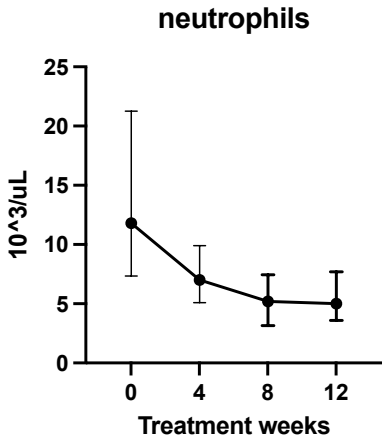


Figure 4.7. Neutrophil count over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

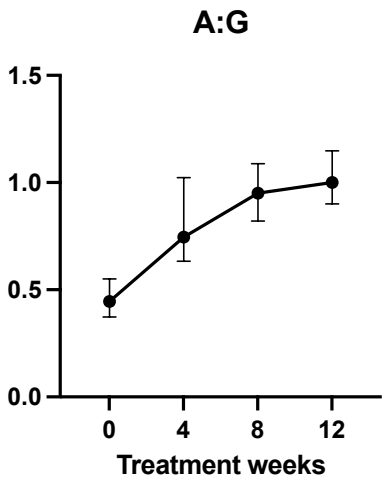


Figure 4.8. Albumin-to-globulin (A:G) ratio over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

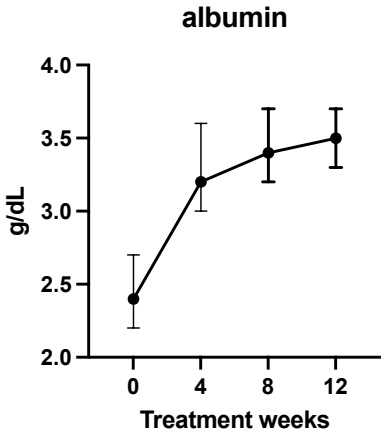


Figure 4.9. Albumin concentrations over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

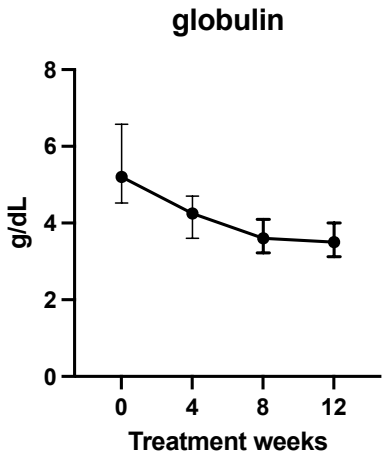


Figure 4.10. Globulin concentrations over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

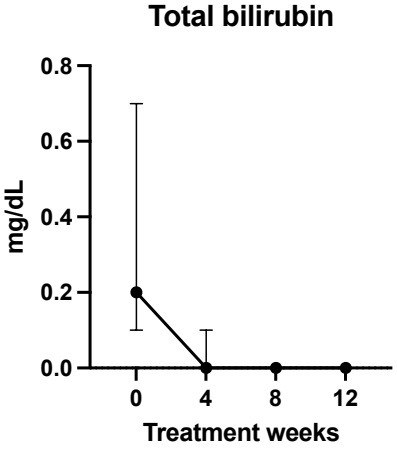


Figure 4.11. Total bilirubin concentrations over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

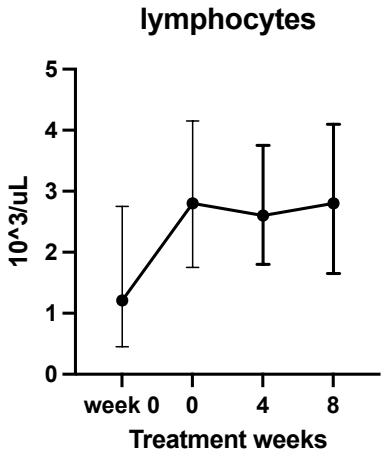


Figure 4.12. Lymphocyte count over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

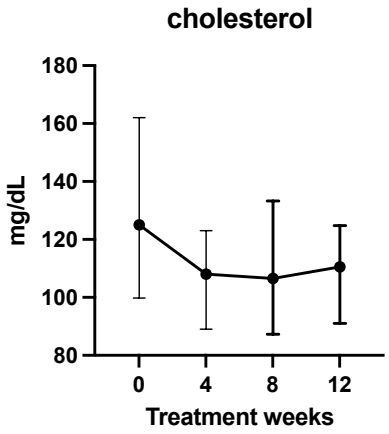


Figure 4.13. Cholesterol concentrations over the 12 weeks of treatment. Each dot represents the median and vertical line is the interquartile range.

Repeated urinalysis was performed in 18/29 (62%) of cats and showed median urine specific gravity of 1.051 (IQR 1.030-1.058), median pH of 6.5 (IQR 6.0-7.0) and median urine protein creatinine ratio of 0.10 (IQR 0.08-0.12). One cat developed worsening dyspnea on day 4 of treatment and needed repeated thoracocentesis, the fluid was negative on RT-PCR. Another cat had repeated abdominocentesis at week 4 due to persistent effusion and the fluid was negative on RT-PCR.

Treatment with immune stimulant vs placebo

The 41 effusive FIP cats were randomized into groups administer the LTC (0.1 ml/kg PO once weekly, 21 cats) vs placebo (0.1 ml/kg PO once weekly, 20 cats). A total of 31/41 (76%) cats survived (17/21 (81%) in LTC group and 14/20 (70%) in placebo group) to 6 months (3 months of therapy and 3 months of observation period) and 10/41 (24%) cats died or were euthanized. The median number of days in which the cats died or were euthanized was 4 (range 1-10). Of the 31 cats that survived, 17 (55%) cats were treated with LTC, and 14 (45%) cats were in the placebo group. Moreover, of the 31 cats that lived, 4 (13%) relapsed and required a secondary course of treatment, when signs referable to FIP (pyrexia, lethargy, and inappetence) recurred. All 4 cats commenced a second course of treatment using a median dosage of 22.4 mg/kg (IQR 21.9-23.1). The cats that relapsed, 3/4 were treated with LTC and 1/4 was in the placebo group, but the difference between the groups was not statistically significant $\chi^2(1, N = 35) = 0.753$, $p=0.443$

4.5 Discussion

In the healthy cat experiment, the most significant changes were seen in the PBMCs at week 2 post LTC administration, with a total of 406 significant DEGs. The top upregulated pathways include those related to protein synthesis, metabolism and a few immune activations. The downregulated gene sets can be categorized as growth factor and signaling related, as well as several antigen-presentation and immune-receptor pathways. However, no major interferon pathways were affected by the LTC immune

stimulant and it is possible that the dose of the LTC was not high enough and more studies are needed to evaluate the dosing of LTC and the effect on immune system of both healthy and cats with FIP.

In this prospective clinical trial with MPV as a first line treatment for 73 cats with naturally occurring FIP, MPV treatment resulted in clinical remission in 78% of the cats enrolled by the end of 6 months. Most cats that died or were euthanized did not survive over 4 days. The treatment with MPV was safe, with no adverse events noted that necessitated discontinuation of the medication but gastrointestinal side effects, mild and mostly transient increases in ALT, mild neutropenia as well as folded ear tips have been observed in this study and also reported previously (7, 9, 11).

Survival rates observed here were similar to the other studies using MPV as a first-line drug for FIP (7, 8, 11). The median dosage of molnupiravir was 13.8mg/kg and the maximum dosage was 21.4mg/kg PO q12h for 12 weeks which is similar to what has been reported previously as first-line drug for FIP (7, 8, 11); however a previous study reported with the nucleoside analogue GS-441524 that 6 week treatment with antivirals may be sufficient for FIP and additional studies evaluating shorter courses of treatment are needed to better define the optimal treatment protocol (32). The survival rate with MPV in this study and other studies is very similar to the ~80-85% success in treatment with GS-441524 and studies have not reported that GS-441524 and MPV have similar effects and safety in cats with FIP (8, 33). However, the mechanism of action of MPV includes introduction of mutations into the viral genome during replication, which raises concern for genetic selection of antiviral resistant strains of FIPV (34). In our study, 12% of the cats relapsed and required a secondary course of treatment, when signs referable

to FIP (pyrexia, lethargy, and inappetence) recurred between 9 and 99 days after discontinuing treatment. All 7 cats commenced a second course of treatment using a higher dosage of MPV (median dosage of 22.4 mg/kg; IQR 21.8-24.8) and all achieved remission during this second course of treatment which makes resistance less likely as a cause of relapse in these cats, but further studies are needed to evaluate the risk of mutations and possible antiviral resistance with MPV in cats with FIP. Host mutagenicity is also a concern with MPV treatment, and long term safety studies monitoring for adverse outcomes and teratogenicity are needed to further assess the safety of MPV treatment in cats (35).

The signalment of the cats in the present study was similar to that of previously reported as most (74%) cats were young, <1 year, at diagnosis. The majority were Domestic shorthairs and longhairs (85%) and only 15% cats were pedigree cats, which is much lower than previously reported; this could be explained by lower number of pedigree cats in the USA (4, 36-40). The presenting clinical signs and physical findings were also consistent with other studies (4, 41, 42) with lethargy and hyporexia were frequently reported as non-specific clinical signs, and pyrexia occurred in around 58% of the cats. More cats (64%) were diagnosed with effusive FIP than non-effusive FIP (36%) and 14%) of cats had ocular involvement, 5% of cats presented with neurological involvement, and 3% of cats had both ocular and neurological involvement.

Rapid resolution of clinical signs associated with FIP was achieved in all cats that survived to 6 months, again consistent with previous studies (4, 42). All presenting clinicopathological abnormalities (e.g. anemia, hyperbilirubinemia, hyperglobulinemia, hypoalbuminemia and low A:G) normalized in the cats receiving MPV treatment but

cholesterol was the only parameter that decreased while on treatment and the reason for this is unclear but it is possible that some of these cats might be developing gastrointestinal disease post recovery from FIP given that several cats had changes in the gastrointestinal tract on ultrasound. The total bilirubin concentrations were higher in cats that died or were euthanized and high bilirubin concentrations have been previously reported in cats as poor prognostic indicator (43). There was a statistical increase in lymphocytes in the cats while on MPV treatment and this has been previously reported in cats being treated with GS-441524 (4, 44), which is likely an immune response in these cats as previous study that also reported lymphocytosis in cats on GS-441524 and performed PCR for Antigen Receptor Rearrangements analysis showed reactive lymphoid cell population (44) but further studies are needed to understand why cats on antiviral therapy develop lymphocytosis. Mild eosinophilia developed in few cats in this study was also reported in previous studies (4, 42) but it is unclear why some cats develop eosinophilia. Fecal examinations were not performed, thus co-infection with parasitic species cannot be excluded and dermatological problems such as allergies cannot be completely ruled out. In humans, increased eosinophil counts are thought to hold positive prognostic value in human patients with COVID-19 (45) and additional studied are needed to understand the significance of eosinophilia in cats receiving antivirals.

Out of the 31 effusive FIP cats that survived 55% cats were treated with LTC and 45% cats were in the placebo group possibly suggesting some benefit in the response to treatment. However, in the effusive FIP cats, more cats in the LTC group relapsed vs placebo group (4 vs 1 respectively) suggesting that LTC does not help in preventing relapses in FIP cats and could potentially be detrimental in effusive FIP treatment.

However, the difference between the groups in number of relapses was not statistically different. While there were some changes in the PBMC transcriptomics using this dose of the LTC, it is possible that the LTC dose was not high enough. More studies are needed to evaluate the effect of LTC both in effusive and in non-effusive FIP cases as previous studies suggested benefit of immune stimulants in FIP treatment (18, 21).

The main limitation of this study is the lack of a control group. Due to the nature of FIP being a lethal disease, it would not be ethical to withhold treatment for this disease. However, the MPV treatment can be compared to historical data as well as GS-441524 treatment. Moreover, RT-PCR was not positive in all cats but presumed diagnosis based on compatible history, signalment, clinical signs, bloodwork abnormalities and positive response to antiviral medications has been recommended by the Advisory Board for Cat Diseases (ABCD) (46). Another limitation of this study was that it was not blinded prospective study; however, blinding to the study drug was not determined to be in the patients' best interest, and an open-label trial was commenced. Future studies comparing MPV to other antiviral treatments should be performed in a randomized, blinded fashion for further evaluation of efficacy. Another limitation is that the first recheck was at 4 weeks and therefore it is not possible to asses the early responses and improvements in these cats with their bloodwork as all cats had marked improvements at the 4 weeks recheck.

4.6 Conclusions

In conclusion, MPV administered at 10 to 20 mg/kg orally q12h for 12 weeks is well tolerated and an effective treatment (78% of cats achieved remission) for all forms of naturally occurring FIP. The risk for relapse is low (~12%) and cats that relapse seem

to respond well to secondary course of treatment at higher dose of MOV. The medication is well tolerated without observation of any adverse events that necessitated drug discontinuation. Additional studies are needed to determine if shorter MPV treatment would be appropriate for different forms of FIP to optimize duration of treatment.

References:

1. Pedersen NC. A review of feline infectious peritonitis virus infection: 1963–2008. Journal of feline medicine and surgery. 2009;11(4):225-58.

2. Tsai H-Y, Chueh L-L, Lin C-N, Su B-L. Clinicopathological findings and disease staging of feline infectious peritonitis: 51 cases from 2003 to 2009 in Taiwan. Journal of Feline Medicine and Surgery. 2011;13(2):74-80.

3. Ritz S, Egberink H, Hartmann K. Effect of feline interferon-omega on the survival time and quality of life of cats with feline infectious peritonitis. Journal of veterinary internal medicine. 2007;21(6):1193-7.

4. Taylor SS, Coggins S, Barker EN, Gunn-Moore D, Jeevaratnam K, Norris JM, et al. Retrospective study and outcome of 307 cats with feline infectious peritonitis treated with legally sourced veterinary compounded preparations of remdesivir and GS-441524 (2020–2022). Journal of feline medicine and surgery. 2023;25(9):1098612X231194460.

5. Murphy B, Perron M, Murakami E, Bauer K, Park Y, Eckstrand C, et al. The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. Veterinary microbiology. 2018;219:226-33.

6. Pedersen NC, Perron M, Bannasch M, Montgomery E, Murakami E, Liepnieks M, et al. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. Journal of feline medicine and surgery. 2019;21(4):271-81.

7. Sase O. Molnupiravir treatment of 18 cats with feline infectious peritonitis: A case series. Journal of Veterinary Internal Medicine. 2023;37(5):1876-80.

8. Reagan KL, Brostoff T, Pires J, Rose A, Castillo D, Murphy BG. Open label clinical trial of orally administered molnupiravir as a first-line treatment for naturally occurring effusive feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2024;38(6):3087-94.

9. Roy M, Jacque N, Novicoff W, Li E, Negash R, Evans SJ. Unlicensed molnupiravir is an effective rescue treatment following failure of unlicensed GS-441524-like therapy for cats with suspected feline infectious peritonitis. Pathogens. 2022;11(10):1209.

10. Cook S, Wittenburg L, Yan VC, Theil JH, Castillo D, Reagan KL, et al. An optimized bioassay for screening combined anticoronaviral compounds for efficacy against feline infectious peritonitis virus with pharmacokinetic analyses of GS-441524, remdesivir, and molnupiravir in cats. Viruses. 2022;14(11):2429.

11. Clark T, Coggins S, Korman R, King J, Malik R. Treatment of feline infectious peritonitis in cats with molnupiravir: clinical observations and outcomes for 54 cases. Australian Veterinary Journal. 2025.

12. de Groot-Mijnes JD, van Dun JM, van der Most RG, de Groot RJ. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. Journal of virology. 2005;79(2):1036-44.

13. Kipar A, Meli M. Feline infectious peritonitis: still an enigma? Veterinary pathology. 2014;51(2):505-26.

14. Kahlon J, Kemp M, Yawei N, Carpenter R, Shannon W, McAnalley B. In vitro evaluation of the synergistic antiviral effects of acemannan in combination with azidothymidine and acyclovir. Molecular biotherapy. 1991;3(4):214-23.

15. Lanford RE, Guerra B, Lee H, Averett DR, Pfeiffer B, Chavez D, et al. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly (i)-poly (c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. Journal of virology. 2003;77(2):1092-104.

16. Zheng B-J, Chan K-W, Lin Y-P, Zhao G-Y, Chan C, Zhang H-J, et al. Delayed antiviral plus immunomodulator treatment still reduces mortality in mice infected by high inoculum of influenza A/H5N1 virus. Proceedings of the National Academy of Sciences. 2008;105(23):8091-6.

17. Kuritz TK, TN, US), inventorMethods and compositions for modulation of innate immunity. USA2018.

18. Legendre AM, Kuritz T, Galyon G, Baylor VM, Heidel RE. Polyprenyl immunostimulant treatment of cats with presumptive non-effusive feline infectious peritonitis in a field study. Frontiers in Veterinary Science. 2017;4:7.

19. Legendre AM, Bartges JW. Effect of Polyprenyl Immunostimulant on the survival times of three cats with the dry form of feline infectious peritonitis. Journal of feline medicine and surgery. 2009;11(8):624-6.

20. Legendre AM, Kuritz T, Heidel RE, Baylor VM. Polyprenyl immunostimulant in Feline rhinotracheitis: Randomized Placebo-controlled experimental and Field safety studies. Frontiers in Veterinary Science. 2017;4:24.

21. Černá P, Ayoob A, Baylor C, Champagne E, Hazanow S, Heidel RE, et al. Retrospective survival analysis of cats with feline infectious peritonitis treated with polyprenyl immunostimulant that survived over 365 days. Pathogens. 2022;11(8):881.

22. Wheat W, Chow L, Coy J, Contreras E, Lappin M, Dow S. Activation of upper respiratory tract mucosal innate immune responses in cats by liposomal toll-like receptor ligand complexes delivered topically. Journal of veterinary internal medicine. 2019;33(2):838-45.

23. Lappin M, Wotman K, Chow L, Williams M, Hawley J, Dow S. Nanoparticle ocular immunotherapy for herpesvirus surface eye infections evaluated in cat infection model. Plos one. 2023;18(1):e0279462.

24. Contreras ET, Olea-Popelka F, Wheat W, Dow S, Hawley J, Lappin MR. Evaluation of liposome toll-like receptor ligand complexes for non-specific mucosal immunoprotection from feline herpesvirus-1 infection. Journal of veterinary internal medicine. 2019;33(2):831-7.

25. Veir JK, Lappin MR, Dow SW. Evaluation of a novel immunotherapy for treatment of chronic rhinitis in cats. Journal of feline medicine and surgery. 2006;8(6):400-11.

26. Cerna P, Dow S, Wheat W, Chow L, Hawley J, Lappin MR. Comparison of Antiviral Immune Responses in Healthy Cats Induced by Two Immune Therapeutics. Pathogens. 2024;13(7):602.

27. Boulder UoC. 2025 [cited 2025 May 24th]. Available from: https://curc.readthedocs.io/en/latest/clusters/alpine/alpine-hardware.html.

28. Partek. Partek Flow™ (Version 11.0) [Computer software], Illumina company (2024); [cited 2025 May 25th]. Available from: https://www.illumina.com/products/by-type/informatics-products/partek-flow.html.

29. Murphy BG, Castillo D, Neely N, Kol A, Brostoff T, Grant CK, et al. Serologic, virologic and pathologic features of cats with naturally occurring feline infectious peritonitis enrolled in antiviral clinical trials. Viruses. 2024;16(3):462.

30. (IRIS) IRIS. Iris Stage Guidelines 2025 [Available from: https://www.iris-kidney.com/iris-guidelines-1.

31. Ettinger SJ, Feldman EC. Textbook of Veterinary Internal Medicine-eBook: Textbook of Veterinary Internal Medicine-eBook: Elsevier health sciences; 2010.

32. Zuzzi-Krebitz A-M, Buchta K, Bergmann M, Krentz D, Zwicklbauer K, Dorsch R, et al. Short treatment of 42 days with oral GS-441524 results in equal efficacy as the recommended 84-day treatment in cats suffering from feline infectious peritonitis with effusion—A prospective randomized controlled study. Viruses. 2024;16(7):1144.

33. Sase O, Iwami T, Sasaki T, Sano T. GS-441524 and molnupiravir are similarly effective for the treatment of cats with feline infectious peritonitis. Frontiers in Veterinary Science. 2024;11:1422408.

34. Sanderson T, Hisner R, Donovan-Banfield Ia, Hartman H, Løchen A, Peacock TP, et al. A molnupiravir-associated mutational signature in global SARS-CoV-2 genomes. Nature. 2023;623(7987):594-600.

35. Waters MD, Warren S, Hughes C, Lewis P, Zhang F. Human genetic risk of treatment with antiviral nucleoside analog drugs that induce lethal mutagenesis: the special case of molnupiravir. Environmental and molecular mutagenesis. 2022;63(1):37-63.

36. Pesteanu-Somogyi LD, Radzai C, Pressler BM. Prevalence of feline infectious peritonitis in specific cat breeds. Journal of feline medicine and surgery. 2006;8(1):1-5.

37. Robison R, Holzworth J, Gilmore C. Naturally occurring feline infectious peritonitis: signs and clinical diagnosis. Journal of the American Veterinary Medical Association. 1971;158(6):981-6.

38. Rohrbach BW, Legendre AM, Baldwin CA, Lein DH, Reed WM, Wilson RB. Epidemiology of feline infectious peritonitis among cats examined at veterinary medical teaching hospitals. Journal of the American Veterinary Medical Association. 2001;218(7):1111-5.

39. Worthing KA, Wigney DI, Dhand NK, Fawcett A, McDonagh P, Malik R, et al. Risk factors for feline infectious peritonitis in Australian cats. Journal of Feline Medicine and Surgery. 2012;14(6):405-12.

40. Norris J, Bosward K, White J, Baral R, Catt M, Malik R. Clinicopathological findings associated with feline infectious peritonitis in Sydney, Australia: 42 cases (1990–2002). Australian Veterinary Journal. 2005;83(11):666-73.

41. Riemer F, Kuehner KA, Ritz S, Sauter-Louis C, Hartmann K. Clinical and laboratory features of cats with feline infectious peritonitis–a retrospective study of 231 confirmed cases (2000–2010). Journal of feline medicine and surgery. 2016;18(4):348-56.

42. Coggins SJ, Norris JM, Malik R, Govendir M, Hall EJ, Kimble B, et al. Outcomes of treatment of cats with feline infectious peritonitis using parenterally administered remdesivir, with or without transition to orally administered GS-441524. Journal of Veterinary Internal Medicine. 2023;37(5):1772-83.

43. Goto S, Kamiyoshi T, Iwasaki R. Predictive factors associated with short-term mortality in cats with feline infectious peritonitis treated with remdesivir or GS-441524 or both. Journal of Veterinary Internal Medicine. 2025;39(1):e17249.

44. Krentz D, Zenger K, Alberer M, Felten S, Bergmann M, Dorsch R, et al. Curing cats with feline infectious peritonitis with an oral multi-component drug containing GS-441524. Viruses. 2021;13(11):2228.

45. Mateos Gonzalez M, Sierra Gonzalo E, Casado Lopez I, Arnalich Fernandez F, Beato Perez JL, Monge Monge D, et al. The prognostic value of eosinophil recovery in COVID-19: a multicentre, retrospective cohort study on patients hospitalised in Spanish hospitals. Journal of clinical medicine. 2021;10(2):305.

46. (ABCD) ABoCD. 2024 [Available from: https://www.abcdcatsvets.org/wp-content/uploads/2024/12/FIP_diagnostic_tool_November-2024.pdf.

Chapter 5: IMMUNE-MEDIATED HEMOLYTIC ANEMIA IN CATS WITH FELINE INFECTIOUS PERITONITIS[3]

5.1 Summary

Feline infectious peritonitis (FIP) is caused by mutated feline coronaviruses. Immune-mediated hemolytic anemia (IMHA) arises due to immune-mediated erythrocyte destruction and can be non-associative or associative with diseases such as FIP. Records of cats with FIP were reviewed to find those with associative IMHA based on exclusion of other causes of anemia and a positive saline agglutination test and/or Coombs test. The inclusion criteria were met for 45 cats (26 cats with effusive and 19 with non-effusive FIP). Median hematocrit was 18% (interquartile range [IQR] 13-20). Anemia was non-regenerative in 36 cats and regenerative in 5 cats, 4 cats had no reticulocyte count available. Concurrent thrombocytopenia was present in 18 cats. All 45 cats were treated with nucleoside analogs and 44 cats with glucocorticoids; in 5 cats, glucocorticoids were added after starting antiviral treatment due to persistent anemia. Median follow-up was 72 days (IQR 14-246); at the time of last follow-up 33 cats had survived while 12 had died or were euthanized. For the 33 cats that survived, FIP and IMHA remission occurred in 17, FIP remission was achieved but IMHA relapsed in 3 cats (in one of these IMHA relapsed twice), FIP relapsed without IMHA in 2 cats, and both FIP and IMHA relapsed in 1 cat. In 9 cats the antiviral and glucocorticoid treatment is still ongoing at the time of the publication. Although FIP is likely an uncommon cause of associative IMHA, as more cats

[3] This chapter includes the complete published manuscript: Černá, P.; Knies, M.; Assink, M.; Evans, S.; Tasker, S.; Gunn-Moore, D.A.; Hartmann, K.; Buchta, K.; Taylor, S.; Meunier, S.; et al. Immune-Mediated Hemolytic Anemia in Cats with Feline Infectious Peritonitis. Pathogens 2025, 14, 660.

with FIP are treated with antiviral therapy, it is important to consider IMHA as a possible cause of anemia in cats with FIP.

5.2 Introduction

Feline infectious peritonitis (FIP) is a disease caused by feline coronavirus (FCoV), although the pathogenesis is still not fully understood (1). Recent studies have reported the use of antiviral drugs like the nucleoside analogs GS-441524 and molnupiravir, to successfully treat FIP (2-8). Immune-mediated hemolytic anemia (IMHA) occurs when the body mounts an immune response against antigens expressed on the surface of erythrocytes (9). In animals with IMHA, the production of antibodies results in the destruction of normal erythrocytes by inappropriate activation of the complement cascade, antibody-dependent cytotoxicity, and/or facilitated phagocytosis in the liver and spleen, resulting in severe anemia. In cats, IMHA can either be non-associative (primary) or be associative (secondary) to an underlying infectious, inflammatory, or neoplastic process (10, 11). Several infections have been documented in cats to cause associative IMHA, including feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), *Leishmania infantum*, *Mycoplasma gatae*, *Mycoplasma haemofelis, Babesia felis* and FIP (10-17). One study also reported 1 FIP and IMHA cat to have co-infection with FeLV (14). While most cats with FIP were euthanized in the past; more cats are now being treated with antiviral drugs. As a result of this, new disease processes that could be associated with FIP are being recognized; for example, IMHA, myocarditis or other cardiac changes, and other comorbidities (18, 19). A case report described a 9-month-old cat diagnosed with FIP that developed left sided congestive heart failure secondary to hypertrophic cardiomyopathy (HCM) phenotype 4 days after initial diagnosis (19).

Humans with SARS-CoV-2 infections present with a spectrum of clinical presentations and complications, with associative IMHA being increasingly recognized in patients, either while infected or shortly after infection (20). It has been proposed that SARS-CoV-2 infection could lead to hemolytic anemia directly through cytopathic injury or indirectly through induction of auto-antibodies (21). The objective of this study was to retrospectively report cases of cats with FIP that presented with concurrent IMHA, including the clinical signs, response to treatment and prognosis.

5.3 Materials and methods

Case inclusion

To be included in this study, the FIP diagnosis had to be either 1) confirmed based on positive immunohistochemistry (IHC) for FCoV antigen or positive reverse transcription polymerase chain reaction (RT-PCR) for FCoV RNA as previously reported (22) , or 2) presumed based on compatible history, signalment, clinical signs, bloodwork abnormalities and positive response to antiviral medications as described by the Advisory Board for Cat Diseases (ABCD) (23). The IMHA diagnosis was made according to the ACVIM consensus statement on the diagnosis of IMHA in dogs and cats (24) by a positive saline agglutination test (persistent microscopic or macroscopic autoagglutination after washing) or, if the saline agglutination test was negative, by positive direct antiglobulin test (DAT, also known as Coombs test). Tests for other infectious causes of anemia were performed according to the standard testing (*e.g.,* FeLV antigen, FIV antibody testing, and Hemotropic *Mycoplasma* spp. (hemoplasma) polymerase chain reaction [PCR]).

The participating clinics were contacted and asked to review medical records for cats meeting the entry criteria. The following data were recorded for each case: signalment; clinical examination findings; form/type of FIP; results of CBC, serum biochemical profile; and whether the cat was positive by DAT or agglutination in saline or both and point of care FeLV antigen and FIV antibody testing. Hemoplasma spp. testing, any cytologic or histologic examinations; findings from thoracic and abdominal imaging; blood type and records of blood product transfusion, and the immunosuppressive drugs or other treatments (e.g. antimicrobials) administered were recorded if available. Follow up information was provided for the cats that included survival (at least 2 weeks after starting antiviral therapy) and response to antiviral and/or glucocorticoid therapy. Relapse was defined for FIP as return of clinical signs or clinicopathological abnormalities when stopping antiviral therapy. Relapse of IMHA was defined as return of clinical signs or clinicopathological abnormalities when stopping or tapering glucocorticoid therapy.

Statistical analysis

All analyses for descriptive statistical analysis were conducted using statistical software (Excel, Microsoft, Redmond, Washington, USA). Variables were assessed for normality by visual assessment of histograms and use of the Shapiro–Wilks test.

5.4 Results

Signalment

A total of 45 cats with FIP met the inclusion criteria for this study from veterinary hospitals in the following countries: Unites States, United Kingdom, The Netherlands, Germany and Switzerland. The median age was 1.2 years (interquartile range [IQR] 0.5-

2.9). There were 34 (76%) males (22 neutered and 12 intact) and 11 females (7 spayed females and 4 intact). Most cats were domestic shorthair (23; 51%) or longhair (1) and there were 21 (47%) pedigree cats (8 British Shorthairs, 5 Maine Coons, 2 Devon Rexes, 2 Siberian, 1 Ragdoll, 1 Sacred Birman, 1 Turkish Angora and 1 Ragdoll/Siamese cross).

Clinical findings

The FIP was effusive in 26 (58%) cats and non-effusive in 19 (42%) cats; 8 cats had ocular involvement, 8 had neurological involvement and 3 had both ocular and neurological involvement. The most common clinical signs were lethargy (34 cats; 76%), hyporexia (29 cats; 64%), enlarged abdomen (11 cats), weight loss (9), vomiting (3), diarrhea (3) and pica (2). Reported ocular signs were uveitis (9 cats) and anisocoria (2). Among neurological signs, the most common sign was ataxia (4 cats), seizures (2), paresis (2), tremors (2), hind limb weakness (2) and generalized weakness (1). Other clinical signs included lack of appropriate weight gain (1 cat), behavioral changes (1), polyuria in (1) and upper respiratory tract signs (1). On physical examination, fever was reported in 22 (49%) cats, pale mucous membranes in 14 cats and jaundice in 14 cats. Dehydration was reported in 13 cats and a fluid wave was palpable in 8 cats. A heart murmur was auscultated in 7 cats and 1 cat was reported to have lymphadenopathy.

Laboratory and diagnostic imaging results

The diagnosis of FIP was confirmed by RT-PCR in 31 (69%) cats and in 1 cat by both PCR and IHC. The other 13 cats had a presumptive diagnosis of FIP. All cats were either positive on saline agglutination (24; 53%) or Coombs test (13; 29%) or both (8;

18%). The titer for the Coombs test was available for 16 of 21 cats tested with a minimum titer of 1:16 to 1:512 for 15 cats and a 1:4 titer in 1 cat. The classification of cats by FIP diagnostic and anti-erythrocyte antibody methods is summarized in Table 5.1.

Table 5.1. Distribution of FIP and IMHA classification results

		IMHA testing positive		
FIP diagnosis		Saline agglutination	Coombs	Both
Confirmed	32	16	10	6
Presumed	13	8	3	2
Total	45	24	13	8

In most cats (40/45; 89%), IMHA was diagnosed at the time of diagnosing FIP; however, 5 cats were diagnosed with IMHA days later when the anemia was not resolving or was progressing despite antiviral therapy (8, 14, 16, 38 and 87 days after FIP diagnosis, respectively). Complete blood cell count was available for all 45 cats. The median hematocrit was 18% (IQR 13-20). However, since the blood samples had been assessed using different instruments with different reference intervals (RI), the degree of anemia was normalized to the percentage below the lower limit of the RI; the median value percentage below the lower limit of the RI was 42% (IQR 30-56). Anemia was non-regenerative in 36 (80%) cats and regenerative in 5 cats; in 4 cats, the reticulocyte count was not available. Concurrent thrombocytopenia was present in 18 (40%) cats, 25 (56%) cats had normal platelet count, and no platelet count was available in 2 cats. Neutrophilia was present in 12 cats; neutropenia was present in 7 cats and 26 (58%) cats had normal

neutrophils counts. Lymphocytosis was present in 4 cats, lymphopenia in 18 (40%) cats, and 23 (51%) cats had normal lymphocyte count. Other abnormalities reported based on microscopic blood smear examination were hypochromasia in 9 cats, anisocytosis in 5 cats, polychromasia in 2 cats and ghost cells in 1 cat. Two cats initially presented with hematocrit at the low end of normal RI but progressed to have anemia within several days of starting antiviral therapy.

Serum biochemistry profiles were available for all 45 cats. Albumin was low in 28 (62%) cats and normal in 17 cats. Hyperglobulinemia was reported in 29 (64%) cats and 16 cats had normal globulin levels. Of the cats positive on saline agglutination, hyperglobulinemia was present in 14/24 (58%), hyperglobulinemia was present in 9/13 (69%) cats that tested positive on DAT alone and in 5/8 (63%) of cats that were positive on both. The median albumin to globulin ration (A:G) was 0.3 (IQR 0.2-0.4). Hyperbilirubinemia was present in 34 (76%) cats and other abnormalities reported were abnormal liver enzymes with increased ALT in 10 (22%) cats and increased ALP in 1 (2%) cat. All cats were tested for FeLV antigen and FIV antibodies on point of care tests and 21 (47%) cats had hemoplasma PCR performed (Table 5.2). One cat was positive for FIV antibodies on point of care test and on Western Blot (this cat was also positive for '*Candidatus* Mycoplasma haemominutum'), and 1 cat each was positive for FeLV antigen, '*Candidatus* Mycoplasma turicensis' by PCR and *Anaplasma* spp. by PCR.

Table 5.2. Distribution of concurrent infectious agent testing results

		Infectious disease testing		
FIP diagnosis		FIV/FeLV negative	Hemoplasma performed	Hemoplasma negative
Confirmed	32	30	12	11
Presumed	13	13	9	8
Total	45	43	21	19

Thoracic radiographs were performed in 11 (24%) cats and showed pleural effusion (2 cats), lymphadenopathy (2), cardiomegaly (2) and pulmonary changes: bronchointerstitial (2), alveolar-interstitial (1), alveolar (1) and bronchial (1) patterns. There was increased pulmonary opacity (1), pericardial effusion (1). Normal findings were described in 1 cat.

Abdominal ultrasound was performed in 37 (82%) cats. The majority of cats (35/37; 95%) had more than one abnormality, with the most common findings being lymphadenopathy (31 cats; 84%), peritoneal effusion (26; 70%), renomegaly (10), splenomegaly (9), medullary rim sign (6), gall bladder thickening or edema (6), mottled spleen (5), heterogenous or hypoechoic liver (5), hyperechoic mesentery (5), thickened intestinal wall (4), hepatomegaly (3), splenic nodules (2), nodular pancreas (2), modular and edematous mesentery (1), enlarged pancreas (1), colonic mass (1) and pyelectasia (1).

Treatment and follow-up

All cats were treated with nucleoside analog antiviral medications; 39 (87%) with GS-441524 (GS), 3 cats with remdesivir and GS-441524, 2 with remdesivir, and 1 cat with molnupiravir. The dosage of remdesivir or GS-441524 was available for 38 (84%) cats with a median dosage of 15 mg/kg/day (IQR 15-20). In the cat that was treated with molnupiravir, the dosage was 16 mg/kg every 12 hours. In most cats (39/45; 87%), prednisolone was started at presentation or within the first week of therapy; however, in 5 cats, prednisolone was added later due to persistent anemia (8, 14, 16, 38 and 87 days, respectively). In 1 cat, the anemia resolved on GS therapy alone and prednisolone was not started (this was the cat with lowest Coombs titer of 1:4). The prednisolone dosage was available for 36/44 (82%) of the cats, with a median dosage of 1.8 mg/kg/day (IQR1.1-2.0). A blood transfusion was given to 23 (51%) cats without any complications.

Other medications that were administered were antimicrobials: amoxicillin clavulanate (9 cats), doxycycline (4), clindamycin (3), pradofloxacin (1), marbofloxacin (1), enrofloxacin (1), secondary immunosuppressive medications: cyclosporine (5) and mycophenolate (2). Additional supportive treatments such as antinausea/antiemetic medications, appetite stimulants and clopidogrel. None of the 3 cats positive for '*Ca.* M. haemominutum', '*Ca.* M. turicensis' and *Anaplasma* spp. were treated with antimicrobials.

Median follow-up was 72 days (IQR 14-246). At the time of latest follow-up, of the 45 cats, 33 (73%) cats had survived and 12 had died or were euthanized. Outcome was available for 31/33 cats that were alive (2 cats were lost to follow up and 1 cat that was euthanized 320 days following diagnosis due to worsening of anemia and not responding to immunosuppressive therapy). Complete remission of FIP and IMHA occurred in 17/32

(53%) cats. In 3 (9.4%) cats, FIP remission was achieved but IMHA relapsed and in 1 of these IMHA relapsed twice. Two (6.2%) cats relapsed with FIP. In 1 (3.1%) cat, both FIP and IMHA relapsed. In 9 (28.1%) cats, the antiviral and glucocorticoid treatment was still ongoing at the time of the publication.

5.5 Discussion

These 45 cats are the largest cohort of FIP cats with presumed evidence of associative IMHA published. It is also the first report of successful treatment of IMHA along with treatment of FIP. The median age of cats in this study was 1.2 years, consistent with the finding that FIP usually occurs in younger cats, but also because cats with IMHA have been reported to be mainly younger to middle aged cats (10, 15, 25, 26). The majority (76%) of cats affected were males, again consistent with previous reports of FIP as well as IMHA previously (10, 15, 25, 26). Almost half of the cats (47%) were purebred cats, with British Shorthair and Maine Coon cats being the most common. It has been reported previously that purebred cats are at higher risk for FIP and these two breeds are amongst the most commonly reported breeds (they are also amongst the most popular cat breeds at present), but no breed predisposition has been reported for IMHA (2, 27).

Associative IMHA was seen with both effusive and non-effusive FIP in this study, as well as with neurological and/or ocular involvement. It therefore appears that IMHA can occur with any form of FIP. The pathogenesis of IMHA in FIP is not known, but it is likely secondary to the severe systemic inflammation that is typical for FIP. On physical examination, the most notable signs reported in the cohort were fever, pale mucous membranes and jaundice.

Clinical, hematologic, and biochemical parameters were similar to those previously reported in cats with FIP and also in cats with IMHA. The median hematocrit was 18%, slightly higher than the mean hematocrits reported in cats with IMHA of 12-15% (15, 25, 28). Lymphocytosis was previously observed in 32% of cats with non-associative IMHA (15), while only 9% of cats in the current study had lymphocytosis at diagnosis. If there is a reduced prevalence of lymphocytosis when IMHA is associated with FIP, it could be because of the associative nature of the IMHA, occurring concurrently with FIP which often results in a lymphopenia (2, 29).

Anemia was non-regenerative in 80% of the cats at the initial evaluation, which was unexpected for a diagnosis of immune-mediated destruction. The lack of regeneration in associative IMHA could be explained by inflammatory disease or concurrent bone marrow disorders as approximately 50% of cats with FIP have non-specific reactive changes in their bone marrow at necropsy (30). Alternatively, it is also possible that the cats had regenerative IMHA but were in an early stage of disease and the bone marrow takes 3-5 days to respond to anemia (30). It is also possible that some cats had precursor-targeted immune mediated anemia (PIMA) caused by FIP which is characterized by persistent non-regenerative anemia secondary to ineffective erythropoiesis (31). Moreover, 40% of the cats had concurrent thrombocytopenia, which was previously reported in cats with IMHA and PIMA (15, 25, 32). In addition to immune-mediated destruction, thrombocytopenia may be caused by sequestration of platelets within the enlarged spleen of these cats, or consumption due to hypercoagulability progressing to disseminated intravascular coagulation (DIC). However, DIC was not

suspected in any of the cats in this study based on their clinical examination and diagnostic investigations.

On serum biochemistry, hyperbilirubinemia and hyperglobulinemia were frequently seen in the cats in this study. Hyperbilirubinemia was present in 76% of the cats and has been previously reported in 68% of cats with IMHA and in around 25% of cats with FIP (15, 25). In IMHA, this could be due to chronic stimulation of the immune system. Hyperbilirubinemia was present in 76% of cats and has been previously reported in around 25% of cats with FIP and around 68% of cats with IMHA (15, 25, 33). This is likely pre-hepatic due to increased breakdown of hemoglobin and/or hepatic due to the severe inflammation in the liver of these cats (increased ALT activity was reported in 22% of the cats in this study, which could reflect hepatic involvement in FIP and/or possible hepatic hypoxemia secondary to anemia). Hyperbilirubinemia has been reported as a poor prognostic indicator in cats with IMHA and FIP (15, 34). Increased ALT activity has been previously reported both in cats with FIP and IMHA as well (25, 29). Hyperglobulinemia was reported in 64% of the cats in this study which has been reported previously in cats with FIP [and also with IMHA (15, 25). In both FIP and IMHA, this could be due to chronic stimulation of the immune system.

Additional infectious agent testing reported co-infections in 4 cats, and it is possible that these infections may have contributed to their anemia and/or triggered associative IMHA as well as FIP; however, according to the ACVIM consensus statement on the diagnosis of IMHA in dogs and cats, there is very limited evidence that '*Ca.* M. hemominutum' or '*Ca.* M. turicensis' can cause associative IMHA in cats (24). Additional infections were unlikely to be the primary cause of the IMHA in the cats in this study as

they were only found in a small number of the cats and not deemed to be the cause of the IMHA in these cats; however, not all cats were tested for hemoplasmas in this study. Given the other abnormalities in the cats not tested for hemoplasmas, as well as the response to antiviral therapy and glucocorticoids, it is very likely that the IMHA was associative to FIP in these cats.

Abdominal ultrasound showed multiple abnormalities in 95% of the cats in which it was performed, with the most common findings being lymphadenopathy, peritoneal effusion, renomegaly and splenomegaly. Other studies have reported splenomegaly with non-associative IMHA; and 60% of cats in 1 study (28) and 42% of cats (with homogeneous parenchyma reported) in another study (25). Diffuse splenomegaly can be caused by inflammatory or infectious processes (*e.g.,* FIP, bartonellosis, toxoplasmosis), infiltration of abnormal cells (*e.g.,* neoplasia), increased extramedullary hematopoiesis, congestion, or hyperplastic splenomegaly (*e.g.,* related to hemolysis) (35).

At the time of publication, 73% of the 45 cats with FIP and associated IMHA survived, which is only slightly lower than in cats with FIP in which IMHA was not described (2, 8, 29). A mortality rate of 27% is similar to the rates of 24.0% in other studies of cats with IMHA (25). This could suggest that IMHA, not FIP, is the outcome-defining factor in these cats with both diseases.

This study has a number of limitations, the main one being that it is a retrospective multicentric study with some missing data or tests not being performed (*e.g.,* follow up information, diagnostic investigation results). There was also variation in the diagnostic imaging and infectious disease testing performed due to its multicentric nature. Therefore, blood samples were submitted to different laboratories which used different

methodologies and RIs, making direct comparisons challenging. This is why the study reports the percentage of cats below the RI for the hematocrit values, as well as describing the median and IQR to give indication of the dispersion of data. However, the multicenter design was necessary to obtain as many case reports as possible, and is a rare example of a worldwide scientific collaboration on a very severe disease to accumulate knowledge and help as many cats as possible.

Another limitation is that not all cats had their diagnosis of FIP confirmed by PCR or IHC; however, a presumptive diagnosis of FIP can be made based on signalment, clinical signs, and diagnostic investigations, as well as response to antiviral therapy.

There are also concerns about the utility of Coombs testing (DAT) and/or agglutination in saline in the diagnosis of IMHA. Single positive test results of these assays in people or cats may be related to factors other than development of antibodies against erythrocytes, including viral infections like human immunodeficiency virus and non-specific erythrocyte binding secondary to multiple causes of hyperglobulinemia (36-39). However, as cats with non-associative IMHA can also present with hyperglobulinemia, this is a general limitation for all cats with IMHA and this study's criteria follows the ACVIM consensus on diagnosis of IMHA (15, 24). That said, Coombs testing for the confirmation of IMHA is controversial in both dogs and cats mainly because it does not distinguish between non-associative and associative IMHA. Positive testing does not verify if there are antiautologous antigens or antibodies against red cell-attached xenoantigens, such as drugs or components of infectious agents or immune-complexed antibodies bound non-specifically to the surface of red blood cells. Another limitation of Coombs testing in a multicentric study is that different reagents may have been used for

different cases. However, false positives are rare; a study looking at Coombs testing for the validation for IMHA found that no healthy or sick nonanemic cats had positive Coombs test, and only 1 of the 56 cats (1.8%) with blood loss anemia or nonregenerative anemia due to renal failure, retrovirus infection or anemia of inflammatory disease had a positive Coombs test(25). Additionally, all of the 7 cats in the same study that were diagnosed with hemolysis not related to immunological processes, such as hypophosphatemia or Heinz body anemia, were Coombs test negative; this included 2 cats with FIP (25). However, it is not clear if cats the other anemic or healthy cats had hyperglobulinemia in this study. In another study, looking at Coombs testing in cats, only 3% of non-anemic cats were Coombs positive at 37°C and these cats both had high titred reactions (at both 4°C and 37°C) with polyvalent antiserum and anti-IgG and both were diagnosed with pancreatitis (26). Further studies are needed to develop better diagnostic testing for confirmation of IMHA in cats such as flow cytometry.

There were several other limitations. None of the cats had bone marrow analysis performed. This was likely due to the severity of the cats' clinical signs precluding more invasive procedures and/or a reluctance to perform this procedure on small patients; because of this bone marrow diseases could not be ruled out. However, IMHA was mostly diagnosed due to the presence of persistent agglutination and/or by the direct antiglobulin Coombs test, according to the ACVIM consensus statement on the diagnosis of IMHA in dogs and cats (24). Pyruvate kinase deficiency or increased osmotic fragility of erythrocytes as hereditary causes for hemolysis were very unlikely because of the positive Coombs test results obtained in many cats, and the breed predisposition as no Abyssinian and Somali cats were in this study. Additional causes of hemolysis (*e.g.,*

hypophosphatemia, Heinz body hemolytic anemia) were excluded by serum biochemistry and evaluation of blood smears. It was not possible to determine what effect the dosages and duration of treatments had on prognosis as different treatments and dosage regimens were involved. Lastly, some cats were lost to follow-up or still in treatment at the time of publication, further limiting survival time data.

5.6 Conclusions

In conclusion, this study is the first to describe a large cohort of cats with both FIP and associative IMHA which appeared to be more common than previously reported in cats with FIP. The findings of this study should ideally be confirmed in a prospective study which would permit assessment of a complete workup for concurrent infections, the efficacy of different treatment protocols, and tapering dosage regimens. Although FIP has been an uncommon cause of associative IMHA, as more cats with FIP are successfully treated with antiviral therapy, it is important to consider IMHA as a possible cause of anemia in cats with FIP.

References:

1. Pedersen NC. A review of feline infectious peritonitis virus infection: 1963–2008. Journal of feline medicine and surgery. 2009;11(4):225-58.

2. Taylor SS, Coggins S, Barker EN, Gunn-Moore D, Jeevaratnam K, Norris JM, et al. Retrospective study and outcome of 307 cats with feline infectious peritonitis treated with legally sourced veterinary compounded preparations of remdesivir and GS-441524 (2020–2022). Journal of feline medicine and surgery. 2023;25(9):1098612X231194460.

3. Murphy B, Perron M, Murakami E, Bauer K, Park Y, Eckstrand C, et al. The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. Veterinary microbiology. 2018;219:226-33.

4. Pedersen NC, Perron M, Bannasch M, Montgomery E, Murakami E, Liepnieks M, et al. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. Journal of feline medicine and surgery. 2019;21(4):271-81.

5. Sase O. Molnupiravir treatment of 18 cats with feline infectious peritonitis: A case series. Journal of Veterinary Internal Medicine. 2023;37(5):1876-80.

6. Reagan KL, Brostoff T, Pires J, Rose A, Castillo D, Murphy BG. Open label clinical trial of orally administered molnupiravir as a first-line treatment for naturally occurring effusive feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2024;38(6):3087-94.

7. Roy M, Jacque N, Novicoff W, Li E, Negash R, Evans SJ. Unlicensed molnupiravir is an effective rescue treatment following failure of unlicensed GS-441524-like therapy for cats with suspected feline infectious peritonitis. Pathogens. 2022;11(10):1209.

8. Zuzzi-Krebitz A-M, Buchta K, Bergmann M, Krentz D, Zwicklbauer K, Dorsch R, et al. Short treatment of 42 days with oral GS-441524 results in equal efficacy as the recommended 84-day treatment in cats suffering from feline infectious peritonitis with effusion—A prospective randomized controlled study. Viruses. 2024;16(7):1144.

9. Mitchell K, Kruth S. Immune-mediated hemolytic anemia and other regenerative anemias. Textbook of veterinary internal medicine. 2010:761-72.

10. Scott D, Schultz R, Post J, Bolton G, Baldwin C. Autoimmune hemolytic anemia in the cat. 1973.

11. Werner LL, Gorman NT. Immune-mediated disorders of cats. The veterinary clinics of North America Small animal practice. 1984;14(5):1039-64.

12. Maede Y, Hata R. Studies on feline haemobartonellosis. II. The mechanism of anemia produced by infection with Haemobartonella felis. 1975.

13. Naik R. Warm autoimmune hemolytic anemia. Hematology/Oncology Clinics. 2015;29(3):445-53.

14. Limlenglert P, Rungsipipat A, Pusoonthornthum R. Application of flow cytometry for early diagnosis and monitoring in cats with immune-mediated hemolytic anemia. The Thai Journal of Veterinary Medicine. 2011;41(2):185-92.

15. Swann J, Szladovits B, Glanemann B. Demographic characteristics, survival and prognostic factors for mortality in cats with primary immune-mediated hemolytic anemia. Journal of veterinary internal medicine. 2016;30(1):147-56.

16. SM N. Immune-mediated hemolytic anemia in cats referring to Veterinary Teaching Hospital of Tehran [2006-2007]. 2009.

17. Piek C, Dekker A, Junius G, Slappendel R, Teske E, editors. Idiopathic immune-mediated hemolytic anemia (IMHA) in cats: differentiation from secondary IMHA and outcome of treatment. 13th European College of Veterinary Internal Medicine Conference, Uppsala, Sweden; 2003.

18. Guarnieri C, Bertola L, Ferrari L, Quintavalla C, Corradi A, Di Lecce R. Myocarditis in an FIP-diseased cat with FCoV M1058L mutation: Clinical and pathological changes. Animals. 2024;14(11):1673.

19. Ernandes MA, Cantoni AM, Armando F, Corradi A, Ressel L, Tamborini A. Feline coronavirus-associated myocarditis in a domestic longhair cat. Journal of Feline Medicine and Surgery Open Reports. 2019;5(2):2055116919879256.

20. Al Khoufi E, Al-Muhainy B, Algadeeb K, Alsalman M. Autoimmune hemolytic anemia: a late presentation of post-COVID-19 syndrome. Oman Medical Journal. 2023;38(1):e467.

21. Al-Kuraishy HM, Al-Gareeb AI, Kaushik A, Kujawska M, Batiha GE-S. Hemolytic anemia in COVID-19. Annals of hematology. 2022;101(9):1887-95.

22. Murphy BG, Castillo D, Neely N, Kol A, Brostoff T, Grant CK, et al. Serologic, virologic and pathologic features of cats with naturally occurring feline infectious peritonitis enrolled in antiviral clinical trials. Viruses. 2024;16(3):462.

23. (ABCD) ABoCD. 2024 [Available from: https://www.abcdcatsvets.org/wp-content/uploads/2024/12/FIP_diagnostic_tool_November-2024.pdf.

24. Garden OA, Kidd L, Mexas AM, Chang YM, Jeffery U, Blois SL, et al. ACVIM consensus statement on the diagnosis of immune-mediated hemolytic anemia in dogs and cats. Journal of veterinary internal medicine. 2019;33(2):313-34.

25. Kohn B, Weingart C, Eckmann V, Ottenjann M, Leibold W. Primary immune-mediated hemolytic anemia in 19 cats: Diagnosis, therapy, and outcome (1998–2004). Journal of veterinary internal medicine. 2006;20(1):159-66.

26. Tasker S, Murray J, Knowles T, Day M. Coombs', haemoplasma and retrovirus testing in feline anaemia. Journal of Small Animal Practice. 2010;51(4):192-9.

27. Barua S, Sarkar S, Chenoweth K, Johnson C, Delmain D, Wang C. Insights on feline infectious peritonitis risk factors and sampling strategies from polymerase chain reaction analysis of feline coronavirus in large-scale nationwide submissions. Journal of the American Veterinary Medical Association. 2025;263(1):82-9.

28. Person J, Sicard M, Pellerin J. Les anemies hemolytiques auto-immunes chez le chat: etude clinique et immunopathologique de cinq cas. Revue de médecine vétérinaire. 1997;148(2).

29. Coggins SJ, Norris JM, Malik R, Govendir M, Hall EJ, Kimble B, et al. Outcomes of treatment of cats with feline infectious peritonitis using parenterally administered remdesivir, with or without transition to orally administered GS-441524. Journal of Veterinary Internal Medicine. 2023;37(5):1772-83.

30. Breuer W, Stahr K, Majzoub M, Hermanns W. Bone-marrow changes in infectious diseases and lymphohaemopoietic neoplasias in dogs and cats—a retrospective study. Journal of comparative pathology. 1998;119(1):57-66.

31. Maldonado-Moreno A, Seth M, Monti P, Miller R. Clinical findings, treatment and outcome in cats diagnosed with precursor-targeted immune-mediated anaemia in a referral hospital in the UK: 30 cases (2010–2021). Veterinary Record Open. 2023;10(2):e70.

32. Black V, Adamantos S, Barfield D, Tasker S. Feline non-regenerative immune-mediated anaemia: features and outcome in 15 cases. Journal of feline medicine and surgery. 2016;18(8):597-602.

33. Sparkes AH, Gruffydd-Jones TJ, Harbour DA. An appraisal of the value of laboratory tests in the diagnosis of feline infectious peritonitis. 1994.

34. Goto S, Kamiyoshi T, Iwasaki R. Predictive factors associated with short-term mortality in cats with feline infectious peritonitis treated with remdesivir or GS-441524 or both. Journal of Veterinary Internal Medicine. 2025;39(1):e17249.

35. Guillermo Couto C. A diagnostic approach to splenomegaly in cats and dogs. 1990.

36. Dunn J, Searcy G, Hirsch V. The diagnostic significance of a positive direct antiglobulin test in anemic cats. Canadian Journal of Comparative Medicine. 1984;48(4):349.

37. Parker V, Tormey CA. The direct antiglobulin test: indications, interpretation, and pitfalls. Archives of pathology & laboratory medicine. 2017;141(2):305-10.

38. Heddle N, Kelton J, Turchyn K, Ali M. Hypergammaglobulinemia can be associated with a positive direct antiglobulin test, a nonreactive eluate, and no evidence of hemolysis. Transfusion. 1988;28(1):29-33.

39. De Angelis V, Biasinutto C, Pradella P, Vaccher E, Spina M, Tirelli U. Clinical significance of positive direct antiglobulin test in patients with HIV infection. Infection. 1994;22(2):92-5.

Chapter 6: ASSESSTMENT OF CARDIAC CHANGES IN CATS WITH FELINE INFECTIOUS PERITONITIS

6.1 Summary

Antiviral drugs such as GS-441524 and EIDD-2801 have been successful in treatment of feline infectious peritonitis (FIP). As more cats are being treated with these new antiviral drugs instead of being euthanized, new disease processes that may be associated with FIP are being recognized. These include the development of myocarditis and myocardial injury, which have also been documented in humans and animals with SARS-CoV-2. The objective of this study was to prospectively document the prevalence of cardiac changes in cats diagnosed with FIP using echocardiography and cardiac troponin I (cTnI). Twenty cats with naturally occurring FIP were enrolled. All cats were diagnosed with FIP and tested negative for concurrent infections associated with myocarditis by testing serum for FIV antibodies, FeLV antigen, *Bartonella* spp. IgG, and *Toxoplasma gondii* IgG and IgM as well as by testing blood for nucleic acids of *Bartonella* spp. and SARS-COV-2. Each cat underwent an echocardiographic examination. All echocardiographic measurements were normalized to patient body weight. The left ventricular posterior wall from right parasternal long axis and short axis was thickened in 55% (11/20) and 25% (5/20) of cats, respectively. It was more common for LVPW to be thickened compared to the interventricular septum in both short and long axis. The short axis left atrium to aortic root ratio measurement was increased in 15% (3/20) of cats and the left atrium in long axis was not increased in any cat. Pleural effusion was present in 40% (8/20) of cats and pericardial effusion in 25% (5/20) of cats, although a cardiac cause

was not identified for these effusions. The median cTnI at initial evaluation was 0.37 ng/ml (IQR 0.20-0.83). Recheck cTnI was performed at 12 weeks following antiviral therapy in 6 cats that initially had elevated cTnI; in all 6 cats, cTnI normalized to <0.20 ng/ml. Cats with FIP can present with elevations in cTnI and ventricular wall thickening, which are suggestive of myocarditis and/or myocardial injury.

6.2 Introduction

Feline infectious peritonitis (FIP) is a disease caused by feline coronaviruses (FCoV) and despite marked efforts and many theories, the pathogenesis of FIP is still not fully understood (1). Left untreated, FIP is almost always fatal, with most cats succumbing within weeks to months of diagnosis (2, 3). Recent studies have reported the use of drugs like the nucleoside analog GS-441524 and molnupiravir as a successful treatment of FIP (4-9). As more cats are being treated with these new antiviral drugs instead of being euthanized as in the past, new disease processes that have may be associated with FIP are being recognized. A recent case report documented a 9-month-old cat diagnosed with FIP that developed left sided congestive heart failure secondary to hypertrophic cardiomyopathy (HCM) phenotype 4 days after initial diagnosis (10). Necropsy performed in this cat confirmed pyogranulomatous lesions affecting multiple organs, including the myocardium. Immunohistochemistry performed on the myocardium revealed feline coronavirus-positive macrophages without evidence of myofiber disarray as typically seen in primary HCM (10). A post-mortem diagnosis of myocarditis associated with feline coronavirus (FCoV) was made. In another study evaluating the efficacy of the nucleoside analog GS-441524 for the treatment of FIP, 2 out of 31 cats being treated died

of cardiac related causes (6). One cat had severe bi-atrial enlargement without increased wall thickness on echocardiogram; this cat was euthanized but no necropsy was performed (6). The second cat in this study was treated for effusive FIP, but was euthanized 4 weeks after; necropsy confirmed the appearance of congenital hypertrophic cardiomyopathy, although results of histologic evaluation of the heart was not mentioned in the study (6). No gross or histologic lesions in the abdomen, chest, eyes brain, or spine associated with FIP were identified in this patient (6). Another case report described fibrinous epicarditis caused by FIP in a three-year-old cat with inflammatory infiltrate observed in the epicardium, composed mainly of macrophages, plasmocytes and lymphocytes; immunohistochemistry was positive for feline coronavirus (11). Recently, there was a reported case of pyogranuloma found in the myocardium in a cat with heart disease; histopathology confirmed myocarditis/myocardial necrosis associated with the presence of *S* gene-mutated FCoV (M1058L biotype) (12).

A study comparing myocardial transcription of cytokines and remodeling enzymes in cats with HCM and cats without cardiac disease, which included 18 cats with FIP, reported the highest myocardial marker transcriptions in cats with multicentric lymphoma, FIP and HCM, followed by diseases likely associated with a systemic inflammatory state (13). Inflammatory marker transcription predominated in the myocardium of cats with systemic inflammatory disease (13). A different study evaluating cytokine production in tissue samples from cats who died of FIP, showed that pyrogenic cytokines IL-6 and TNF-a were upregulated in cardiomyocytes (13). Although the heart was not directly affected by FIP lesions in any of these cases, the upregulation of these cytokines suggested that cardiomyocytes likely played a role in the systemic inflammation caused by FCoV (14).

Apart from the limited reported cases above, a clear association between myocarditis and FIP has not been documented, which is likely in part due to previously limited treatment options. Feline coronavirus shares similarities with another coronavirus, SARS-CoV-2, the etiologic agent for the COVID-19 pandemic. Myocardial injury in patients with SARS-CoV-2 has also been documented (15-17). One study in humans identified up to 27.8% cases of COVID-19 having evidence of myocardial injury based on elevated cardiac troponin levels, which has been supported by definitive diagnosis of myocarditis via endomyocardial biopsy and autopsy (15). Humans with SARS-CoV-2 associated myocarditis often have echocardiographic evidence of systolic dysfunction, myocardial edema on cardiac magnetic resonance imaging, and electrocardiographic changes including ST segment changes and ventricular tachycardia (15, 16). COVID-19 patients with suspected or confirmed myocarditis have a high (27%) mortality rate (15, 16). At this time, there is no evidence that myocarditis is directly caused by SARS-CoV-2 in humans, but it is suspected that myocardial inflammation is secondary to a generalized inflammatory reaction induced by cytokines (15, 16). Additionally, myocarditis has now been documented in a limited number dogs and cats diagnosed with SARS-CoV-2 in the United Kingdom (18).

The primary aim of the study was to determine the prevalence of cardiac changes in cats with FIP including elevations in cardiac biomarkers (Troponin I) and changes in echocardiographic parameters (including increased ventricular wall thickness, atrial enlargement, and/or pericardial effusion).The secondary aim was to re-evaluate patients with FIP who exhibit cardiac changes or evidence of myocardial injury following treatment with antiviral drugs for FIP.

6.3 Materials and methods

Study design

This prospective clinical study included cats with an antemortem diagnosis of FIP. Each cat was systemically evaluated with a complete blood count, serum biochemical panel, and urinalysis. Diagnosis of FIP was made if patients were showing clinical signs consistent with FIP (eg. body cavity effusion, lethargy, hyporexia and pyrexia), having clinicopathological changes consistent with FIP (eg. hyperglobulinemia and low albumin:globulin ratio) and/or positive RT-PCR.

Echocardiograms were performed with the same ultrasound unit (Philips EPIQ 7C, Philips Healthcare, Andover, MA, USA) equipped with multiple cardiac transducers by a cardiology resident under supervision of a board-certified cardiologist. If necessary, cats were administered sedation with butorphanol 0.25 mg/kg IV. Simultaneous ECG was performed based on patient compliance. Choice of carrier frequency and techniques to optimize two-dimensional image quality were at the discretion of the operator (ER and RE). Data were captured digitally for measurements at an off-cart workstation with analysis software (TomTec Imaging Systems GmbH, Chicago, IL). The same imaging protocol was used for each echocardiographic examination by each operator. Care was taken to optimize spectral Doppler recordings and the cursor was aligned as parallel as possible to blood flow. Sweep speed (≥100 mm/sec), baseline, scale, and gain were all manually adjusted to optimize the velocity spectra. All echocardiographic measurements were performed by the individual who acquired the images. All measurements were performed from three consecutive cycles, and averaged with the highest quality images or spectra were selected for measurement. Maximum left ventricular (LV) wall thickness

was also recorded for both the interventricular septum (IVS) and LV posterior wall (LVPW).

Imaging planes were acquired based on standard imaging planes for cats (19). 2-D images were obtained for all cats in this study as optimal lineup for M-mode imaging was challenging in many juvenile cats. The interventricular septum (IVS) was measured between the distance from the blood-tissue interface to the blood-tissue interface (19-21). For the LVPW, the distance from the blood-tissue interface to the tissue-pericardial interface (19-21). IVS and LVPW measurements were obtained from both the right parasternal long-axis 4-chamber view (Lx) and right parasternal short-axis view (Sx) (19-21). Maximum end-diastolic measurements of the IVS, LVPW, and LV dimension (LVDd) were obtained at end diastole (19-21). Maximum left atrial wall diameter (LADmax) was measured from the Lx view in the cranial-caudal dimension at end-systole, just prior to mitral valve opening (19-21). The short axis left atrial to aorta diameter (LA:Ao) was obtained from the Sx view at the base timed to aortic valve closure (19-21). Maximum right ventricular outflow tract velocity ($Vmax_{PV}$) and left ventricular outflow tract velocity ($Vmax_{AV}$) were obtained using continuous wave Doppler from the Sx view at the base (optimized for the RV outflow tract) and the left apical 5 chamber view respectively.[1] Color flow doppler was utilized to identify areas of turbulence, dynamic obstructions to blood flow, and valve regurgitations. Systolic anterior motion of the mitral valve or chordae tendinae was identified as anterior motion of the mitral valve/chordae toward the IVS in systole, identified on either 2-D or M-Mode examination. The presence of cavitary effusions was noted and described subjectively in quantity (19).

Given the wide variety in ages and sizes of cats utilized in this study with naturally

occurring FIP, standard reference ranges for cats were not applicable (21). Instead, reference intervals derived from allometric scaling of healthy cats was utilized (21). For allometric scaling, the 2-D measurement (in mm) was divided by the patient's body weight raised to the corresponding scaling exponent. The allometric scaling exponents used are as follows: MaxLAD = 0.177, IVSd,Lx = 0.158, LVDd,Lx = 0.202, LVPWd,Lx = 0.278, IVSd,Sx = 0.158, LVDd,Sx = 0.247, and LVPWd,Sx = 0.212 (21). Each measurement was defined as being enlarged/thickened if the allometrically scaled echocardiographic measurement exceeded the upper 97.5% prediction interval of the derived proportionality constant (21). The measurement was decreased/thin if it was below the 2.5% prediction interval of the proportionality constant (21). La:Ao>1.6 was defined as increased in size (19, 20).

All cats had blood obtained at the initial visit for complete blood cell count and serum chemistry. Leftover serum was spun and frozen at -80°C until analysis. High-Sensitivity Cardiac Troponin I (cTnI) was measured to assess evidence of ongoing myocardial injury. In cats, a cutoff value for cTnI of <0.06 ng/mL has been established as screening for cats (22). Each cat was screened for other infectious diseases previously shown to be associated with myocarditis in cats, including FeLV (antigen), FIV (antibodies), *Bartonella* spp. (antibodies and PCR on blood), *Toxoplasma gondii* (IgG and IgM antibodies), and SARS-COV-2 (PCR on whole blood) (23-25). For cats that had increased cTnI on initial testing (cTnI > 0.2 ng/ml), repeat cTnI was performed 12 weeks following treatment with an antiviral therapy for FIP.

The study followed ethical guidelines and was approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol number 3965, approval

date December 2, 2022).

Statistical analysis

Data were recorded in Excel (Excel, Microsoft, Redmond, Washington, USA). Variables were assessed for normality by visual assessment of histograms and use of the Shapiro–Wilks test. Descriptive statistics were used to report the data. Data were reported as median (range) and interquartile range (IQR). For echocardiography, descriptive statistics were performed utilizing commercially available software (MedCalc Statistical Software, Ostend, Belgium). Normality testing was performed using the Shapiro-Wilk test. Mean and standard deviation is presented for normally distributed data; median and interquartile range is presented for non-normally distributed data; Categorical data was presented as proportions exceeding upper reference limits for both the average of three measurements and if a single measurement was increased.

6.4 Results

Study cohort

Twenty cats with FIP were recruited between November 2022 and September 2024. Of these, the majority were Domestic shorthairs (15/20; 74%) or longhairs (1/20; 11%) and only 4/20 (15%) cats were pedigree breeds: Maine Coon (1 cat), Sphynx (1), Bengal (1) and Siberian (1). There were 14/20 (53%) neutered males, 4/20 (37%) spayed females, 2/20 (5%) entire females. The majority (13/20; 74%) of cats were aged <2 year at diagnosis, the median age was 0.9 years (IQR 0.5-4.9) and the oldest cat enrolled in the study was 9.6 years old.

Clinical findings

Most cats (11/20; 64%) were diagnosed with effusive FIP: pleural effusion (5/20; 25%), peritoneal effusion (3/20; 15%), or both (3/20; 15%). Non-effusive FIP was diagnosed in 8/20 (36%) cats and 5/20 (14%) cats had ocular involvement and 3/20 (5%) cats had neurological involvement. Out of these cats, 13/20 (59%) had confirmed FIP by RT-PCR, while 7/20 (41%) cats had presumptive FIP with 5 (30%) cats PCR negative and in 2 (11%) cats no PCR was performed. Only 6/20 (%) cats presented with a heart murmur which ranged between grade I/VI to IV/VI. A total of 16/20 (80%) had blood pressure measured by Doppler according to ACVIM consensus statement on systemic hypertension in dogs and cats (26). The median systolic blood pressure was 97mmHg (IQR 82-151).

Infectious disease testing

All cats were negative for feline leukemia virus antigen, feline immunodeficiency virus antibody, and *D. immitis* antigen. All cats were negative for SARS-CoV-2 nucleic acids from whole blood by PCR and toxoplasma serology. None of the 20 cats were positive for *Bartonella* spp. in blood by PCR. Of the 18 cats tested for *Bartonella* spp antibodies, 12 were negative, 5 cats were seropositive at 1:64, and 1 cat was seropositive at 1:128.

Troponin measurements

The cTnI was increased in 11/20 (55%) of cats and the median value at initial evaluation was 0.37 ng/ml (IQR 0.20-0,83; upper reference range 0.20 ng/ml). Recheck cTnI was performed at 12 weeks following antiviral therapy in 10 cats with elevated values and the cTnI had normalized to <0.20 ng/ml in all.

Echocardiography results

Twenty cats had echocardiograms performed. The values for each echocardiographic variable (following allometric scaling, where applicable) are provided in Table 6.1. A total of 3 cats (15%) had LA:Ao that was increased while LADmax was not increased in any cat. It was more common for LVPW to be thickened compared to the IVS. For the average of three measurements, the LVPWd,Lx was increased in 6 (30%) cats and LVPWd,Sx increased in 4 (20%) of cats. For maximum LV wall thickness, the LVPWd,Lx was increased in 11 (55%) of cats and LVPWd,Sx increased in 5 (25%) of cats. The average IVSd was not increased in any cat and 1 cat had an maximum IVSd,Sx that was increased (Figure 6.1). 2 (10%) cats had a decreased LVIDd Lx and 1 (5%) cat had a decreased LVIDd Sx.

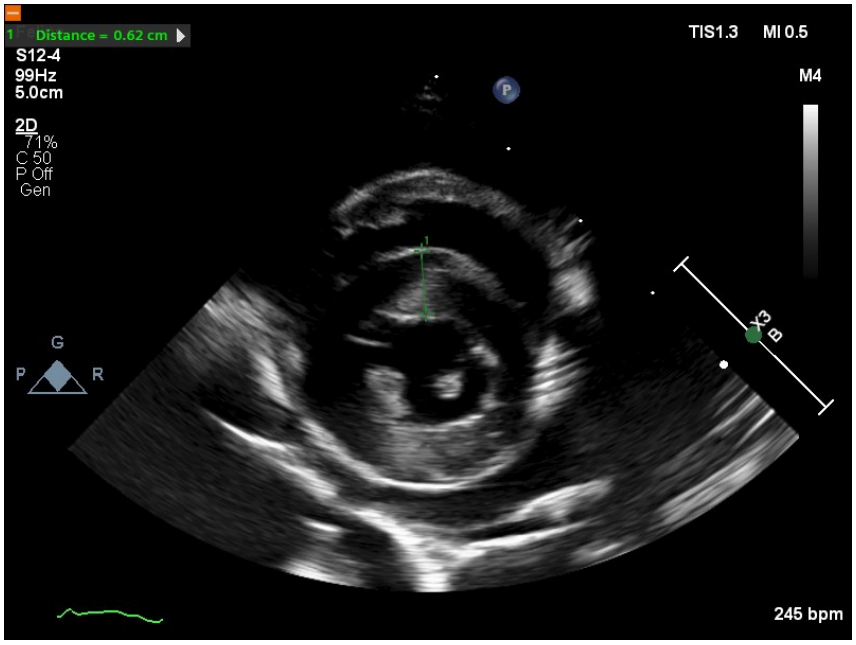


Figure 6.1. Right parasternal short-axis view at the level of the papillary muscle with an increased interventricular septal thickness and a moderate amount of pleural effusion.

There was pleural effusion present in 8 cats, which was characterized as moderate volume in 3 cats (Figure 6.2) and mild in the remaining 5 cats (all cats with mild effusion had echocardiogram performed post thoracocentesis). Pericardial effusion was present in 5 cats, which was moderate in volume in one cat and scant in the remainder; 2 cats had both pleural and pericardial effusion. A primary cardiac cause for the effusions was not identified in any cat. Four cats had mild mid-left ventricular obstruction, and 2 cats had systolic anterior chordal motion of the mitral valve. LVOT velocity did not exceed 2.1 m/s in any cat. Trace tricuspid regurgitation was seen in 6 cats, trace mitral regurgitation in 2 cats, trace aortic insufficiency in 1 cat, and dynamic right ventricular outflow tract obstruction in 2 cats.

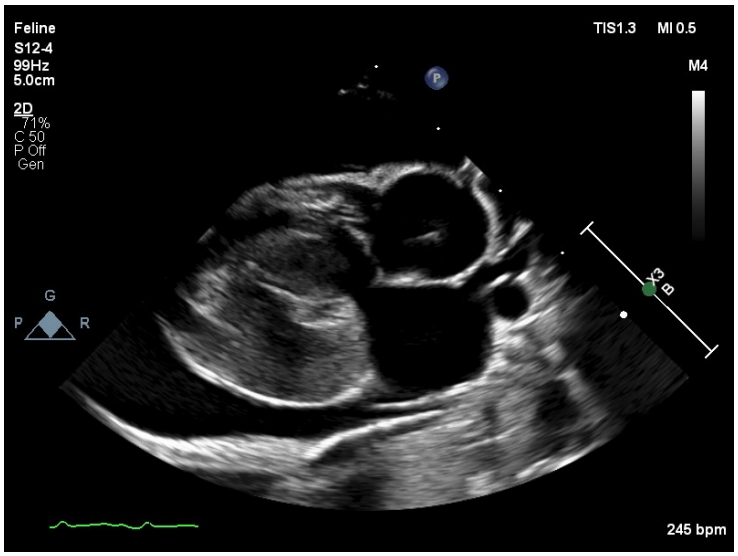


Figure 6.2. Right parasternal long-axis 4-chamber view including a moderate amount of pleural effusion.

Table 6.1. Descriptive statistics of echocardiographic variables and proportions of wall thickening/chamber in cats with FIP.

Echocardiographic variable	Mean	Upper reference limit[3]	Thickened/dilated (average value)	Thickened/dilated (single maximum value)
Heart Rate (bpm)	213.4 ± 30.0 bpm			
MaxLAD (mm/kg$^{0.177}$)	10.60 ± 1.58	13.65	0%	0%
LA:Ao	1.40 ± 0.18	1.6	15%	15%
IVSd,Lx (mm/kg$^{0.158}$)	3.33 (0.75)	4.93	0%	0%

LVIDd,Lx (mm/kg$^{0.202}$)	10.03 ± 1.64	13.93	0%	0%
LVFWd,Lx (mm/kg$^{0.177}$)	3.43 ± 0.44	3.51	30%	55%
IVSd,Sx (mm/kg$^{0.158}$)	3.57 ± 0.54	5.01	0%	5%
LVIDd,Sx (mm/kg$^{0.247}$)	10.00 ± 1.54	12.82	0%	0%
LVFWd, Sx (mm/kg$^{0.212}$)	3.45 (0.76)	3.97	20%	25%
VmaxPV (m/s)	0.98 ± 0.26			
VmaxAV (m/s)	1.21 ± 0.40			

Data summarized as mean (standard deviation) for normally distributed data and median (±IQR) for non-normally distributed data. The upper reference limit for allometrically scaled data is based on the URL (97.5) of the proportionality constant derived from healthy control cats. Abbreviations: MaxLAD, maximum long axis left atrial dimension; LA:Ao left atrium to aortic root ratio in short axis; IVSd,Lx, interventricular septal thickness in diastole, long-axis view; LVIDd,Lx, left ventricular internal diameter in diastole, long-axis view; LVFWd,Lx, left ventricular free wall thickness in diastole, long-axis view; IVSs,Lx, interventricular septal thickness in systole, long-axis view; LVIDs,Lx, left ventricular internal diameter in systole, long-axis view; LVFWs,Lx, left ventricular free wall thickness in systole, long-axis view; IVSd,Sx, interventricular septal thickness in diastole, short-axis view; LVIDd,Sx, left ventricular internal diameter in diastole, short-axis view; LVFWd,Sx, left ventricular free wall thickness in diastole, short-axis view; IVSs,Sx, interventricular septal thickness in systole, short-axis view; LVIDs,Sx, left ventricular internal diameter in systole, short-axis view; LVFWs,Sx, left ventricular free wall thickness in systole, short-axis view; PV, pulmonary valve; AV, aortic valve.

Treatment and follow up

All cats were treated with antiviral therapy. A total of 14/20 (70%) cats were treated with MPV (median dose 14.3mg/kg BID (IQR 12.2-17.4) for 12 weeks and the remaining 6/20 (30%) were treated with nucleoside analogue GS-441524 (doses were not available) for 12 weeks. A total of 17 cats (85%) were alive after 12 weeks of therapy and 3 cats died or were euthanized by that time point. The cats that died had increased troponin levels but, based on the post-mortem findings, it did not appear that myocarditis was the cause of death. Post-mortem examination was performed in all 3 cats and all three cats were reported to have mildly increased heart weight: 0.48%, 0.56%, and 0.82% of the body weight (normal 0.3-0.45%). Two cats had no significant histologic lesions in the heart. The third cat had patchy perivascular edema along with small numbers of circulating neutrophils and mononuclear cells and had positive immunoreactivity to FIP on immunophistochemistry (IHC) within infiltrating mononuclear cells throughout the subpleura and adjacent pulmonary parenchyma.

6.5 Discussion

Based on data collected in humans with SARS-CoV-2, documented myocarditis in a cat with FIP, and newly developing therapies for FIP that prolonged survival of these patients, further investigation of possible development of myocardial injury in cats with FIP was warranted. The goal of this prospective study was to evaluate echocardiographic, electrocardiographic, and cardiac biomarker changes in cats diagnosed with FIP to better understand the prevalence of cardiac changes associated with this disease.

Myocarditis is a form of myocardial disease characterized by the presence of

inflammation in response to physical, chemical and infectious agents. Reports of cats with infectious myocarditis caused by systemic diseases, such as protozoa (*Hepatozoon species, Toxoplasma gondii*), viruses (FIV), bacteria (*Bartonella* species) and, in some cases, opportunistic fungi have been described (23, 27-30). Clinical and laboratory findings associated with *Toxoplasma gondii* and *Bartonella* spp. associated myocarditis can be confused with those of non-effusive FIP (24, 31). In particular, *T. gondii*, *Bartonella* spp., and non-effusive FIP commonly share the findings of fever and hyperglobulinemia (32). As bartonellosis and toxoplasmosis are inexpensively treated with antibiotics, it was important to exclude these agents from the differential list before making an antemortem suspect diagnosis of coronavirus associated myocarditis.

All cats were retroviral negative and none of the cats included in the study had toxoplasmosis or bartonellosis; however, the diagnosis of *Bartonella* infection can be challenging. Culture of the bacterium is the gold standard method of diagnosing *Bartonella* infection and a combinational approach with pre-enrichment culture and PCR increases sensitivity (33). Sensitivity can also be increased by repeated blood cultures or PCR performed on several biological samples (blood, lymph node, oral swab, tissues) (34), which was not perfomed in this study and is a limitation. Some of the cats in this study had positive serology; however, serology is more useful for exclusion than for confirmation of the disease, because of the high negative predictive value but low positive predictive value (35). Because of the high prevalence of infection in healthy cats in endemic areas, a positive culture or PCR are not confirmatory of disease in cats with compatible signs, and other compatible diagnoses must be ruled out. All cats had FIP that was confirmed by positive RT-PCR for feline coronavirus or had clinical signs

consistent with FIP as well as response to antiviral therapy as recommended by the Advisory Board for Cat Diseases (ABCD) (36). None of these cats were treated with antibiotics and it is therefore possible to make suspect diagnosis of coronavirus associated myocarditis since they responded to antiviral therapy and the troponin levels normalized on antiviral therapy in all the cats that were retested. *Bartonella henselae* has been reported post COVID-19 in humans with lymphadenopathy following close contact with cats (37, 38) and more studies are needed to see if cats after recovery from FIP might be more prone to developing bartonellosis. All the cats in this study that survived are doing well and did not develop any signs of bartonellosis following the antiviral treatment while being in FIP remission.

There have been few reported cases showing the link between myocarditis and FIP. Feline coronavirus shares similarities with another coronavirus, SARS-CoV-2, the etiologic agent for the COVID-19 pandemic. Myocardial injury in patients with SARS-CoV-2 has been documented (15-17) and myocarditis has now been associated with SARS-CoV-2 in a limited number of dogs and cats in the United Kingdom (18). All cats in this study were tested for SARS-CoV-2 and were all negative and the cause of myocarditis by SARS-CoV-2 is therefore unlikely.

Given the wide variety in ages and sizes of cats utilized in this study with naturally occurring FIP, standard reference ranges for cats were not applicable (21). Instead, reference intervals derived from allometric scaling of healthy cats was utilized (21). In this study, 15% had LA:Ao that were increased while LADmax was not increased in any cat. It was more common for LVPW to be thickened compared to the IVS. For the average of three measurements, the LVPWd,Lx was increased in 30% cats and LVPWd,Sx

increased in 20% of cats. For maximum LV wall thickness, the LVPWd,Lx was increased in 55% of cats and LVPWd,Sx increased in 25% of cats. There was pleural effusion present in 8 cats, which was characterized as moderate volume in 3 cats and mild in the remaining 5 cats (all cats with mild effusion had echocardiogram performed post thoracocentesis). Pericardial effusion was present in 5 cats, which was moderate in volume in one cat and scant in the remainder but a primary cardiac cause for the effusions was not identified in any cat.

High-Sensitivity cTnI was measured to assess evidence of ongoing myocardial injury. In cats, a cutoff value for cTnI of <0.06 ng/mL has been established as screening for cats (22). Elevations in cTnI can be seen in cats with hypertrophic cardiomyopathy, although these elevations are generally mild in primary cardiac disease (<1.0 ng/mL) (22, 39). Higher elevations in cTnI are more suggestive of ongoing myocardial injury/myocarditis (39, 40).

The multifocal systemic inflammation caused by FIP infection suggests that chemotaxis and immune cells migration occur due to the inflammatory process to limit FIP spread throughout the body and the infection increases pro-inflammatory factors, such as tumor necrosis factor alpha (TNF-α) cytokine in infected macrophages and areas of inflammation, causing cell apoptosis (41). Myocarditis in these cats can be a consequence of viral spread throughout the body or secondary to the systemic inflammation. A previous study evaluating myocardial transcription of cytokines and remodeling enzymes in cats reported the highest myocardial marker transcriptions in cats with multicentric lymphoma, FIP and HCM and inflammatory marker transcription predominated in the myocardium of cats with systemic inflammatory disease (13).

Another study evaluating cytokine production in tissue samples from cats who died of FIP showed that pyrogenic cytokines IL-6 and TNF-a were upregulated in cardiomyocytes and in this study, the heart was not directly affected by FIP lesions in any of these cases, the upregulation of these cytokines suggested that cardiomyocytes likely played a role in the systemic inflammation caused by FCoV (14). There is however evidence that FIP virus can affect the heart with one case report describing fibrinous epicarditis caused by FIP in a three-year-old cat where inflammatory infiltrate was observed in the epicardium, composed mainly by macrophages, plasmocytes and lymphocytes and immunohistochemistry performed was positive for feline coronavirus (11). Another case reported pyogranuloma found in the myocardium in a cat with heart disease and the histopathological finding was the myocarditis/myocardial necrosis was associated with the presence of *S* gene-mutated FCoV (M1058L biotype) (12).

There were limitations to this study. Cats did not have repeated echocardiograms at the end of the study. Blood pressure measurements were missing in 20% of the cats at the initial visit unfortunately because cats had to be sedated. Moreover, not all cats had repeated troponin levels but in 50% of cats where troponin was measured at 12 weeks, the cTnI normalized to <0.20 ng/ml. Additional studies in measuring troponin levels in cats with FIP before and after antiviral therapy are needed. Not all cats had their diagnosis of FIP confirmed by PCR or IHC; however, a presumptive diagnosis of FIP can be made based on signalment, clinical signs, and diagnostic investigations, as well as response to antiviral therapy as recommended by the Advisory Board for Cat Diseases (ABCD) (36).

In regard to echocardiographic data, it is challenging to determine increased ventricular wall thickeness in this cohort of patients that were a wide variety of ages and

sizes. The cutoff for increased left ventricular wall thickeness is generally considered to be >6 mm, although this applies to a normal sized adult cat (20). For this reason, allometric scaling was utilized in determination of wall thickening and chamber enlargement. However, this method has not be utilized broadly for determination of wall thickness in cats, especially young or growing cats. Thus, false positives are possible (especially in underweight cats). This is a potential limitation in any data that requires allometric scaling. Additionally, increased LV wall thickness can be due to more than just myocarditis and can be associated with primary hypertrophic cardiomyopathy (especially in the older cats in this study), systemic hypertension, hyperthyroidism, other infiltrative diseases, and pseudohypertrophy. For the cats with increased LV wall thickness, it is not definitive that this is directly related to the presence of FIP. The clinical implication of increased LV wall thickeness is unclear in this study, especially since no cats demonstrated cardiovascular signs associated with FIP. Finally, it is not definitive that myocarditis would solely manifest with LV wall thickening and it is likely that some cats with myocarditis would not have LV walls that measure thickened on echocardiogram.

6.6 Conclusions

In conclusion, this study suggests that cats with FIP can present with elevations in cTnI and increased ventricular wall thickness, both of which could be suggestive of myocarditis and/or myocardial injury. Antiviral therapy may reduce ongoing myocarditis in patients with FIP, and additional studies are needed to further evaluate the involvement of the myocardium in FIP.

References:

1. Pedersen NC. A review of feline infectious peritonitis virus infection: 1963–2008. Journal of feline medicine and surgery. 2009;11(4):225-58.

2. Tsai H-Y, Chueh L-L, Lin C-N, Su B-L. Clinicopathological findings and disease staging of feline infectious peritonitis: 51 cases from 2003 to 2009 in Taiwan. Journal of Feline Medicine and Surgery. 2011;13(2):74-80.

3. Ritz S, Egberink H, Hartmann K. Effect of feline interferon-omega on the survival time and quality of life of cats with feline infectious peritonitis. Journal of veterinary internal medicine. 2007;21(6):1193-7.

4. Taylor SS, Coggins S, Barker EN, Gunn-Moore D, Jeevaratnam K, Norris JM, et al. Retrospective study and outcome of 307 cats with feline infectious peritonitis treated with legally sourced veterinary compounded preparations of remdesivir and GS-441524 (2020–2022). Journal of feline medicine and surgery. 2023;25(9):1098612X231194460.

5. Murphy B, Perron M, Murakami E, Bauer K, Park Y, Eckstrand C, et al. The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. Veterinary microbiology. 2018;219:226-33.

6. Pedersen NC, Perron M, Bannasch M, Montgomery E, Murakami E, Liepnieks M, et al. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. Journal of feline medicine and surgery. 2019;21(4):271-81.

7. Sase O. Molnupiravir treatment of 18 cats with feline infectious peritonitis: A case series. Journal of Veterinary Internal Medicine. 2023;37(5):1876-80.

8. Reagan KL, Brostoff T, Pires J, Rose A, Castillo D, Murphy BG. Open label clinical trial of orally administered molnupiravir as a first-line treatment for naturally occurring effusive feline infectious peritonitis. Journal of Veterinary Internal Medicine. 2024;38(6):3087-94.

9. Roy M, Jacque N, Novicoff W, Li E, Negash R, Evans SJ. Unlicensed molnupiravir is an effective rescue treatment following failure of unlicensed GS-441524-like therapy for cats with suspected feline infectious peritonitis. Pathogens. 2022;11(10):1209.

10. Ernandes MA, Cantoni AM, Armando F, Corradi A, Ressel L, Tamborini A. Feline coronavirus-associated myocarditis in a domestic longhair cat. Journal of Feline Medicine and Surgery Open Reports. 2019;5(2):2055116919879256.

11. Araujo G, Matta E, Lallo M, Machado G, Rocha P. Epicarditis in a cat caused by feline infectious peritonitis virus: case report. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2020;72(03):823-6.

12. Guarnieri C, Bertola L, Ferrari L, Quintavalla C, Corradi A, Di Lecce R. Myocarditis in an FIP-diseased cat with FCoV M1058L mutation: Clinical and pathological changes. Animals. 2024;14(11):1673.

13. Fonfara S, Kitz S, Monteith G, Hahn S, Kipar A. Myocardial transcription of inflammatory and remodeling markers in cats with hypertrophic cardiomyopathy and systemic diseases associated with an inflammatory phenotype. Research in Veterinary Science. 2021;136:484-94.

14. Malbon AJ, Fonfara S, Meli ML, Hahn S, Egberink H, Kipar A. Feline infectious peritonitis as a systemic inflammatory disease: Contribution of liver and heart to the pathogenesis. Viruses. 2019;11(12):1144.

15. Ho JS, Sia C-H, Chan MY, Lin W, Wong RC. Coronavirus-induced myocarditis: a meta-summary of cases. Heart & Lung. 2020;49(6):681-5.

16. Mele D, Flamigni F, Rapezzi C, Ferrari R. Myocarditis in COVID-19 patients: current problems. Internal and emergency medicine. 2021;16(5):1123-9.

17. Irabien-Ortiz Á, Carreras-Mora J, Sionis A, Pàmies J, Montiel J, Tauron M. Fulminant myocarditis due to COVID-19. Revista espanola de cardiologia (English Ed). 2020;73(6):503.

18. Ferasin L, Fritz M, Ferasin H, Becquart P, Corbet S, Ar Gouilh M, et al. Infection with SARS-CoV-2 variant B. 1.1. 7 detected in a group of dogs and cats with suspected myocarditis. Veterinary Record. 2021;189(9):no-no.

19. Boon JA. Veterinary echocardiography: John Wiley & Sons; 2011.

20. Luis Fuentes V, Abbott J, Chetboul V, Côté E, Fox PR, Häggström J, et al. ACVIM consensus statement guidelines for the classification, diagnosis, and management of cardiomyopathies in cats. Journal of veterinary internal medicine. 2020;34(3):1062-77.

21. Karsten S, Stephanie S, Vedat Y. Reference intervals and allometric scaling of two-dimensional echocardiographic measurements in 150 healthy cats. Journal of Veterinary Medical Science. 2017;79(11):1764-71.

22. Hertzsch S, Roos A, Wess G. Evaluation of a sensitive cardiac troponin I assay as a screening test for the diagnosis of hypertrophic cardiomyopathy in cats. Journal of veterinary internal medicine. 2019;33(3):1242-50.

23. Varanat M, Broadhurst J, Linder K, Maggi R, Breitschwerdt E. Identification of Bartonella henselae in 2 cats with pyogranulomatous myocarditis and diaphragmatic myositis. Veterinary Pathology. 2012;49(4):608-11.

24. Simpson KE, Devine BC, Gunn-Moore D. Suspected toxoplasma—Associated myocarditis in a cat. Journal of feline medicine and surgery. 2005;7(3):203-8.

25. Ware WA, Bonagura JD. Cardiovascular Disease in Companion Animals: Dog, Cat and Horse: CRC Press; 2021.

26. Acierno MJ, Brown S, Coleman AE, Jepson RE, Papich M, Stepien RL, et al. ACVIM consensus statement: guidelines for the identification, evaluation, and management of systemic hypertension in dogs and cats. Journal of Japanese association of veterinary nephrology and urology. 2020;12(1):30-49.

27. Donovan T, Balakrishnan N, Barbosa IC, McCoy T, Breitschwerdt E, Fox P. Bartonella spp. as a possible cause or cofactor of feline endomyocarditis–left ventricular endocardial fibrosis complex. Journal of Comparative Pathology. 2018;162:29-42.

28. Joseph J, Oxford E, Santilli R. Transient myocardial thickening in a Bartonella henselae–positive cat. Journal of Veterinary Cardiology. 2018;20(3):198-203.

29. Kegler K, Nufer U, Alic A, Posthaus H, Olias P, Basso W. Fatal infection with emerging apicomplexan parasite Hepatozoon silvestris in a domestic cat. Parasites & vectors. 2018;11:1-5.

30. Rolim VM, Casagrande RA, Wouters ATB, Driemeier D, Pavarini SP. Myocarditis caused by feline immunodeficiency virus in five cats with hypertrophic cardiomyopathy. Journal of Comparative Pathology. 2016;154(1):3.

31. Bradbury CA, Lappin MR. Evaluation of topical application of 10% imidacloprid–1% moxidectin to prevent Bartonella henselae transmission from cat fleas. Journal of the American Veterinary Medical Association. 2010;236(8):869-73.

32. Whittemore J, Hawley J, Radecki S, Steinberg J, Lappin M. B artonella Species Antibodies and Hyperglobulinemia in Privately Owned Cats. Journal of Veterinary Internal Medicine. 2012;26(3):639-44.

33. Duncan AW, Maggi RG, Breitschwerdt EB. A combined approach for the enhanced detection and isolation of Bartonella species in dog blood samples: pre-enrichment liquid culture followed by PCR and subculture onto agar plates. J Microbiol Methods. 2007;69(2):273-81.

34. Drummond MR, Lania BG, Diniz P, Gilioli R, Demolin DMR, Scorpio DG, et al. Improvement of Bartonella henselae DNA Detection in Cat Blood Samples by Combining Molecular and Culture Methods. J Clin Microbiol. 2018;56(5).

35. Pennisi MG, Marsilio F, Hartmann K, Lloret A, Addie D, Belák S, et al. Bartonella species infection in cats: ABCD guidelines on prevention and management. J Feline Med Surg. 2013;15(7):563-9.

36. (ABCD) ABoCD. 2024 [Available from: https://www.abcdcatsvets.org/wp-content/uploads/2024/12/FIP_diagnostic_tool_November-2024.pdf.

37. Dong Y, Alhaskawi A, Zou X, Zhou H, Ezzi SHA, Kota VG, et al. Post-COVID reactivation of latent Bartonella henselae infection: a case report and literature review. BMC Infect Dis. 2024;24(1):422.

38. Aubry A, Corvilain E, Ghelfenstein-Ferreira T, Camelena F, Meignin V, Berçot B, et al. Unmasking Bartonella henselae infection in the shadows of long COVID thanks to clinical metagenomics. Eur J Clin Microbiol Infect Dis. 2024;43(5):1025-9.

39. Langhorn R, Willesen J. Cardiac troponins in dogs and cats. Journal of veterinary internal medicine. 2016;30(1):36-50.

40. Gavazza A, Marchegiani A, Guerriero L, Turinelli V, Spaterna A, Mangiaterra S, et al. Updates on Laboratory Evaluation of Feline Cardiac Diseases. Veterinary Sciences. 2021;8(3):41.

41. Vermeulen BL, Devriendt B, Olyslaegers DA, Dedeurwaerder A, Desmarets LM, Favoreel HW, et al. Suppression of NK cells and regulatory T lymphocytes in cats naturally infected with feline infectious peritonitis virus. Veterinary microbiology. 2013;164(1-2):46-59.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Feline coronavirus (FCoV) belongs to the order *Nidovirales*; family *Coronaviridae*; subfamily *Coronavirinae*; genus *Alphacoronavirus*; species *Alphacoronavirus* 1. Feline coronavirus exist in two biotypes; feline enteric coronavirus (FECV), which mainly replicates in the enteric epithelial cells, and feline infectious peritonitis virus (FIPV), which results in lethal infection with efficient systemic replication in monocytes or macrophages (1). FIPV mainly replicates systemically; however, FECV has also been shown to replicate systemically in healthy cats and those without FIP (2-4). Efficient FCoV replication in activated monocytes and macrophages is a key event in FIP pathogenesis (5). Fortunately, only a small proportion of FCoV-infected cats go on to develop FIP and the virulence of the virus, the viral load and the cat's immune response determine whether FIP will develop (6-11). More studies looking into the genetics of the cats with FIP as well as the reasons why only some cats develop FIP while FCoV is ubiquitous virus present in cats worldwide are needed.

The effusive form of disease generally is believed to develop in cats with poor cell-mediated immune responses, and the non-effusive form develops in cats with partial cell-mediated immunity and effective immunotherapeutic agents for use in cats are needed to aid in the management of FIP. We compared two different immune stimulants for antiviral activity in cats: a TLR 2/6-activating compound polyprenyl immunostimulant; (PI) and a liposome Toll-like receptor 3/9 agonist complexes (LTC) to determine relative abilities to stimulate immune responses in vitro and to study the effects of treatment on immune responses in healthy cats. We have shown that both LTC and PI protocols induced

immune enhancing effects, suggesting a possible clinical use for the management of chronic infectious diseases like FIP. We concluded that activating the TLR 3 and 9 pathways (LTC) induced superior broad interferon production in vitro than the activation of the TLR 2 and 6 pathways (PI). In the second study with oral LTC in healthy cats no major interferon pathways were affected by the LTC immune stimulant and it is possible that the dose of the LTC was not high enough. More studies are needed to evaluate the dosing of LTC and the effect on immune system of both healthy and cats with FIP. Moreover, when LTC vs placebo was used in cats with effusive FIP, more cats in the LTC group relapsed vs placebo group (4 vs 1 respectively) suggesting that LTC does not help in preventing relapses in FIP cats and could potentially be detrimental in effusive FIP treatment. However, the difference between the groups in number of relapses was not statistically different and more studies are needed to evaluate the effect of LTC both in effusive and in non-effusive FIP cases as previous studies suggested benefit of immune stimulants in FIP treatment (12, 13).

We also determined the pharmacokinetics of molnupiravir in cats with naturally occurring FIP by measuring MPV and EIDD-1931 (β-D-N4-hydroxycytidine; NHC) serum levels in seven cats diagnosed with naturally occurring FIP treated with MPV. The mean NHC concentrations at all time points were at least 4 times the reported in vitro EC_{50} for feline coronavirus strains and twice daily dosing for seven days did not lead to accumulation of drug within the serum. Minimal accumulation of drug was seen in trough concentrations following twice-daily dosing for 7 days. These results supported the use of MPV in the treatment of FIP. Additional studies evaluating NHC triphosphate

concentrations in cats with FIP and therapeutic drug monitoring are also needed to improve success with therapy and develop individualized patient care.

We conducted a prospective, open-label longitudinal single-center clinical trial with MPV in cats with FIP. A total of 78% cats survived to 6 months and 22% cats died or were euthanized. We also found that the median total bilirubin concentrations were significantly different (p= 0.0007) between the survivors vs non-survivors. Relapses occurred in 12% of the cats and all achieved remission during a second course of treatment. No adverse effects necessitated discontinuation of the treatment. This study showed effectiveness of using 12 week therapy with MPV in treatment of FIP; however, other studies using the nucleoside analogue GS-441524 reported success with only a 6-week GS-441524 monotherapy in treatment of FIP (14). Further studies are needed to evaluate the effectiveness of shorter therapy of MPV in treatment of FIP as well as combination therapy with protease inhibitors such as nirmatrelvir and GC376 in cats with FIP.

While most cats with FIP were euthanized in the past; more cats are now being treated with antiviral drugs. As a result of this, new disease processes that could be associated with FIP are being recognized; for example, immune-mediated hemolytic anemia (IMHA), myocarditis or other cardiac changes, and other comorbidities and the goal of this study was to evaluate the development of IMHA and myocarditis in cats with FIP and its impact on survival and prognosis of these cats.

We have evaluated 45 cats with associative IMHA to FIP in a multicentric study. The median hematocrit for these cats was 18% and anemia was non-regenerative in 36/45 cats with concurrent thrombocytopenia was present in 18/45 cats. All 45 cats were treated with nucleoside analogs and 44 cats with glucocorticoids and at the time of last

follow-up 33 cats had survived while 12 had died or were euthanized. Although FIP is likely an uncommon cause of associative IMHA, as more cats with FIP are treated with antiviral therapy, it is important to consider IMHA as a possible cause of anemia in cats with FIP. More studies are needed to evaluate the causes of anemia in cats with FIP.

We documented the cardiac changes in cats diagnosed with FIP using echocardiography and cardiac troponin I (cTnI). Twenty cats diagnosed with FIP and tested negative for concurrent infections associated with myocarditis underwent an echocardiographic examination. The left ventricular posterior wall from right parasternal long axis and short axis was thickened in 55% (11/20) and 25% (5/20) of cats, respectively. The median cTnI at initial evaluation was 0.37 ng/ml (IQR 0.20-0.83) and recheck of cTnI performed at 12 weeks following antiviral therapy in 6 cats that initially had elevated cTnI showed normalized cTnI to <0.20 ng/ml. Cats with FIP can present with elevations in cTnI and ventricular wall thickening, which are suggestive of myocarditis and/or myocardial injury.

Feline infectious peritonitis is an inflammatory disease that can result in multiple comorbidities and additional studies looking into coagulation problems in these cats are required as well as studies evaluating critically ill cats with FIP that might be suffering from sepsis or severe inflammatory response syndrome in order to understand the pathogenesis of these comorbidities more as well as to improve the treatment success for these cats as currently about 15-20% of cats with FIP do not survive despite treatment with antiviral therapy (14-20).

The findings from this study provide important new insights into the use of antivirals and immune stimulants in FIP. In addition, this work provides an important understanding of possible comorbidities in cats with FIP that can be used to support future investigations.

References:

1. Gao Y-Y, Wang Q, Liang X-Y, Zhang S, Bao D, Zhao H, et al. An updated review of feline coronavirus: Mind the two biotypes. Virus research. 2023;326:199059.

2. Fish EJ, Diniz PPV, Juan Y-C, Bossong F, Collisson EW, Drechsler Y, et al. Cross-sectional quantitative RT-PCR study of feline coronavirus viremia and replication in peripheral blood of healthy shelter cats in Southern California. Journal of Feline Medicine and Surgery. 2018;20(4):295-301.

3. Kipar A, Baptiste K, Barth A, Reinacher M. Natural FCoV infection: cats with FIP exhibit significantly higher viral loads than healthy infected cats. Journal of feline medicine and surgery. 2006;8(1):69-72.

4. Meli M, Kipar A, Müller C, Jenal K, Gönczi E, Borel N, et al. High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. Journal of Feline Medicine and Surgery. 2004;6(2):69-81.

5. Malbon AJ, Michalopoulou E, Meli ML, Barker EN, Tasker S, Baptiste K, et al. Colony Stimulating Factors in Early Feline Infectious Peritonitis Virus Infection of Monocytes and in End Stage Feline Infectious Peritonitis; A Combined In Vivo And In Vitro Approach. Pathogens. 2020;9(11):893.

6. Dewerchin HL, Cornelissen E, Nauwynck HJ. Replication of feline coronaviruses in peripheral blood monocytes. Arch Virol. 2005;150(12):2483-500.

7. Rottier PJ, Nakamura K, Schellen P, Volders H, Haijema BJ. Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is

determined by mutations in the feline coronavirus spike protein. Journal of virology. 2005;79(22):14122-30.

8. Malbon AJ, Russo G, Burgener C, Barker EN, Meli ML, Tasker S, et al. The Effect of Natural Feline Coronavirus Infection on the Host Immune Response: A Whole-Transcriptome Analysis of the Mesenteric Lymph Nodes in Cats with and without Feline Infectious Peritonitis. Pathogens. 2020;9(7).

9. Hsieh LE, Chueh LL. Identification and genotyping of feline infectious peritonitis-associated single nucleotide polymorphisms in the feline interferon-γ gene. Vet Res. 2014;45(1):57.

10. Pedersen NC, Liu H, Gandolfi B, Lyons LA. The influence of age and genetics on natural resistance to experimentally induced feline infectious peritonitis. Vet Immunol Immunopathol. 2014;162(1-2):33-40.

11. Mustaffa-Kamal F, Liu H, Pedersen NC, Sparger EE. Characterization of antiviral T cell responses during primary and secondary challenge of laboratory cats with feline infectious peritonitis virus (FIPV). BMC veterinary research. 2019;15:1-15.

12. Černá P, Ayoob A, Baylor C, Champagne E, Hazanow S, Heidel RE, et al. Retrospective survival analysis of cats with feline infectious peritonitis treated with polyprenyl immunostimulant that survived over 365 days. Pathogens. 2022;11(8):881.

13. Legendre AM, Kuritz T, Galyon G, Baylor VM, Heidel RE. Polyprenyl immunostimulant treatment of cats with presumptive non-effusive feline infectious peritonitis in a field study. Frontiers in Veterinary Science. 2017;4:7.

14. Zuzzi-Krebitz A-M, Buchta K, Bergmann M, Krentz D, Zwicklbauer K, Dorsch R, et al. Short Treatment of 42 Days with Oral GS-441524 Results in Equal Efficacy as the

Recommended 84-Day Treatment in Cats Suffering from Feline Infectious Peritonitis with Effusion—A Prospective Randomized Controlled Study. Viruses. 2024;16(7):1144.

15. Taylor SS, Coggins S, Barker EN, Gunn-Moore D, Jeevaratnam K, Norris JM, et al. Retrospective study and outcome of 307 cats with feline infectious peritonitis treated with legally sourced veterinary compounded preparations of remdesivir and GS-441524 (2020–2022). Journal of Feline Medicine and Surgery. 2023;25(9):1098612X231194460.

16. Jones S, Novicoff W, Nadeau J, Evans S. Unlicensed GS-441524-Like Antiviral Therapy Can Be Effective for at-Home Treatment of Feline Infectious Peritonitis. Animals. 2021;11(8):2257.

17. Pedersen NC, Perron M, Bannasch M, Montgomery E, Murakami E, Liepnieks M, et al. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. Journal of feline medicine and surgery. 2019;21(4):271-81.

18. Coggins SJ, Norris JM, Malik R, Govendir M, Hall EJ, Kimble B, et al. Outcomes of treatment of cats with feline infectious peritonitis using parenterally administered remdesivir, with or without transition to orally administered GS-441524. Journal of Veterinary Internal Medicine. 2023;37(5):1772-83.

19. Green J, Syme H, Tayler S. Thirty-two cats with effusive or non-effusive feline infectious peritonitis treated with a combination of remdesivir and GS-441524. Journal of Veterinary Internal Medicine. 2023;n/a(n/a).

20. Zwicklbauer K, Krentz D, Bergmann M, Felten S, Dorsch R, Fischer A, et al. Long-term follow-up of cats in complete remission after treatment of feline infectious

peritonitis with oral GS-441524. Journal of Feline Medicine and Surgery. 2023;25(8):1098612X231183250.