Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Review

An update on feline infectious peritonitis: Virology and immunopathogenesis

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ARTICLE INFO

ABSTRACT

Feline infectious peritonitis (FIP) continues to be one of the most researched infectious diseases of cats. The relatively high mortality of FIP, especially for younger cats from catteries and shelters, should be reason enough to stimulate such intense interest. However, it is the complexity of the disease and the grudging manner in which it yields its secrets that most fascinate researchers. Feline leukemia virus infection was conquered in less than two decades and the mysteries of feline immunodeficiency virus were largely unraveled in several years. After a half century, FIP remains one of the last important infections of cats for which we have no single diagnostic test, no vaccine and no definitive explanations for how virus and host interact to cause disease. How can a ubiquitous and largely non-pathogenic enteric coronavirus transform into a highly lethal pathogen? What are the interactions between host and virus that determine both disease form (wet or dry) and outcome (death or resistance)? Why is it so difficult, and perhaps impossible, to develop a vaccine for FIP? What role do genetics play in disease susceptibility? This review will explore research conducted over the last 5 years that attempts to answer these and other questions. Although much has been learned about FIP in the last 5 years, the ultimate answers remain for yet more studies.

Introduction

Feline infectious peritonitis (FIP) continues to be a major killer of young cats and is one of the most researched infections of the species. Over 100 published articles concerning FIP have appeared in the world's literature since the last extensive review of the disease (Pedersen, 2009). Many of these and earlier publications have been covered in excellent clinical (Addie et al., 2009; Drechsler et al., 2011) and scientific (Myrrha et al., 2011; Kipar and Meli, 2014) reviews. The goal of the present review is not to retrace old tracks, but rather to present old and new discoveries on FIP in a different light.

FIP is a relatively new disease of cats and only became clinically significant in the late 1950s (Pedersen, 2009). It is uncertain where the first FIP virus (FIPV) came from, but one possibility is that feline coronaviruses originated within the century from another host species. Alternatively, it is possible that contemporary feline coronaviruses are genetic variants of a preexisting and somewhat different virus species that was less prone to undergo biotype conversion. Both scenarios are known to occur with coronaviruses. The intestinal form of coronavirus in pigs has been replaced worldwide by a much less pathogenic pneumotropic strain (Rasschaert et al., 1990), while several coronaviruses have entered human beings from other mammalian hosts (Chan et al., 2013).

We also cannot discount the important changes that occurred in the status and husbandry of cats in the period after World War II and how they affect FIP incidence. Cats have grown steadily in numbers as pets, and pedigreed cats and catteries have increased greatly in popularity. The post-war urban and suburban sprawl has greatly increased the numbers of feral and semi-feral (community) cats, and resulted in large numbers of kittens and cats coming into shelters and other foster/rescue organizations. An increasing proportion of our pet cats now come from these types of multi-cat environments, which can be stressful and favor fecal–oral transmission at a very young age. It should not be surprising that coronaviruses are now the most common pathogen identified in the feces of cats (Sabshin et al., 2012).

Coronaviruses have adapted themselves over thousands of years to virtually every species of mammals and birds, and are a common cause of transient enteritis and respiratory disease. Prior to the last decade, research interest in coronaviruses as pathogens has largely been limited to poultry, pigs, cattle and cats (Hagemeijer et al., 2012). However, with the emergence of severe acute respiratory syndrome (SARS) and Middle Eastern respiratory syndrome (MERS), interest in coronaviruses as pathogens has dramatically increased, along with levels of research funding. There are commonalities between what has been learned with animal coronaviruses and these

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emerging and sometimes fatal human viruses. They are continuously adapting themselves to new hosts, they readily recombine with closely related species to form new viruses, and they even change cell tropisms and virulence within the same host.

Although there is still no cure or sure prevention for FIP, a great deal has been learned about the disease in the last 5 years. The internal mutation theory, whereby a ubiquitous feline enteric coronavirus (FECV) mutates into a FIPV, has been reconfirmed and at least three specific mutations have now been associated with the FECV-to-FIPV biotype conversion. Much more is known about the interaction of enteric and FIP biotypes with their specific and very different host cells, and the radically different host response and clinical outcomes they provoke. A number of new reagents have been developed, allowing researchers to better understand these two biotypes and their diseases, and hopefully more are forthcoming. Nevertheless, FIP remains one of the most complex of all viral infections of cats.

Origin of feline infectious peritonitis viruses

Viral replication

It is not possible to understand the interrelationship of FECVs and FIPVs without understanding how enveloped, positive, single-stranded RNA viruses replicate (Hagemeijer et al., 2012). The genome of feline coronaviruses consists of > 29,000 nucleotides and 11 open reading frames (ORFs) encoding structural, non-structural and accessory genes. Coronaviruses attach to specific cell receptors through a complimentary ligand on the spike or surface (S) protein. Once attachment occurs, fusion with the cell membrane is dependent on a separate fusion domain and a fusion peptide comprising two heptad repeat regions (HR1 and HR2). The virus is then internalized and the single positive strand of RNA is released into the cytosol.

The 5' two thirds of the feline coronavirus genome consists of two ORFs, ORF 1a and ORF 1b. Ribosomes initiate translation at the beginning of ORF 1a and a proportion undergo frame shifting at the junction of ORF 1a and 1b, resulting in polyprotein pp1ab. Ribosomes that do not frame shift produce polyprotein pp1a. These polyproteins consist of approximately 16 non-structural proteins involved in proteolytic processing, genome replication and subgenomic mRNA synthesis.

The non-structural proteins of feline coronaviruses interact with components of the endoplasmic reticulum and Golgi apparatus to produce a replication–transcription complex. A RNA-dependent RNA polymerase makes negative sense copies of the genome, as well as subgenomic RNAs, which in turn serve as templates for the production of positive strand mRNAs. Only positive-stranded RNAs are capped and polyadenylated. The 3' polyadenylation and 5' cap structures mimic those of cellular mRNAs, enabling the virus to use the cell's own machinery for viral protein synthesis.

The nucleocapsid (N) protein plays an essential role in viral RNA and protein synthesis, and virion assembly (Verheije et al., 2010). Viruses undergoing assembly make their way to the cell surface within membrane structures, where they are released by exocytosis as mature virions (Almazán et al., 2004). In the process of maturation, the viral envelope also incorporates proteins acquired from various cell compartments. These added host constituents might aid survival in the face of host defenses.

This strategy to produce viral proteins from nested subgenomic mRNAs is highly efficient. However, like any process involving RNA polymerases, an error rate in the order of 1/10,000 nucleotides is expected. To minimize errors, the large genome encodes a number of non-structural proteins that ensure a higher fidelity of replication (Hagemeijer et al., 2012). Nonetheless, mutations do occur with some frequency. Chang et al. (2012) compared whole virus sequencing of 11 FIPV-FECV pairs and observed substitutions in at least 2963

nucleotides, representing 10% of the genome. Of these 2963 nucleotides, 1187 occurred in ORF 1ab, 1246 in ORF S (encoding the S glycoprotein), 248 in ORF 3abc, 22 in ORF E (encoding the small envelope protein), 42 in ORF M (encoding the integral membrane protein), 113 in ORF N (encoding the N protein) and 106 in ORF 7ab. Other types of mutations, leading to insertions, deletions and premature stop codons, as well as recombinants, have also been observed in vivo, both within and between hosts (Pedersen et al., 2009, 2012).

Phillips et al. (2013) studied the mutation rate of FIPV strain WSU-79-1146 in vitro after 1, 8 and 50 passages, followed by whole viral genome sequencing at each passage level. They observed 21 predicted amino acid changes in ORF 1a/1b during this period in culture, one predicted change in the S protein (which reverted back after additional passages), four changes in ORF 3c and one each in ORFs 3a, M, N and 7a, and calculated the mutation rate to be $5–6 \times 10^{-6}$ nucleotides/site/passage. This suggested that the genome was relatively stable in vitro and in the absence of host and environmental selection pressures.

Feline coronavirus mutations that do not have a negative impact on survival accumulate with time and can become dominant within micro- and macro-environments, such as catteries and geographically distinct regions (Pedersen et al., 2009). Such mutations can be used to track a specific coronavirus back to its most likely origin. Recombination also adds to genetic variation between coronaviruses and is common within clades, within the same cat and between cats (Pedersen et al., 2009, 2012). Recombination can even occur between related coronaviruses from different animal species. The type (serotype) II feline coronaviruses are an example of cross-species recombination that has occurred between the S gene region of type I feline coronaviruses and canine coronavirus.

The proportion of types I and II FECVs, and therefore types I and II FIPVs, varies across the world, although type I strains predominate. Duarte et al. (2009) studied the distribution of types I and II FIPVs in a Portuguese cat population using a reverse transcriptase (RT)-PCR assay that amplified the 3' end of the genome encompassing the region of feline/canine coronavirus S gene recombination. In cats with FIP, type I coronavirus was present in 79% and type II coronavirus in 3.5%, whereas the remaining 17.5% could not be typed. These viral sequences were further analyzed using a heteroduplex mobility assay, which detected quasi-species in 17% of samples. Phylogenetic analysis of type I sequences revealed high genetic diversity among Portuguese and previously characterized strains, while the tree for type II strains had higher genetic homogeneity than the tree for type I strains (Duarte et al., 2009).

The internal mutation theory

There is a general consensus that FIPVs arise by internal mutation from FECVs in the same environment (Pedersen et al., 2009, 2012; Harley et al., 2013). Except in unusual circumstances (Wang et al., 2013), the causative mutations occur independently within each cat and each FIPV strain has unique genetic features (Pedersen et al., 2009, 2012; Chang et al., 2012; Barker et al., 2013; Licitra et al., 2013). Currently, three different genes have been associated with the FECV-to-FIPV mutation or biotype conversion. Each mutation is a result of positive selection pressures, initially for a switch from enterocyte to monocyte/macrophage tropism, then ultimately for infection, replication and survival in peritoneal macrophages in the face of host immunity.

Mutations in the ORF 3c accessory gene

The ORF 3c accessory gene was the first gene to be implicated in FECV-to-FIPV conversion (Vennema et al., 1998), and these findings have been corroborated in subsequent studies (Poland et al., 1996; Chang et al., 2010; Pedersen et al., 2012). Two thirds or more

of FIPVs have ORF 3c mutations that lead to a truncated protein product, i.e. nucleotide deletions and insertions leading to frame shifting, and single nucleotide polymorphisms causing premature stop codons (Pedersen et al., 2009; Chang et al., 2012; Hsieh et al., 2013). The one third that do not have truncating mutations have an increased number of nucleotide changes, leading to an accumulation of non-synonymous amino acid changes in the 3' terminus of the gene (Pedersen et al., 2012). However, only truncating mutations have a known effect on host cell tropism. Two independent studies have confirmed the absolute requirement of an intact ORF 3c for replication of feline coronavirus in the intestinal epithelium (Chang et al., 2012; Pedersen et al., 2012). FIPVs with truncating ORF 3c mutations will not replicate in the gut epithelium, but will efficiently replicate in macrophages. FIPVs with non-truncating mutations in the 3' terminus of ORF 3c will replicate in the intestine, but they do not appear to be infectious under experimental conditions (Pedersen et al., 2012).

The function of the protein expressed by ORF 3c is unknown. Hsieh et al. (2013) transiently expressed ORF 3c in macrophage-like cells (Fcwf-4) and found that the protein was distributed mainly in the perinuclear region. They then infected Fcwf-4 cells expressing the intact ORF 3c protein with FIPV NTU156 (Lin et al., 2013), which has a functionally mutated ORF 3c gene. A significant inhibition of viral replication was observed relative to cells not expressing the ORF 3c protein; this inhibition did not involve autophagy. The authors concluded that loss of a functional ORF 3c protein in FIPVs could enhance viral replication in macrophage-like cells.

It might also be possible to infer functions to the ORF 3c gene product by studying similar and better studied genes in other coronaviruses. A GenBank BLAST[1] search demonstrated 30% genetic homology between feline coronavirus ORF 3c and SARS coronavirus ORF 3a (Pedersen et al., 2009). Although the genetic homology is low, the ORF 3c protein of feline coronavirus has a similar hydrophilicity profile to its own membrane (M) protein, and to the M and ORF 3a proteins of SARS coronavirus (Oostra et al., 2006).

The SARS ORF 3a protein has pro-apoptotic properties involving both cells death receptor and mitochondrial pathways (McBride and Fielding, 2012). Similar to FIPV ORF 3c, mutants of the SARS ORF 3a gene are frequently identified in diseased tissues and encode proteins with shorter N termini than found in wild type (i.e. bat coronavirus) forms (Tan et al., 2004). The most recent studies on the ORF 3a protein of SARS coronavirus indicate that it forms a cation-selective channel that is expressed in infected cells and is involved in release of virions (Schwarz et al., 2011). Is it also possible that mutations in FIPV ORF 3c protein inhibit apoptosis of infected macrophages? Programmed cell death is a major factor in immunity to viral infection of a cell.

Mutations in the S gene

The first mutation in the S gene that was associated with the FIPV biotype was reported by Chang et al. (2012), who sequenced and compared the complete genomes of 11 FIPV-FECV pairs. A single nucleotide change within the S gene encoding the fusion peptide was present in 124/129 (96%) FIPVs from cats with the wet and dry forms of FIP, but absent from 11 FECVs. A second mutation, two nucleotides away, was also strongly associated with FIPVs. Either or both of these two single base pair mutations were observed in most FIPVs, but not in FECVs. These mutations, designated M1058L and S1060A, caused minor changes in single amino acids within the S protein in FIPVs (i.e. methionine to leucine at position 1058 and serine to alanine at position 1060). The authors postulated that even

minor changes in amino acids might be responsible for the increased macrophage tropism of FIPVs. Since these mutations were only observed in FIPVs in diseased tissue and not in virus in feces, it can also be assumed that these particular mutations occur outside the intestine, possibly in monocytes/macrophages. Their role in causing disease is unknown, but they are more likely to be involved in macrophage infectivity than in subsequent host–virus immune interactions.

A second set of S gene mutations was reported by Licitra et al. (2013), who examined mutations at the S1/S2 cleavage site of feline coronaviruses. This site is cleaved by furin, a protein that processes normal cellular precursor proteins into biologically active forms and is found in large quantities in the Golgi network. Compared to FECVs, all FIPVs had at least one single base mutation in and around the S1/S2 cleavage domain. These mutations were unique to each FIPV and, depending on the number of mutations and exact amino acids that were changed, affected the efficiency of cleavage of the S protein by furin; in most cases, the efficiency was increased, while in some cases it was decreased and in others it remained unchanged. Mutations in the region of the S1/S2 cleavage site were not universal to all FIPVs. Unlike the two single base pair mutations described in the S region encoding the fusion peptide, which were present in 96% of FIPV genomes examined (Chang et al., 2012), only two thirds of the same genomes had mutations at the S1/S2 cleavage site (Licitra et al., 2013).

There is evidence from a single cat that mutations in the region of the S1/S2 cleavage site can occur early in the conversion of FECVs to FIPVs (Licitra et al., 2013). Two cats in this study shed FECVs in their feces for 2–3 years before one of them developed FIP. The same single base pair mutation in the S1/S2 cleavage region was identified in the fecal FECV and the FIPV from diseased tissues of the cat that developed FIP, whereas this mutation was not present in the cat that remained unaffected. These particular mutations may have resulted from positive selection pressures related to the adaptation of FECV for better replication in monocyte–macrophages, but not in later processes involving host–virus interactions.

The above studies indicate that regions within the C terminus of the protein encoded by the S gene may be important in the conversion of FECVs to FIPVs. A type I FIPV (strain C3663) remained fully virulent despite a 735 base pair deletion encoding a predicted 245 amino acid sequence in the N terminus (Terada et al., 2012); this deletion would not affect the fusion peptide or S1/S2 cleavage regions of the S protein. This begs the question as to what is the role of the N terminus of the S protein.

It is too soon to say whether all of the mutations relevant to the FECV-to-FIPV transition have been found. Although we do not fully understand how these mutations function in disease, they do suggest potential targets for antiviral drugs, such as protease inhibitors (Kim et al., 2013) or viral ion channel blockers (Schwarz et al., 2011).

Mutations associated with feline infectious peritonitis virus virulence

Loss of virulence in FIPVs such as strain WSU-79-1146 has been associated with large mutations encompassing almost the entire ORF 7b accessory gene (Herrewegh et al., 1995, 1998), as well as small mutations, including those of only two nucleotide changes (Takano et al., 2011a). Lin et al. (2009) sequenced and compared feline coronavirus ORF 7b mutations in ascites or pleural effusions from 20 cats with effusive FIP and in feces from 20 clinically healthy FECV infected cats. Thirty-two of the 40 sequences had an intact ORF 7b, whereas 8/40 sequences had deletions of either three or 12 nucleotides. However, only 3/8 viruses with deletions were from cats with FIP. Therefore, deletions in ORF 7b can occur naturally, as well as in tissue culture, and can occur in both FECVs and FIPVs.

Loss of virulence associated with ORF 7b mutations in FIPVs has also led to false assumptions regarding the importance of this ORF

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[1] See: http://blast.ncbi.nlm.nih.gov/Blast.cgi.

in FECV-to-FIPV conversion and the designation of feline coronavirus strain WSU-79-1683 as a prototypic FECV, which was originally based on cat infection studies comparing type II WSU-79-1683, which does not cause FIP, with WSU-79-1146, the prototypic type II FIPV (Pedersen et al., 1984). The authors concluded that WSU-79-1683 did behave as a FECV. However, this conclusion is counterintuitive, since according to the mutation theory the ORF 7b mutation should have been in WSU-79-1146. Furthermore, WSU-79-1683 grew readily in Crandell Rees feline kidney (CRfk) cells, while the enteric coronavirus could not be cultured from infectious feces in any cell line at the time. Sequencing of WSU-79-1683 also demonstrated a mutated ORF 3c, whereas the ORF 3c of FECVs is always intact (Chang et al., 2010). These findings led to the conclusion that the WSU-79-1683 strain was not a true FECV (Pedersen, 2009). This finding altered the conclusions of many previous studies using WSU-79-1683 and WSU-79-1146 as prototypic FECVs and FIPVs, respectively.

Dedeurwaerder et al. (2013a) tried to link ORF 7b with macrophage tropism and hence with the evolution of FIPVs by measuring the replication kinetics of FIPV WSU-79-1146 and several specific deletion mutants in monocyte cultures. The mutants lacked either the ORF 3abc accessory genes (FIPV-Δ3), ORF 7ab (FIPV-Δ7) or both ORF 3abc and ORF 7b (FIPV-Δ3Δ7). Growth of FIPV-Δ7 and FIPV-Δ3Δ7 could not be sustained in monocyte cultures, whereas sustained growth was observed in monocyte cultures infected with intact FIPV WSU-79-1146. FIPV-Δ3 replicated in monocyte cultures, but at a lower level than intact FIPV WSU-79-1146. The authors concluded that ORF 7 is crucial for FIPV replication in monocytes/macrophages. They also concluded that their findings support the role of ORF 7b in the development of FIP and provide an explanation as to why ORF 7b is almost always conserved in field strains of FIPV. However, they failed to note that ORF 7b is also highly conserved in field strains of FECV (Pedersen et al., 2009; Chang et al., 2010). Therefore, it is true that loss of the integrity of ORF 7b (almost always occurring during cell culture passage) will cause FIPVs to lose virulence, but it is not true that ORF 7b mutations are involved in FECV-to-FIPV transformation.

Alternatives to the internal mutation theory

A study by Brown et al. (2009) temporarily shook the foundations of the internal mutation theory and was supported in theory, but not in fact, by at least one other group (Licitra et al., 2013). Using phylogenetic analyses of coronaviruses identified in a small number of FIPV-infected and healthy cats in a regional shelter in New England, USA, the authors purported to show the existence of two distinct types of feline coronaviruses circulating independently in the population tested; one type causing FIP and the other not. The authors identified five non-contiguous amino acids in the M protein of feline coronavirus that differentiated these two types. However, the numbers of cats used to construct the phylogenetic trees were inadequate and the authors failed to consider that many of the cats originated from different geographic regions, thus introducing genetic drift and population bias.

Several subsequent experiments using larger numbers of cats and taking into consideration geographic bias failed to confirm the existence of distinct FECV and FIPV types. A study from the Netherlands failed to show that the five amino acid sequence defined distinct virus types and concluded that FIPVs originate from FECVs in the same environments (Chang et al., 2011). Additional studies on a large population of cats, taking into consideration genetic differences related to geographic origins, also failed to find evidence for the 'two virus' theory or any significant genetic differences between FIPVs and FECVs from the same environments (Pedersen et al., 2012). A phylogenetic analysis of fecal and tissue coronaviruses from an outbreak of FIP in a group of 20 cats also confirmed that strains of FIP-associated feline coronaviruses were very closely related

to viruses identified in the feces of healthy cats in the same environment (Barker et al., 2013). This study also provided no evidence that genetically distinct virulent (FIPV) and avirulent (FECV) strains of feline coronavirus were present during this outbreak.

Recombinant feline infectious peritonitis virus

Considerable information has been gained about mutations affecting biotypes and virulence of feline coronaviruses using reverse genetics. Haijema et al. (2003) generated a mutant of FIPV WSU-79-1146 in which the S gene was replaced by that of murine hepatitis virus (MHV), which allowed recombinant virus to be selected for in murine cells. In a second reverse process, the FIPV S gene was reintroduced to permit growth in feline cells. The growth characteristics of this reverse recombinant virus in tissue culture and its virulence for cats were indistinguishable from that of the parental virus, FIPV WSU-79-1146. This technique was then used to develop an avirulent deletion mutant of FIPV WSU-79-1146 that was assessed as a potential vaccine (Haijema et al., 2004).

Tekes et al. (2008, 2010, 2012) used a similar reverse genetics technique, but with a replication competent molecular clone of the type I FIPV Black strain, as well as the type II FIPV WSU-79-1146. In their first study, ORF 3abc of FIPV Black was replaced by a green fluorescent protein and luciferase so that the virus could be tracked in CD14^{+} blood monocytes and dendritic cells (Tekes et al., 2008). In a subsequent study, Tekes et al. (2010) demonstrated that recombinant feline coronaviruses expressing the type II S protein acquire the ability to efficiently use feline aminopeptidase N for host cell entry, while type I viruses use another host cell receptor. They also noted that the recombinant virus containing the type II S gene produced high levels of large plaque virus identical to wild type FIPV WSU-79-1146. Therefore, both the growth kinetics and the efficient usage of aminopeptidase N as a cellular receptor for type II feline coronaviruses were attributed solely to the S protein.

In their most recent study, Tekes et al. (2012) studied the virulence of their earlier molecular clones in specific pathogen free (SPF) cats inoculated via the intraperitoneal route. Both engineered viruses established productive infection in cats, as determined by the detection of viral RNA in feces and the induction of specific antibodies, but neither induced FIP. Unfortunately, neither control wild type FIPV Black, nor its recombinant, caused FIP. The authors deduced that the mutation(s) responsible for the loss of virulence occurred upon prior tissue culture passage and resided in ORFs 1a, M, N or 7ab, or the 3'-untranslated region (3'-UTR). In contrast, SPF cats infected with wild type FIPV WSU-79-1146 and its replication competent molecular clone exhibited clinical signs starting at 2–3 weeks post infection and one cat in each group had to be euthanased after 7 weeks. These two cats, along with two others, had typical lesions of FIP at postmortem examination.

A full length genome sequence was obtained from one of the cats infected with wild type FIPV WSU-79-1146. Nine nucleotide differences were observed in the infecting virus, with three non-synonymous mutations in the S gene, one in the gene encoding non-structural protein 3 (replicase gene) and one in the E gene. Significantly, the stop codon in the ORF 3c accessory gene was changed to encode a glutamine residue, thus yielding an intact ORF 3c protein. A full-length genomic sequence was also obtained from the spleen and kidney of one cat infected with the molecular cloned virus. Two non-synonymous nucleotide changes were identified in the virus sequence from the spleen, while seven non-synonymous and three silent changes were identified in the virus sequence from the kidney. Restoration of ORF 3c was observed once again and viral RNA with this genotype was also detected in the feces of both cats. This finding appeared to contradict earlier reports suggesting that only enteric biotypes (FECVs) require an intact ORF 3c for intestinal replication (Chang et al., 2010; Pedersen et al., 2012). However, FIPVs with intact

ORF 3c genes have been found in the feces of cats with FIP, and these viruses often have an increase in non-synonymous mutations in the 3' terminus of ORF 3c (Pedersen et al., 2012). Tekes et al. (2012) demonstrated the importance of extending this reverse genetics approach to pathogenic type I feline coronaviruses and the need to generate recombinant FECVs that could be used to study early events in natural FECV-to-FIPV evolution.

Feline enteric coronavirus infection studies

Three studies from different groups have addressed the behavior of FECV infection and immunity in laboratory cats (Pedersen et al., 2008; Kipar et al., 2010; Vogel et al., 2010). These experiments confirmed what had been previously observed in cats naturally infected with FECVs. Primary FECV infection is largely subclinical or associated with a transient and usually mild diarrhea and is centered in the lower small intestine and colon (Kipar et al., 2010; Vogel et al., 2010). Large amounts of virus are shed in the feces for many weeks and even months after initial infection, but with time most cats cease shedding. There also appears to be a low level of FECV in blood monocytes during initial infection (Kipar et al., 2010). However, immunity is not always solid and, as antibody levels in the blood decrease, many cats become susceptible to reinfection (Pedersen et al., 2008). These secondary infections closely resemble the primary infection.

The comparative importance of various feline enteric pathogens, including FECV, was studied by Sabshin et al. (2012) in 100 shelter cats. Fecal samples were collected within 24 h after admission in 50 normal cats and 50 cats with diarrhea, and tested by fecal flotation, antigen assays, PCR and electron microscopy for pathogens. Twelve enteric pathogens were identified, including coronavirus, *Clostridium perfringens* enterotoxin A, *Cryptosporidium* spp., *Giardia* spp., *Cystoisospora* spp., hookworms, ascarids, *Salmonella* spp., astrovirus, feline panleukopenia virus, calicivirus and *Spirometra* spp. Interestingly, cats with diarrhea were no more likely to be infected with one or more of these enteropathogens than cats with normal feces (84% in both groups). Only FECV was significantly more prevalent in cats with diarrhea than in normal cats (58% and 36%, respectively). FECV was also the most common fecal pathogen, being several times more common than any other enteric pathogen studied.

Although numerous strains of FECV are found among cats throughout the world, at least one population has remained surprisingly free of infection. Cats in the Falkland Islands have no signs of infection and attempts are being made to quarantine these cats from an inadvertent introduction of the virus (Addie et al., 2012). The long isolation of cats in the Falklands is indirect evidence that coronaviruses might not have existed in cats at the time of their transit to the island.

Research investigating the FECV-to-FIPV pathotype transformation has been hampered by an inability to grow FECV in tissue culture. A group from Belgium might have solved this problem by creating a long-term feline ileocyte/colonocyte cell line using simian virus 40 T antigen/human telomerase reverse transcriptase for immortalization (Desmarets et al., 2013). This cell line readily propagated two strains of type I FECVs, while no infection was seen in cultures inoculated with FIPV tissue homogenates. The fact that FIPVs would not grow in this cell line supports the theory that FIPVs lose tropism for the intestinal epithelium and that FECV is the main biotype circulating among cats. The creation of this cell line will fill a large hole in the list of reagents needed to study the pathogenic properties of FIPV.

Types I and II feline coronaviruses

FECVs, and therefore FIPVs, exist as two distinct types based on both serology and sequencing. Type I FECVs/FIPVs are unique to cats, while type II FECVs/FIPVs appear to be recombinants between type I FECVs and the closely related canine coronaviruses. Serologic differences are due to canine coronavirus S protein sequences encoded by the ORF 3a and S gene regions (Pedersen et al., 2009). Type I FECVs/FIPVs predominate in Europe and the Americas, while up to 25% or more feline coronaviruses from Asian countries are type II (Sharif et al., 2010; An et al., 2011; Amer et al., 2012). The exact origin of type II feline coronaviruses is unknown, but their genetic variability suggests that they are continuously being generated. The independent nature of these recombination events is indicated by the varying genetic makeup of the 'hybrid' viruses. These recombination events are often highly complex. This is demonstrated by strains such as NTU156 from Taiwan (Lin et al., 2013). This strain not only contains an in-frame deletion of 442 nucleotides in ORF 3c, a mutation associated with the FECV-to-FIPV biotype change, but also two crossover events with recombination sites located in the RNA-dependent RNA polymerase and M genes. This produced a new virus with one-third of its genome originating from canine coronavirus. Although not proven, it is widely assumed that type II FIPVs are more virulent than type I FIPVs (Lin et al., 2013; Wang et al., 2013), possibly associated with the ease with which they are propagated in a number of cat and dog cell lines. Type II FIPVs might also be more likely to be transmitted between cats and to be associated with outbreaks of FIP, such as one described in a Taiwanese shelter (Wang et al., 2013).

Transmission of feline infectious peritonitis virus

The question of whether FIPV is transmitted cat-to-cat (horizontal transmission) or by internal mutation from FECV (vertical transmission) has also been a topic of interest. There is no solid evidence that cats with FIP readily transmit FIPV directly to other cats, although the possibility has been suggested as an explanation for rare mini-outbreaks of FIP (Pedersen, 2009). FIPV can cause disease when fed to laboratory cats, and some experimentally infected cats will shed low levels of FIPV-like virus (Pedersen et al., 2009, 2012). However, such virus does not appear to be infectious when fed to other cats (Pedersen et al., 2012). Some cats with naturally acquired FIP can also shed either FECV or a coronavirus that appears genetically similar to the FIPV within the same cats (Chang et al., 2010). An outbreak of FIP in Taiwan associated with a type II FIPV appeared to be transmissible, but was self-limiting, either due to quarantine measures or more likely to genetic drift (Wang et al., 2013). Sequential samples collected over time from this outbreak documented the replacement of the original virus, which had an intact ORF 3c, with viruses having functionally mutated ORF 3c. This provided indirect evidence that ORF 3c mutations are more likely to be associated with disease than with infectivity.

Immunopathogenesis

Our knowledge of the pathogenesis of FIP remains at a very basic level (Pedersen, 2009; Myrrha et al., 2011). The working hypothesis is that FECVs mutate in a manner that causes them to lose tropism for enterocytes, while gaining tropism for macrophages. The exact site where these mutations occur is unknown, but it is apparently at some point between the intestine (enterocyte) and FIP lesions (macrophage). A possible intermediate site for this transformation would be blood monocytes/macrophages, which are known to be infected during FECV infection (Kipar et al., 2010). FECV-to-FIPV transition appears to involve positive selection for mutants that are increasingly fit for replication in macrophages and unfit for replication in enterocytes. The ultimate target cell is not just any macrophage, but rather a distinct population of precursor monocytes/macrophages that have a specific affinity for the endothelium of venules in the serosa, omentum, pleura, meninges and uveal tract.

It is widely assumed that immunity, when it occurs, is largely cell-mediated and that the production of antibodies is counterproductive. Antibodies enhance the uptake and replication of FIPVs in macrophages and also contribute to a type III hypersensitivity (antibody-mediated or Arthus-type) vasculitis (Pedersen, 2009). It is also assumed that much of the pathology occurring in FIP is associated with how macrophages respond to viral infection and how the immune system of the host responds to the infected cells. In this scenario, the effusive form of FIP results from a failure to mount T cell immunity in the face of a vigorous B cell response. At the opposite extreme, cats that resist disease presumably mount a vigorous cell-mediated immune response that is able to overcome any negative effects of antibodies. Cats with the dry form of FIP represent an intermediate state involving a cellular response that is partially effective in containing the virus to a relatively small number of macrophages in a few focal sites within specific target organs. The two forms of FIP are somewhat interchangeable; when it has been observed in experimental infection, the dry form always follows a brief bout of effusive disease. In the terminal stages of naturally occurring dry FIP, immunity can completely collapse and the disease reverts to a more effusive form. Although this scenario fits what is known about FIP, it must be emphasized that much of this scheme awaits confirmation and there are large gaps to be filled.

B cell vs. T cell immunity

An imbalance in T cell vs. B cell immune responses has been evoked as one reason for the inability of cats to resist FIPV infection. One study demonstrated that the ratio of peripheral blood surface immunoglobulin positive cells (sIg^+) to CD21+ cells was higher in cats with FIP than in SPF cats, and that cells strongly expressing mRNA of the plasma cell master gene encoding B lymphocyte-induced maturation protein 1 (*Blimp-1*) were increased (Takano et al., 2009a). The mRNAs of interleukin 6, CD40 and B cell activating factor (BAFF) were also overexpressed in macrophages previously shown to be associated with antibody-dependent enhancement.

Vermeulen et al. (2013) studied the role of natural killer (NK) cells and regulatory T cells (Tregs) in the innate and adaptive cell-mediated immunity, respectively, in cats with naturally occurring FIP. NK cells and Tregs were drastically depleted from the peripheral blood, mesenteric lymph nodes and spleen in cats with FIP, while the mesentery and kidneys from cats with FIP did not show any differences when compared to healthy uninfected control cats. Other regulatory lymphocytes of the $CD4^+CD25^-Foxp3^+$ and $CD3^+CD8^+Foxp3^+$ phenotypes were depleted from both blood and lymph nodes. NK cells in cats with FIP were upregulated for the activation markers CD16 and CD25, and migration markers CD11b and CD62L, while NK cells from lymph nodes had upregulation of CD16 and CD62L only. NK cells from the lymph nodes of cats with FIP also exhibited less cytotoxic activity than NK cells from the lymph nodes of healthy cats. Therefore, it appears that infection with FIPV is associated with severe depletion of both NK cells and Tregs, and reduced NK cell function. This could reduce the capacity of the innate immune system to attack the virus and to suppress the associated immunologic and inflammatory responses.

The most compelling evidence for the role of T cell immunity in FIP might actually come from research with a distantly related coronavirus infection of another species. Mouse hepatitis virus (MHV) is one of the most common virus models in laboratory mice. MHV, like FIPV, exists as two biotypes, designated enterotropic and polytropic. Polytropic strains of MHV are mainly of laboratory origin and cause a variety of hepatic and neurologic syndromes, depending on the laboratory of origin, in vitro and in vivo passage history, and inbred mouse strain used for the infection study (Homberger, 1997). In contrast, enterotropic biotypes tend to predominate in nature.

Most coronavirus contamination in experimental mice colonies is associated with enterotropic strains of MHV brought in from wild mice.

Interestingly, certain polytropic laboratory strains of MHV cause a disease syndrome analogous to FIP of cats when they infect an interferon (IFN)-γ deficient strain of mice (Kyuwa et al., 1998). This prompted additional comparative studies of enterotropic and polytropic strains of MHV (Compton et al., 2004). Immunocompetent, as well as B and T cell deficient strains of laboratory mice were infected with an enterotropic strain of MHV, MHV-Y. The infection in immunocompetent mice was limited to the small intestine for the first week and then to the cecum and colon for the next 2 weeks. B cell deficient mice also developed chronic subclinical infection restricted to the gastrointestinal tract, but lasting for 7–8 weeks. The pattern of infection in these two strains of mice was reminiscent of FECV infection in laboratory cats (Pedersen et al., 2008; Kipar et al., 2010). In contrast, T cell deficient mice infected with MHV-Y developed a multi-systemic lethal infection, with virus detected in the intestine during the first week; the mice then developed peritonitis by week 2, with virus detected in mesentery and visceral peritoneum, before the mice died by weeks 3–4. These findings with MHV strains in inbred strains of mice support the importance of both IFN-γ and T cell immunity in resistance to FIP. In the mouse model, it was the host's response to MHV and not the biotype that determined the disease form (enteritis or FIP-like disease), while in the case of FIP, genetic differences between the FECV and FIPV biotypes determine the host response and disease form (enteritis or FIP).

The pyogranuloma

The classical lesions of FIP are the pyogranuloma of the effusive form and the somewhat more typical granuloma of the dry form. As the name implies, the pyogranuloma is an accumulation of macrophages, neutrophils, lymphocytes and occasional plasma cells that tend to form small aggregations around venules in the target tissues. These cells arrive from the bloodstream as a result of upregulation of both adhesion proteins and their receptors. Olyslaegers et al. (2013) studied the altered expression of the transmigration adhesion molecules CD11a, CD11b, CD15s, CD18, CD49d and CD54 on blood leukocytes from cats with naturally occurring FIP. They found that T and B lymphocytes and monocytes expressed higher levels of CD11a and CD18, while B lymphocytes had increased levels of expression of CD49d and granulocytes had increased levels of expression of CD11b. It was concluded that all of these blood leukocytes exhibited features of systemic activation in cats with FIP and that this contributed to their transmigration into the pyogranulomas seen in the disease.

The pyogranuloma is also strongly associated with edema and the effusion of large volumes of a proteinaceous fluid that is rich in plasma proteins, hemoglobin breakdown products, inflammatory proteins of many types and activated clotting factors. The factors responsible for this outpouring of fluid have not been identified fully, but at least one of these factors appears to be vascular endothelial growth factor (VEGF; Takano et al., 2011a). This is produced by FIPV infected monocytes and macrophages. Other proteins, such as tumor necrosis factor (TNF)-α, granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) are also produced by macrophages in cats with FIP and are thought to contribute to increased survival of neutrophils (Takano et al., 2009b). The p38 mitogen-activated protein kinase (MAPK) pathway has been shown to play an important role in the over production of pro-inflammatory cytokines in FIPV infected peripheral blood mononuclear cells (PBMCs) (Regan et al., 2009).

Cytokines and feline infectious peritonitis

Clinically normal cats living in catteries with enzootic FECV infection overall had higher serum IFN-γ concentrations than cats in catteries experiencing losses due to FIP and catteries free of infection (Giordano and Paltrinieri, 2009). IFN-γ concentrations were high in the sera of cats with FIP, and even higher in body cavity effusions, the latter thought to reflect production by local inflammation. It was concluded that FECV-infected cats have a strong systemic IFN-γ response, while cats with FIP have a strong IFN-γ response at the tissue level.

A test to measure the ability of leukocytes in whole blood to produce IFN-γ (protein and mRNA) in vitro in response to eight immunogenic peptides derived from the N protein of virulent (FIPVs) and avirulent (FECVs) feline coronaviruses has been developed for cats (Rossi et al., 2011). The sequences used were obtained from an earlier study by Battilani et al. (2010) that compared a small number of N gene sequences from lesions of cats with FIP and feces of clinically healthy cats, and purported to show N epitopes that would differentiate the two. The test was applied to a group of healthy cats and another group with chronic diseases other than FIP. Cats with chronic diseases other than FIP responded with increases in both IFN-γ protein and mRNA when exposed to peptides that were presumed to be specific for avirulent (FECV) or a mixture of avirulent (FECV) and virulent (FIPV) epitopes. Unfortunately, no results were given for cats with FIP.

Satoh et al. (2011a) developed monoclonal antibodies against feline IFN-γ and used them to study immune responses to FIPV. PBMCs from cats experimentally infected with FIPV that did not develop clinical disease had significantly increased levels of IFN-γ production after exposure to heat–inactivated FIPV compared to cats that died of FIP; the increased IFN-γ levels were more marked in CD8+ than CD4+ T cells. It was concluded that cell-mediated immunity was important in resistance to FIP.

Dedeurwaerder et al. (2013b) studied the role of the ORF 7a gene product in antiviral defense. Deletion of ORF 7a and ORF 7b of FIPV WSU-79-1146 rendered the virus more susceptible to IFN-α treatment in cell culture. They then separately added ORF 7a and ORF 7b back into the mutant virus; ORF 7a, but not ORF 7b, rescued normal sensitivity to IFN-α. When these insertions were performed in a mutant of FIPV WSU-79-1146 with deletions of ORF 3abc and ORF 7ab, sensitivity to IFN-α was not restored. It was concluded that the ORF 7a protein acted as a type I IFN antagonist in the presence of ORF 3abc protein(s) and protected FIPV against the effects of this particular host cell antiviral response.

One of the newest approaches to studying immune responses involves transcriptional profiling, wherein the various cellular mRNAs triggered by a viral infection can be monitored for up- or down-regulation. Harun et al. (2013) demonstrated the feasibility of such an approach using CRfk cells exposed to FIPV WSU-79-1146 for 3 h. They were able to detect 18,899 of the 19,046 annotated feline genes. Based on results from their culture studies and Kal's Z test, 44 genes were upregulated, 61 genes were not affected by virus infection and the remaining genes were down-regulated. This transcriptional profile was used to identify three genes (A3H, PD-1 and PD-L1) that were both upregulated and potentially relevant to FIP. A real-time (RT)-PCR was than developed for each gene, and the level of expression in PBMCs from seven cats with confirmed FIP was measured. Upregulation of cDNAs from the three genes was observed, but it tended to be low and highly variable between individual genes, as well as cats. Although this study did not report details on the number of replicates used in various parts of the study, and could have used actual normal and diseased tissues/cells in preference to experimentally infected and non-infected CRfk cells, it is an approach that will be applied much more in the future.

Antibody-dependent enhancement

There is ample evidence from experimental studies that FIPV infection is greatly enhanced in the presence of coronavirus antibodies, whether they are actively elicited by exposure to FECV or avirulent FIPVs, or passively administered in the form of immune serum from either FECV- or FIPV-infected cats (Pedersen, 2009). This antibody-dependent enhancement (ADE) is best demonstrated when the antibody and challenge strain of FIPV are of similar type (Takano et al., 2008). This ADE is not unique to feline coronaviruses and has been the bane of vaccines used or tested in the field against dengue virus, feline immunodeficiency virus and human immunodeficiency virus type 1 (HIV-1) (Huisman et al., 2009).

The exact anti-coronavirus epitopes responsible for ADE have been somewhat elusive, but they appear to reside in the S protein. Satoh et al. (2011b) synthesized 81 peptides from the S2 region of the S protein of a type I FIPV (KU-2) and type II FIPV (WSU-79-1146), and from the N protein of FIPV KU-2. They used these peptides to stimulate IFN-γ production from PBMCs of cats experimentally infected with FIPV and identified a number of linear immunodominant antibody-binding epitopes at different positions in the S2 and N regions within both type I and type II FIPVs. Satoh et al. (2010) also studied three baculovirus expressed proteins derived from different regions of the S2 domain of the S protein of the type I FIPV KU-2 strain. One of these proteins, which spanned the region from the fusion peptide to the heptad repeat 2 region, induced a vigorous T helper type 1 (Th1) and T helper type 2 (Th2) immune response in mice. Out of 30 peptides derived from this region that were tested in mice, four peptides that included both Th1 and Th2 epitopes were identified; the authors concluded that these regions should be explored in cats as potential immunogens.

Takano et al. (2011b) also studied the putative ADE epitopes in FIPV WSU-79-1146 with a battery of monoclonal antibodies against the virus neutralizing epitope on the S protein. Virus cultures exposed to one of these monoclonal antibodies eventually yielded a virus with two amino acid changes in the neutralizing epitope of the S protein that rendered it resistant to neutralization. In the process, the monoclonal antibody used to produce this mutant also lost its ability to enhance the replication of homologous virus, thus suggesting that virus neutralization and ADE were present in the same region of the S protein. The mutant virus used in the study also replicated to lower levels in monocyte/macrophages.

Evasion of antibody-dependent, complement-mediated lysis has been studied in CRfk cells and monocytes infected with FIPV WSU-79-1146 and an ORF 3abc/ORF 7b deletion mutant (Cornelissen et al., 2009). Surface expression of viral proteins by infected cells, whether by wild type FIPV or the deletion mutant, prevented complement-mediated lysis, even though controls showed it to be functional. The authors postulated that this was a new mechanism for FIPV to evade the immune system.

A different evasion route was proposed by Dewerchin et al. (2008). Monocytes infected with FIPV express viral proteins on their plasma membranes; upon binding of antibodies, these proteins are rapidly internalized through a clathrin- and caveolae-independent pathway, which is also dependent on dynamin (Van Hamme et al., 2008). In doing so, the infected monocytes can escape antibody-dependent cell lysis. In a subsequent study, Dewerchin et al. (2014) identified kinases and cytoskeletal proteins that were involved in internalization and subsequent intracellular transport of FIPV. Myosin light chain kinase and myosin 1 were crucial for the initiation of the internalization process, as shown by the co-localization of both proteins with antigens prior to virus internalization. One minute after internalization commenced, the virus-laden vesicles had co-localized with microtubules and accumulated at the microtubule organizing center after 10–30 min. This activity of myosin light chain

kinase was effectively inhibited by ML-7, thus identifying a possible target for an anti-viral drug.

How do feline coronaviruses enter their host cells?

The mechanism by which the various feline coronaviruses attach to their host cells is not known. Type II FIPVs appear to utilize aminopeptidase N as their principal receptor. While the primary receptor for type I feline coronaviruses is unknown, the lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209) can act as a co-receptor (Regan and Whittaker, 2008; Regan et al., 2010). These receptors are undoubtedly important for FECV binding to target enterocytes, but whether they play a role in infection of macrophages by FIPVs is unknown. It is possible that FIPVs could enter their target macrophages using less specific means, such as through the Fc-receptors used for complement binding.

Vaccines against feline infectious peritonitis

A spate of papers describing possible vaccines against FIP has appeared over the last three decades (Pedersen, 2009). Several reportedly showed protection, but only one of doubtful efficacy went into commercial production. However, recently there has been a renewed interest in FIP vaccines. Bálint et al. (2013) developed a vaccine based on a pair of recombinant viruses derived from the type II FIPV strain DF2, a virus nearly identical to FIPV WSU-79-1146. One strain was avirulent and the other of low virulence. SPF cats appeared to be fully protected against virulent FIPV DF2 after two oronasal and two intramuscular injections 2 weeks apart. Surprisingly, purebred British shorthair cats were not protected following the same vaccine regimen, although experimental and control groups were small. Vaccination with the avirulent virus induced ADE and, following challenge-exposure, all cats developed FIP; 40% of cats had prolonged survival, while 60% died from fulminant disease. The researchers evoked differences between conventional and SPF cats, as well as heterologous virus pre-exposure, as reasons for the various experimental outcomes.

Takano et al. (2014a) immunized cats with Th1 stimulating peptides identified previously in the N protein of the type I FIPV strain KU-2 using feline CpG-oligodeoxynucleotides (fCpG-ODNs) as adjuvants. Some immunity was demonstrated against challenge-exposure with the type II FIPV strain WSU-79-1146, but immune tolerance was also observed at higher vaccine dosages. The researchers concluded that further investigations on the combination and concentrations of the peptides and fCpG-ODNs, along with dose, frequency and route of administration, were needed. Takano et al. (2014b) identified strong Th1 epitopes within the S1 domain of the S protein and in the M protein of both type I (KU-2) and type II (WSU-79-1146) FIPVs. Interestingly, no immunodominant antibody binding Th1 epitope-containing peptide was identified in the primary antibody binding S1 domain of the type II FIPV. Eleven Th1 epitope-containing peptides were identified in the S, M and N proteins that were common to both type I and type II FIPVs. Three of these peptides were administered with CpG-ODNs to SPF cats; two of these peptides from the S1 and M proteins induced Th1 activity to peptide stimulation of PBMC cultures, but no cat infection results were reported.

Is there a genetic susceptibility to FIP disease?

Early research strongly suggested that susceptibility to FIP might be controlled to some extent by genetic factors (Pedersen, 2009). A genome-wide association study of a relatively large number of Birman cats identified five regions on four different chromosomes that could harbor genes involved in susceptibility (Golovko et al., 2013). Five candidate genes (ELMO1, RRAGA, TNSF10, ERAP1 and ERAP2) were identified in these four regions; all are associated with processes relevant to FIP, such as cellular migration, phagocytosis, apoptosis and virus–host interactions. These associations require confirmation and further study, as they might only be relevant to one breed. If genetics are involved in the disease, as it appears, the exact genes and pathways involved appear to be numerous and, therefore, inheritance is likely to be complex.

Conclusions

We are rapidly gaining a better understanding of the mutational events that cause FECVs to become FIPVs, but we lack knowledge of how these mutations are involved in immunopathogenesis. At least three separate types of mutations have been associated with the acquisition of FIP virulence and more are likely to be discovered. Cell lines that will replicate FECVs in vitro have been developed and this, coupled with the creation of infectious molecular clones of FIPV, will hopefully allow us to assess the importance of these and any future mutations. A number of new reagents and procedures have given us a window into the cells involved in the inflammatory and immune responses that characterize the two distinct, yet overlapping, clinical forms of FIP. Other environmental and host factors undoubtedly play a role in determining the outcome of exposure, but their roles are not understood. The elaboration of various cytokines and other inflammatory proteins in the FIP disease process has been extensively studied, but how these various factors are stimulated and their role in pathology is poorly understood. A possible role for genetic susceptibility has been identified in at least one breed of cats, but the genetics appear to be highly complex and cannot explain the entire disease incidence. A more parsimonious conclusion is that FIP results from a confluence of numerous viral, host and environmental factors akin to the 'perfect storm'. Although we are heartened by our increasing knowledge of this disease, we are continually reminded of the words of Robert Frost in his poem 'The Secret Sits': '*We dance round in a ring and suppose, but the secret sits in the middle and knows.*'

Conflict of interest statement

The author has no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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