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Feline infectious peritonitis: still an enigma?

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Abstract: Feline infectious peritonitis (FIP) is one of the most important fatal infectious diseases of cats, the pathogenesis of which has not yet been fully revealed. The present review focuses on the biology of feline coronavirus (FCoV) infection and the pathogenesis and pathological features of FIP. Recent studies have revealed functions of many viral proteins, differing receptor specificity for type I and type II FCoV, and genomic differences between feline enteric coronaviruses (FECVs) and FIP viruses (FIPVs). FECV and FIP also exhibit functional differences, since FECVs replicate mainly in intestinal epithelium and are shed in feces, and FIPVs replicate efficiently in monocytes and induce systemic disease. Thus, key events in the pathogenesis of FIP are systemic infection with FIPV, effective and sustainable viral replication in monocytes, and activation of infected monocytes. The host's genetics and immune system also play important roles. It is the activation of monocytes and macrophages that directly leads to the pathologic features of FIP, including vasculitis, body cavity effusions, and fibrinous and granulomatous inflammatory lesions. Advances have been made in the clinical diagnosis of FIP, based on the clinical pathologic findings, serologic testing, and detection of virus using molecular (polymerase chain reaction) or antibody-based methods. Nevertheless, the clinical diagnosis remains challenging in particular in the dry form of FIP, which is partly due to the incomplete understanding of infection biology and pathogenesis in FIP. So, while much progress has been made, many aspects of FIP pathogenesis still remain an enigma.

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Review for the Special Issue of *Veterinary Pathology* on the topic of infectious diseases

Feline Infectious Peritonitis, still an enigma?

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Abstract

Feline infectious peritonitis (FIP) is currently the most relevant fatal infectious disease of cats. Despite intensive research in the field, its pathogenesis is still not completely revealed. FIP is a challenging clinical disease, with progressively worsening general symptoms, i.e. fluctuating fever, anorexia, and weight loss, in combination with a variety of additional clinical features that reflect the type and distribution of the pathological changes. Several, also very recent reviews, have been published that thoroughly cover all relevant clinical aspects of the disease.[41,71,131] In the present review, we therefore focus on the pathological features of FCoV infection and FIP and the various aspects that are relevant for the pathogenesis of the disease.

Keywords: Diagnosis, Feline Coronavirus, Feline infectious peritonitis, Feline infectious peritonitis virus, Feline enteric coronavirus, pathogenesis

History

Feline infectious peritonitis (FIP) has first been thoroughly described and named in 1966, when experimental infections of healthy cats with organ material of diseased animals confirmed it as a specific, fatal infectious disease of cats, and a viral etiology was suspected.[172] However, the disease syndrome had already been observed in the 1950s and 60s in the USA, and even earlier, a very similar disease had been reported from cats in Naples.[17,83]

In 1968, the viral etiology was demonstrated.[165] The virus morphology suggested a coronavirus (CoV), which was finally confirmed in 1976.[116,121,167] The virus was first grown in peritoneal cells of experimentally infected cats, and, after propagation in cell culture, was shown to cause FIP in 100% of intraperitoneally infected animals.[123] Subsequently, the macrophage cell line *Felis catus* whole fetus-4 (Fcwf-4) has predominantly been used for virus propagation.[87,123]

In his recent review article, based on more than 40 years of work in the field, Pedersen speculated on the reason for the likely emergence of FIP in the 20th century. He considered as the most relevant potential factors the evolution of the feline CoV (FCoV) alongside CoV of pigs and dogs, the development of virulent FIP virus mutations from enteric FCoV that had only evolved at that stage, and the changes in keeping and especially breeding of cats due to their increasing popularity as pets.[131]

At present, despite decades of research on its etiology, pathogenesis, transmission and prevention, FIP is still the most frequent fatal and infectious feline disease for which there is so far no effective cure.

Feline Coronaviruses (FCoV)

63 *Virology*

64 FCoV are pleiomorphic enveloped, single-stranded positive sense RNA viruses with
65 an almost 30kb non-segmented genome and 11 putative open reading frames
66 (ORF). They belong to the family *Coronaviridae*, order *Nidovirales*, and, together with
67 Canine Coronavirus (CCV) and Transmissible Gastroenteritis Virus (TEGV) of pigs,
68 belong to the subfamily *Coronavirinae*, genus *Alphacoronavirus*, species
69 *Alphacoronavirus 1*.[61]

70 At the 5' end of the FCoV genome, approximately 20kb comprise the two overlapping
71 ORF 1a and 1b that encode for two polypeptides which are subsequently
72 enzymatically cleaved into 16 non-structural functional proteins mainly involved in the
73 synthesis of the viral RNA (viral replicase). The remaining genome contains nine
74 ORF that encode for four structural proteins (spike [S], nucleocapsid [N], membrane
75 [M], and envelope [E]) and five group-specific, accessory proteins (3a-c, 7a and b).
76 These are expressed individually from a nested set of subgenomic mRNAs that each
77 contain a leader RNA sequence derived from the 5' end of the genome, and are
78 generated by discontinuous transcription from the 3' end of the genome.[44,46] The CoV
79 envelope is formed by the S protein, a 180-200 kDa glycoprotein arranged in
80 peplomers that induces the antibody response and cell mediated immunity in the
81 host. The S peplomers are 12-24 nm long, dome shaped, and arranged like a crown;
82 they are the key determinants of cell tropism.[13] The S protein is a type I
83 transmembrane protein with a very short C-terminal cytoplasmic tail and a long N-
84 terminal ectodomain that is divided into a N-terminal (S1) domain responsible for
85 receptor binding, and a C-terminal (S2) domain containing the fusion peptide which
86 mediates fusion with the target cell membrane.[18] The M and E proteins are smaller
87 surface glycoproteins and important for virus maturation, assembly, budding and

88 interaction with the host cell. The M protein, with a mass of approximately 29 kDa,
89 penetrates the envelope and connects it to the capsid, and participates in the RNA
90 packaging. E proteins are type III membrane proteins of about 9 kDa that interact
91 with the M protein in the budding compartment of the host cell.[44] In mouse hepatitis
92 virus (MHV), they can induce apoptosis.[7] The N proteins have a molecular weight of
93 approximately 50 kDa. Together with the viral RNA, they form the flexible, helical
94 nucleocapsid and seem to be critical for viral transcription.[114] Vaccine studies based
95 on the N protein indicate that it induces cell mediated immunity and can play a
96 protective role.[82] So far, no specific function could be ascribed to the accessory
97 proteins. The 71-72 amino acid long 3a and b proteins are well conserved among
98 subspecies 1 alphacoronaviruses. Since they lack predicted hydrophobic segments,
99 both are thought to be located and exert their function in the cytoplasm. ORF 3c is
100 very well conserved among the alphacoronavirus genus and its predicted sequence
101 indicates that it is a class III triple spanning membrane protein of 238-244 residues,
102 with a topology similar to that of the M protein.[68,115] ORF 7a encodes for a small
103 membrane protein of approximately 10 kDa with N-terminal cleavable signal
104 sequence and a C-terminal transmembrane domain. A recent study with deletion
105 mutants of the homologous TGEV protein has found evidence that 7a impairs the
106 host's antiviral response.[32] The ORF 7b is present only in FCoV, CCV and ferret CoV
107 and encodes for a soluble glycoprotein of 207 residues (approximately 24 kDa) that
108 has been shown to induce antibodies in naturally infected cats.[76,90]
109 CoV occur in many mammalian species including humans, and in birds. They lead to
110 acute or chronic infections and, depending on their cell tropism, induce highly
111 variable diseases in their hosts. The host and tissue specificity is dependent on
112 sequence variations of the S gene as well as receptor usage and distribution.[46]

113 RNA viruses have high error rates in their replication and therefore occur as
114 quasispecies, i.e. groups of related genotypes.[37] With every RNA replication of CoV,
115 several point mutations occur. Even virus "stocks" prepared from plaques form
116 quasispecies. Genetic diversity within a quasispecies has been suggested to
117 contribute to pathogenesis by cooperative interactions among variant viruses within a
118 population.[163] On the other hand, proofreading or repair mechanisms, mediated by
119 the exoribonuclease in the replicase complex, allow RNA viruses to evolve whilst
120 keeping a balance between adaptation and viral fitness.[37] Also, homologous RNA
121 recombination during mixed infections of closely related CoV strains in the same
122 group promote cross-species transmission and pathogenesis; the cat might represent
123 a "mixing vessel" as *in vitro* studies showed that feline aminopeptidase N can be
124 used as a functional receptor by closely related alphacoronaviruses, such as FCoV,
125 CCV, TGEV, and human coronavirus HCV-229E.[158]
126 Genome sequences and subsequent phylogenetic analysis showed that FCoV
127 isolates form geographical clusters.[10-12,42,102,132] FCoV from cats of the same
128 household exhibit over 95% genetic identity, suggesting infection from a common
129 virus.[5,102,162] Focussing on the S gene, one study specifically examined the evolution
130 of virus strains in cohorts of naturally infected cats over several years. It
131 demonstrated very high conservation of the virus in persistently infected and
132 (recurrently) shedding animals, but also showed that cats can become transiently
133 infected and subsequently re-infected with the same or a different strain. There was
134 also evidence of super- or co-infection of persistently infected cats with other strains.[5]
135
136 *Serotypes*

137 As shown by virus neutralizing antibody reaction and amino acid sequences of the S
138 protein, FCoV form two antigenetically distinct serotypes, type I FCoV which are
139 difficult to grow in cell culture, and type II FCoV which are the consequence of a
140 double recombination between type I FCoV and CCV.[79,112,127,143] *In vitro*, the growth
141 kinetics of both serotypes appear to be solely related to the S protein, as determined
142 using a recombinant type I FCoV encoding a type II S protein.[157] For type II FCoV,
143 like for several other alphacoronaviruses, the cell receptor is aminopeptidase N
144 (APN, CD13) which upon binding to the S protein, mediates the viral internalization
145 into the target cells.[81,157] Antibody blockage of APN has been shown to severely
146 reduce the infection of bone marrow derived macrophages (BMDM) with the type II
147 FIP strain 79-1146.[140] However, it has so far not been confirmed that APN is also the
148 receptor for FIPV II in infected animals. Furthermore, the receptor for serotype I
149 FCoV is not known. Interestingly, however, isolated feline monocytes rapidly
150 internalize both serotype I and II FIPV and accumulate the virus particles in
151 endosomes, followed by particle disassembly.[160] Both serotypes can use dendritic
152 cell (DC)-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-
153 SIGN, CD209), a C-type lectin, which recognizes high-mannose oligosaccharides as
154 ligands, to infect monocyte-derived dendritic cells.[138] Co-localization and binding
155 inhibition studies confirmed that DC-SIGN and not APN is involved in the entry
156 process of serotype I FCoV in monocytes, whereas for serotype II FCoV, both APN
157 and DC-SIGN play a role in infection of monocytes, i.e. binding is mediated by APN
158 but DC-SIGN is important for either internalization or a subsequent step.[161] In both
159 models, a role of an unknown co-receptor cannot be excluded.
160 Both FCoV serotypes can cause FIP, but serological and, more recently, molecular
161 studies confirmed that type I FCoV dominate by far in the cat population worldwide,

162 with a prevalence of up to 98%.[5,80,105,107,127,143] Type I FCoV were shown to induce
163 higher antibody titres than type II FCoV, and were more frequently associated with
164 clinical signs and/or FIP.[105] A higher type II prevalence, partly together with type I
165 infection, has been reported for cats with FIP, ranging from 10% to more than 30%,
166 the latter in an older study in Japan.[15,42,107,143]
167
168 *Feline Enteric Coronavirus versus Feline Infectious Peritonitis Virus*
169 FCoV occur as two pathotypes, Feline Enteric Coronavirus (FECV), defined as the
170 "ubiquitous enteric biotype", and Feline Infectious Peritonitis Virus (FIPV), the
171 "virulent biotype that causes FIP in individual cats".[131] FECV and FIPV cannot be
172 distinguished serologically or morphologically, and for many years, the search for
173 markers that can discern the two pathotypes remained unsuccessful.
174 In the past, it was assumed that the main difference between FECV and FIPV was
175 that FECV exclusively infect the intestinal epithelium and do not pass the intestinal
176 mucosal barrier, while FIPV infect and replicate in monocytes/macrophages and can
177 therefore gain access to the blood and induce the disease.[124,125,128] When more
178 sensitive molecular methods became available, this hypothesis was proven too
179 simple. It was shown that also FECV can infect monocytes and that FCoV generally
180 spread from the initial site of infection, the intestine, via monocyte-associated
181 viremia.[62,94,98,109] Indeed, approximately 80% of cats, healthy or with FIP, in
182 households with endemic FCoV infection were shown to harbor FCoV RNA in their
183 blood monocytes, and healthy cats remained viremic over the 12 month test period.[62]
184 Furthermore, it was shown recently that intraperitoneal inoculation with FECV can,
185 albeit only occasionally, lead to virus shedding with the feces, which confirms FECV

186 spread also from extraintestinal sites, again most likely via monocyte-associated
187 viremia.[133]
188 In order to test whether the difference between FECV and FIPV is based on the
189 exclusive capability of FIPV to replicate in feline monocytes, a new method was
190 developed that demonstrates replicating virus in the blood, through the specific
191 detection of viral M protein mRNA.[145] The protocol was applied in two studies on
192 naturally infected cats and showed that FCoV can replicate within monocytes in
193 healthy cats. However, while the first study found a strong correlation between virus
194 replication in the blood and FIP, the second did not confirm this finding.[24,145]
195 Subsequently, a quantitative method detected high levels of viral replication in cats
196 suffering from FIP.[84] Furthermore, a recent experimental study showed that after
197 oronasal infection with known FECV isolates, only very few cats develop viremia, and
198 without evidence of viral replication.[164]
199 Initial molecular studies on FECV and FIPV isolates identified deletions in the 3c, 7a
200 and 7b genes in FIPV and indicated that FECV are the ancestors of FIPV.[89,162] Also,
201 deletion of the 3a-c and/or 7a/7b genes from wild type FIPV II (79-1146) in an
202 attempt to generate FIP vaccines led to the loss of virulence in experimental
203 infections.[67] This initial work was followed by a more rigorous search for virulence
204 markers. The sequencing of structural (S, E, M, N) and accessory (3a-c, 7a and b)
205 genes of FCoV from feces and diseased tissues of cats with FIP identified significant
206 mutations only in the 3c gene; these resulted in variable truncation of the 3c protein.
207 Virus with the mutated 3c gene was identified in diseased tissues, whereas the FCoV
208 in the feces generally exhibited an intact 3c gene, and only in some cases also the
209 mutated form.[132] Together with the results of a previous study, this indicated a role of
210 3c gene deletions in the viral switch from FECV to FIPV.[132,162] However, the fact that

211 3c gene deletions were not consistently observed with FIP suggested that additional
212 factors are essential for the acquisition of the FIPV pathotype.[162]
213 Two larger subsequent studies compared the 3c gene of FCoV from healthy cats
214 (FECV) and cats with FIP (FIPV) to further assess its role as a virulence marker.
215 Almost all FECV carried an intact 3c gene.[28,133] After oronasal inoculation with such
216 FECV isolates, cats became infected and shed the virus with the feces.[133] In
217 contrast, the majority (71%) of FCoV from cats with FIP exhibited 3c mutations, i.e.
218 deletions and insertions that were sometimes associated with severe truncation and
219 loss of function. Interestingly, FCoV identified in the feces of cats with FIP generally
220 exhibited an intact 3c gene, which was interpreted as an indication of FECV
221 superinfection.[26] Nonetheless, similar to a previous study, both these studies
222 identified FIPV with an intact 3c gene in diseased tissues in a substantial proportion
223 of cats with FIP (29% and 40% respectively).[26,133,162] Furthermore, the FCoV with an
224 intact 3c gene were shown to be indeed FIPV, since they induced FIP after both
225 oronasal and intraperitoneal inoculation.[133] Interestingly, when shed with the feces,
226 the latter was not infectious to cats.[133] These findings lead to the following
227 conclusion: FCoV need to carry an intact 3c gene to be able to sustainably replicate
228 in the intestinal epithelium and be infective to other cats. However, an intact/non-
229 truncated 3c gene does not prevent a FCoV from inducing FIP even after oronasal
230 inoculation. The potential significance of the observed higher frequency of non-
231 synonymous amino acid changes towards the 3' end of FIPV with intact 3c genes
232 needs to be further investigated.[133]
233 A non-targeted approach has recently been taken in an attempt to identify further
234 mutations that might be responsible for the change in virulence. Each 11 randomly
235 selected FECV (from healthy cats) and FIPV (from cats with FIP confirmed by post

236 mortem examination) were compared, based on full genome sequencing. Differences
237 were found scattered along the entire genome, but a larger genetic variation with two
238 hot spots was identified in the S gene. Subsequent sequencing and phylogenetic
239 analysis of more isolates identified the two alternative codons in the S gene in more
240 than 95% of the examined FIP cases. Both mutations occur in the supposed fusion
241 peptide of the S protein, but without any evidence of potential functional
242 consequences.[28] However, due to the mutation rate of RNA viruses and the relative
243 rarity of FIP, the authors concluded that the identified S gene mutations are unlikely
244 solely responsible for the FECV-FIPV virulence switch.[28]
245 A group that has worked extensively on antibody mediated enhancement (ADE) in
246 FIP used FCoV that are resistant to virus neutralizing monoclonal antibodies to
247 search for virulence markers. Their so-called mar-mutant viruses all exhibit mutations
248 in several amino acids in the S1 region.[91] However, when orally administered to cats,
249 only some were found to induce FIP. None of the latter carried mutations in ORF 2-7
250 other than those observed in the S1 region, whereas the avirulent viruses also
251 showed deletions in the 7b gene.[154]
252 A further molecular study on natural cases identified a relatively high diversity of the
253 N protein in endemically infected cat groups, but without any pattern or relation to
254 virulence.[12] Furthermore, analysis of nucleotide substitutions identified residues in
255 the N protein that were subjected to positive selection. These could represent
256 antigenic immunodominant sites, indicating the antigenic role of the N protein in
257 stimulating cell-mediated immunity.[12]
258 *In vitro* studies complement the *in vivo* approaches and provide strong evidence that
259 FCoV virulence requires the ability to productively and sustainably infect feline
260 monocytes. This was first indicated in an older study which demonstrated less

261 effective and shorter replication of avirulent FCoV than FIPV in feline peritoneal
262 macrophages and was more recently confirmed in isolated feline monocytes, in
263 which a FIPV (79-1146) established sustainable replication, whereas a FECV (79-
264 1683) could replicate, but not sustainably.[38,149] It needs to be emphasized though that
265 despite rapid virus binding, internalization and disassembly, even the replication of
266 FIPV is limited to a very small proportion of macrophages and monocytes.[38,149,160]
267 This strongly suggests that most monocytes/macrophages are resistant to the virus
268 at the time of infection, most likely due to inhibition of genome release and/or
269 translation.[160]
270 Attempts have been made to relate the monocyte/macrophage tropism to differences
271 in the viral protein structure. In BMDM cultures, FECV (79-1683) was shown to infect
272 fewer cells than FIPV (79-1146) and appeared unable to spread the infection.[140] This
273 was determined by the S protein alone, and, interestingly, by the membrane-proximal
274 S2 domain involved in virus mediated membrane fusion, and not the receptor binding
275 S1 region.[140] In agreement with these findings, a recent study found that deletions in
276 the S1 gene region did not affect the viral capacity to productively infect feline
277 monocytes. However, a N-terminal 29 amino acid deletion in the 7b gene lead to a
278 decrease in virulence, although one of these mutants still retained the capacity to
279 productively infect macrophages, suggesting that this ability is not mediated by ORF
280 7b.[154]
281 Full sequencing of the FIPV type II strain DF2 has shown that it carries a 338-nt
282 deletion in the ORF3abc, resulting in the truncation of 3a and 3c and the complete
283 loss of ORF3b. This virus replicated efficiently in isolated feline monocytes. When the
284 DF2 ORF3abc was replaced with a genetically closely related, intact CCV ORF3abc
285 region, the recombinant virus was able to replicate in feline monocytes, but yielded

286 significantly lower virus titers.[9] These results are in contrast to those of an
287 investigation into the relevance of the ORF3 and 7 proteins for the replication of
288 FCoV in monocytes published a year later, using the type II FIPV 79-1146, which is
289 thought to obtain its virulence through its S protein structure but has also been shown
290 to have a truncated 3c.[35,140] The use of genetically modified viruses with deletion of
291 ORF 3abc (FIPV-Δ3), 7ab (FIPV-Δ7) or both (FIPV-Δ3Δ7) demonstrated a lower, but
292 sustainable replication capacity in the absence of 3, whereas viruses lacking 7ab
293 could only undergo one replication cycle.[35,67] Since ORF 7 are located at the 3' end
294 of the genome where transcription begins, 7a and 7b are produced very early in viral
295 replication; it was therefore concluded that they might neutralize the innate immune
296 response to the virus during the early phase of infection, for example by
297 counteracting the IFN-mediated induction of an antiviral state, resulting in inhibition of
298 viral replication.[35,44] A contradiction remains to be clarified since Rottier and co-
299 workers showed in their study that mutants lacking the 7b gene could still
300 productively replicate in macrophages and mentioned, without showing the results,
301 that the same applied for mutated viruses lacking both 7a and 7b.[140] This difference
302 might be related to the use of different cells in both experiments, since Rottier
303 infected BMDM, whereas the other study used peripheral blood-derived
304 monocytes.[35,140]
305 In summary, while promising, the above results from several studies do not yet
306 provide a conclusive picture (Table 1). This is likely also due to the general diversity
307 of the study material, in particular with regard to the virus isolates, but also the
308 methodological approaches that have been taken.[131]
309
310 *Prevalence*

311 FIP is currently the leading infection cause of death in cats.[131] However, despite the
312 generally high prevalence of FCoV infection in the cat population, which can exceed
313 90% in multicat environments, FIP morbidity is low and rarely surpasses 5% of
314 infected cats.[41,131] In larger cat groups, the proportion of chronic shedders and the
315 overall frequency of virus shedding represent risk factors.[50] FIP is a disease of young
316 (6 months to 2 years), purebred, male intact cats.[139] Purebred cats appear to be
317 more susceptible also to FCoV infection in general, since they were overrepresented
318 when healthy mixed populations were screened.[48,101] A recent study indicates that
319 the breed predilection is restricted. While Abyssinians, Bengals, Birmans,
320 Himalayans, Ragdolls and Rexes were found to have a significantly higher risk for
321 the development of FIP, Burmese, Exotic Shorthairs, Manxes, Persians, Russian
322 Blues and Siamese cats did not exhibit an increased risk.[134]
323
324 *Transmission, shedding, and persistence of FCoV*
325 FCoV are transmitted via the fecal-oral route and primarily infect enterocytes.[129] Cats
326 can become persistently infected and generally remain healthy, despite systemic
327 infection, indicating that healthy (FECV) carriers play a key role in the epidemiology
328 of FIP.[3,4,62,77,94,100,109] FCoV are shed with the feces, and carrier animals have been
329 shown to shed the virus intermittently for months.[4,49,69,78,109] Furthermore, there is
330 evidence of a correlation between shedding frequency and intensity and high
331 antibody titres.[71]
332 Experimental studies with type I FECV isolates have demonstrated consistent
333 shedding as early as two days and for up to 2 weeks post infection (pi), with a
334 subsequent decline in fecal viral loads and intermittent shedding up to 20 weeks after
335 this period.[100,109,164] They confirmed previous studies in which oral administration of

336 cell culture-adapted FIPV (Wellcome strain) led to viral antigen expression in the
337 small and large intestine between day 1 and 7 pi, and restriction to cecum and colon
338 on day 14.[148] After clearance from the small intestine, FCoV can apparently spread
339 from the persistently infected colon at later stages, leading to renewed shedding.[100]
340 In natural FIP cases, shedding can occur until death.[3,147] However, compared to
341 diarrhoeic or healthy shedders, the amount of replicating virus shed by cats with FIP
342 is only very low, and it is significantly lower in the gut than in organs.[84,132]
343 Despite the generally strong evidence that only FECV and not FIPV are transmitted
344 between cats, a recent study confirmed that the FCoV with a truncated 3c gene
345 found in diseased tissues are occasionally also present in the feces of cats with
346 FIP.[132] While this would suggest that horizontal transmission is possible, a
347 subsequent study indicated that oronasal uptake of shed, fecal FIPV does not lead to
348 FIP.[133] It remains to be clarified whether this is a universal characteristic of FIPV.
349 This could be of relevance for epizootic FIP outbreaks, which are defined by the
350 occurrence of FIP in more than 10% of cats in high prevalence establishments, the
351 percentage can be lower in an environment with generally very low FIP
352 prevalence.[41,131,136] In this context, a closer look into an "artificially induced" FIP
353 outbreak that we monitored a number of years ago is of interest. As part of a trial to
354 investigate the efficacy of a FIP vaccine, a multicat environment similar to an animal
355 shelter was created. This housed 40 specific pathogen free (SPF) cats (20 female,
356 20 male neutered) to which 10 clinically healthy cats (aged 6 months to 3 years) from
357 different animal shelters were introduced. The latter had been selected since they
358 tested positive for circulating FCoV immune complexes.[94,96,98] Within one week after
359 introduction of the shelter cats and in which fighting for the establishment of
360 hierarchies occurred, several animals developed transient cat flu symptoms. These

subsided, but the first FIP cases occurred in week 6 (n=4), followed by a peak in weeks 7, 8 (each n=4) and 9 (n=3), with further individual cases in weeks 14 through to 22; a total of 23 animals (45%; 22 (55%) SPF cats, 1 shelter cat) succumbed to the disease. Most SPF cats that died with FIP (18; 82%) had shown previous or concurrent flu symptoms.[106] Only 14 (35%) SPF cats survived the challenge period of 21 weeks. They had all become infected, had shed virus at least intermittently, and exhibited histological features in the hemolymphatic tissues that indicate a strong immune response to the virus.[94,96,99] The characteristics of this experimental FIP epidemic, using natural infections with field viruses, support assumptions that the occurrence of outbreaks is associated with factors related to the environment (such as crowding, concurrent infections, long-term exposure to shedders), the virus (such as virulence, replication rate and mutation rate of the strain) and the host (individual differences in the immune response to FCoV).[41] It also appears likely that horizontal FIPV transmission played a role in this particular case. It might indeed be the generally low amount of FIPV that is shed with the feces if at all, and the inability of at least some FIPV shed with the feces to induce FIP that prevent more frequent horizontal transmission of the disease.[132,133]

The main site of FCoV persistence is the colon, where viral antigen has been found in differentiated enterocytes.[78,100,164] However, virus can also be detected in other tissues in the absence of viremia, and has been shown to infect tissue macrophages.[100] Also, there is evidence of recurrent systemic spread.[100] These findings suggest that viremia and, ultimately, FIP can develop in infected animals at any stage after initial viremia, even when the virus is cleared from the gut.[100]

386 **Pathologic features of FCoV infection and FIP**

387

388 *FIP*

389 The name given to the disease in the 1960s acknowledges the consistent main gross
390 pathological finding, a peritonitis (Figs. 1, 2). Upon gross post mortem examination,
391 FIP is typically characterized by a fibrinous and granulomatous serositis, protein rich
392 serous effusions and/or pyogranulomatous lesions in several organs (Fig. 1). The
393 latter, however, are often very small and only identified by histological examination
394 (Fig. 1e, f). Clinically, a rather clear distinction is made between an effusive (wet or
395 non-parenchymatous) and a non-effusive (dry or parenchymatous) form of the
396 disease, with a proportion of cases being considered in a transition stage between
397 the two forms.[41,71,131] However, the post mortem examination often identifies
398 extensive serosal and parenchymatous granulomatous lesions in organs alongside
399 effusions of a variable quantity (Fig. 1a-d), indicating that mixed forms are indeed
400 more common than clinically appreciated.
401 When the disease was first observed, several reports provided histopathological
402 descriptions of both spontaneous and experimental cases.[19,73,74,166,169-172] Several
403 years later, a few studies attempted to categorize FIP lesions.[16,92,118] Based on
404 distribution, cellular composition, and viral antigen expression, four types of lesions
405 were described: diffuse alterations on serosal surfaces; granulomas with and without
406 areas of necrosis; focal and perivascular B cell and plasma cell infiltrates, and a
407 granulomatous to necrotizing vasculitis; these can be found alongside each other.[92]
408 The distribution of lesions varies in each individual case, but shows a consistent
409 general pattern.[92,96,131,172] Detailed gross, histological and immunohistological
410 examinations that we performed on a large cohort of diagnostic cases to identify all

411 potential lesions, confirmed peritoneal involvement in 75% of the cases, in the
412 majority (69%) associated with abdominal effusion (Fig. 2), sometimes also with
413 effusion in the thorax. Among organs, the kidneys were affected most often, followed
414 by brain and eyes (Fig. 2). The latter were always involved alongside the brain, and
415 in a subsequent study that thoroughly examined confirmed FIP cases for the
416 presence of ocular lesions, 29% (25/86) showed involvement of the eye, in the
417 majority of cases (68%) bilateral (M. Weber, unpublished data), suggesting that the
418 actual involvement of the eyes is generally underestimated.[131] FIP lesions are
419 occasionally seen at unusual sites, such as the tunica vaginalis in cats with
420 peritonitis, the skin or the testicle.[23,34,144]
421 Interestingly, the general distribution of the FIP vasculitis is relatively limited. It mainly
422 affects small and medium sized veins in leptomeninges, renal cortex (stellate veins)
423 and eyes (mainly venules in iris, chorioidea and retina), less frequently in lungs and
424 liver.[97]
425 For natural FIP cases, an incubation period is not known. However, we gathered
426 some information on the time log between the onset of FCoV exposure and overt
427 disease; when we introduced several FCoV carriers into a large group of SPF cats
428 housed together in a shelter-like multicat environment, the first clinical signs of FIP
429 were detected after 6 weeks.[106] After experimental infection, however, the incubation
430 period has been shown to range between 2 and 14 days for the effusive and several
431 weeks for the dry form.[36,123]
432 The clinical course of FIP in natural cases is usually quite rapid for the wet form, but
433 can take several weeks in particular for the dry form.[131] We observed a clinical
434 course of 6 to 42 days (average: 14 days) prior to death in the above mentioned
435 group of naturally infected SPF cats.[106] However, evidence of subclinical or

436 protracted disease over a period of weeks to months has been reported.[131] Also, an
437 experimental longitudinal study demonstrated recurring waves of clinical disease,
438 where fever and weight loss coincided with T cell depletion and increased viral loads
439 in the blood.[36] From such data, histological findings suggestive of "disease waves"
440 would be expected, and indeed, these can be observed. As reported, the typical
441 serosal FIP lesions often exhibit an underlying layer of B cells and plasma cells, and
442 some of the latter contain FCoV-specific antibodies.[92] We observed occasional
443 natural cases with serosal lesions dominated by a thick plasma cell layer and
444 evidence of granulation tissue formation with superficial granulomas and/or a
445 fibrinous exudate (Figs. 3, 4). There is histological evidence that in FIP granulomas,
446 macrophages are progressively replaced by B cells and plasma cells and that the
447 typical FIP perivasculitis can develop into the focal and perivascular B cell and
448 plasma cell infiltrates that are frequently observed, for example, in leptomeninx and
449 mesentery.[92,97] These findings indicate that the humoral immune response can limit
450 disease progression at least to some extent or for a limited time.
451 The typical FIP vasculitis is a phlebitis, mediated and dominated by activated virus
452 infected monocytes, with only few T cells and neutrophils. On the basis of these
453 features it can be distinguished from an immune mediated vasculitis, including an
454 immune complex vasculitis.[97] However, in fulminant cases, necrosis of the vessels
455 has been seen.[19,73,169] Interestingly, such an acute necrotizing vasculitis can
456 occasionally be observed in veins with apparent previous changes (Fig. 5) which
457 further confirms the multiphasic nature of the disease. However, due to the
458 morphological features of the acute vascular lesions, it also provides evidence that a
459 type III hypersensitivity reaction does contribute to the pathogenesis at least in some
460 cases.[19,73,111,169,170]

461 It has long been suspected that some cats can survive clinical FIP.[126] In the above-
462 mentioned longitudinal experimental study, the majority of cats died from FIP, but
463 several animals that had undergone one or more episodes of clinical disease
464 survived the 4-month study period and were seen to be "free of lesions" post
465 mortem.[36] Similarly, in our longitudinal study undertaken on naturally infected SPF
466 cats, a proportion of SPF cats became infected but survived the experiment despite
467 consistent direct contact with virus shedding carriers for 7.5 months, from the age of
468 21 weeks onwards.[94,106,109] All survivors had remained clinically healthy throughout
469 the experiment, apart from two cats that had developed uveitis. This did not resolve
470 in one animal which also showed transient non-specific clinical symptoms. The post
471 mortem examination of the latter revealed a moderate chronic B cell and plasma cell
472 dominated leptomeningitis and perivascular encephalitis as well as a severe
473 mononuclear conjunctivitis, iridocyclitis and perineural leptomeningitis with FCoV
474 antibody-positive plasma cells in the infiltrates (Fig. 6). These findings support the
475 results of an older experimental vaccine study and provide further evidence that
476 lesions can remain limited, and that macrophages in lesions can be replaced by B
477 cells and plasma cells with time.[8,126]
478 Some animals can apparently confine the disease locally, at least for some time. One
479 case series reported FIP lesions restricted to the mesenteric lymph nodes, and
480 another single mural intestinal lesions, partly in association with local lymph node
481 involvement.[72,95] These findings suggest a strong local response to the virus. In the
482 latter cases, however, progression to overt FIP was generally observed despite
483 surgical removal of the intestinal lesions.[72]
484 The lymphatic tissue of cats with FIP generally exhibits B and T cell depletion.
485 However, in the majority of cats, this occurs with previous follicular hyperplasia and is

486 associated with markedly increased numbers, proliferation and activation of
487 macrophages in the splenic red pulp, lymph node sinuses and bone marrow.[92,96,99]
488
489 *Coronavirus enteritis*
490 FECV is generally regarded as the avirulent pathotype of FCoV and indeed, in older
491 cats, oral FECV infection does lead to no or only very mild, non-specific clinical
492 symptoms, such as transient anorexia.[164] However, in young SPF kittens, at an age
493 when animals would usually be protected by maternal antibodies, oral FECV infection
494 can induce severe enteritis.[1,125] There have also been reports of fatal coronavirus
495 enteritis in naturally infected juvenile and adult cats. Affected cats presented with
496 catarrhal to hemorrhagic enteritis and immunohistology confirmed that the virus
497 infected the fully differentiated villous epithelial cells.[75,93,108]
498
499 *Coronavirus infection without FIP*
500 In an environment of high infection pressure, such as a FIP outbreak, cats become
501 FCoV-infected and develop monocyte associated viremia and a systemic immune
502 response to the virus, as reflected by the development of antibody and circulating
503 immune complex titres. This is associated with distinct T and B cell hyperplasia in
504 lymphatic tissues and the presence of plasma cells expressing FCoV-specific
505 antibodies which does not prevent virus shedding and viral spread to
506 tissues.[94,96,100,109] A similar reaction of the lymphatic tissue has also been described
507 after experimental FECV infections.[66,109] Interestingly, it is associated with
508 macrophage proliferation in hemolymphatic tissues, similar to, but less intense than
509 that seen in cats with FIP.[99]
510

511

512 **Pathogenesis**

513 The pathogenesis of FIP has been a research focus for several groups in Europe, the
514 USA, and Japan. Although the picture is still not clear, the results of both *in vivo* and
515 *in vitro* studies, though sometimes controversial, have contributed more and more
516 pieces to the jigsaw. At present, three key features have been identified as essential
517 prerequisites for the development of FIP lesions: systemic infection with virulent
518 FCoV, i.e. FIPV; effective and sustainable FIPV replication in monocytes; and
519 activation of FIPV-infected monocytes.

520

521 *Systemic infection with virulent FIPV*

522 Two theories have been proposed for the infection of the host; the "*in vivo* mutation
523 transition" or "internal mutation" hypothesis and the "distinct circulating avirulent and
524 virulent strains" hypothesis.[21] The first model assumes that FIPV arise *in vivo* from
525 mutations of FECV in infected animals, and there is indeed strong evidence that the
526 initially acquired FCoV of most cats is not a FIPV *per se*. Initial comparative genome
527 analyses of FECV and FIPV laboratory and field strains has shown these to occur as
528 closely related pairs.[76,124,162] Also, an experiment performed in chronically Feline
529 Immunodeficiency Virus (FIV)-infected cats showed that FIPV arise *de novo* from the
530 FECV inoculum.[135] Furthermore, many studies demonstrated phylogenetic clustering
531 of FIPV and FECV according to geographic distribution rather than disease
532 phenotype.[10,27,132,135,162] Finally, it is well known that although FECV are endemic in
533 cat populations, FIP develops only sporadically, providing further strong evidence
534 that FIPV are generally not transmitted horizontally from cat to cat, but emerge *de*
535 *novo* in each cat that succumbs to FIP.[131]

536 FCoV exhibit *in vivo* genetic diversity, as shown by the frequent occurrence of viral
537 quasispecies both in individual infected animals and in infected cats from the same
538 household.[11,42,64,102] Indeed, experimental infection with fecal matter of naturally
539 FECV infected healthy cats leads to the occurrence of quasispecies in the large
540 intestine of individual animals.[102] In natural infections, cats with FIP showed more
541 extensive viral quasispecies formation than healthy animals, suggesting that a higher
542 viral mutation rate is relevant for the generation of virulent mutants.[11] Surprisingly,
543 however, recent phylogenetic analyses indicated that the observed genetic diversity
544 mainly applies to type I FCoV, whereas type II viruses are relatively
545 homogenous.[42,107] If this were indeed the case, the results of many recent studies
546 would be called at least partly into question.
547 The "distinct circulating avirulent and virulent strains" hypothesis is based on
548 phylogenetic analyses and suggests that both virulent and avirulent strains circulate
549 in the feline population and that, independently of geographic location, sequences
550 tend to cluster with disease phenotype.[20] The occurrence of occasional FIP
551 epidemics indicates that this theory applies sporadically, while in the majority of
552 cases, evidence supports the internal mutation theory.
553
554 *Effective and sustainable FIPV replication in monocytes*
555 The second essential prerequisite for FIP appears to be the viral capacity to replicate
556 effectively and sustainably in monocytes of the infected host. *In vitro*, both FECV and
557 FIPV can replicate in isolated feline peritoneal macrophages, BMDM, and
558 monocytes, but only FIPV undergo sustainable replication and spread the infection in
559 the culture.[38,140,149] These results support *in vivo* studies which have shown that
560 FCoV infection generally leads to monocyte associated viremia, but that viral

561 replication in the blood (i.e. in monocytes) and viral loads in tissues are generally
562 significantly higher in association with FIP.[62,84,98,109,145] Based on older experimental
563 studies, Pedersen suspected that viral replication in monocytes is very slow at least
564 during the first two weeks after FIPV infection, but then increases rapidly, around the
565 time when specific antibodies occur.[120,131,170] So far, this hypothesis has not been
566 tested in experimentally infected cats. However, it has been shown that FCoV
567 infection of cats induces macrophage/monocyte proliferation in hemolymphatic
568 tissues.[96,99] This is not associated with upregulation of cytokines that stimulate
569 macrophage proliferation in these tissues, and could therefore represent a systemic
570 effect of infected monocytes which we found to transcribe granulocyte-monocyte
571 colony stimulating factor (GM-CSF) and interleukin (IL-)6, cytokines that both induce
572 proliferation and differentiation of monocyte and neutrophil precursors, within hours
573 after *in vitro* FIPV infection (Kipar, unpublished data). The proliferation of
574 monocytes/macrophages likely ensures the supply of viral target cells, i.e. mature
575 circulating monocytes or tissue macrophages. Proliferating macrophage populations,
576 such as macrophages in the splenic red pulp or myelomonocytic cells in the bone
577 marrow, appear not to replicate the virus, since viral antigen cannot been detected in
578 these cells.[96,100] Higher blood cytokine levels due to cytokine release from a larger
579 number of infected monocytes and from macrophages in FIP lesions could account
580 for the more pronounced proliferation and generalised activation of macrophages in
581 hemolymphatic tissues observed in cats with FIP.[99]
582 Based on the fact that FECV can also replicate at least briefly in monocytes, it was
583 recently suggested that monocytes, rather than the intestinal epithelial cells, might be
584 the cells in which the FECV-FIPV mutations occur.[133] Given the high mutation rate of
585 the virus, this would allow positive selection for macrophage tropism and progressive

586 viral adaptation to replication in monocytes/macrophages.[133,149] It would also suggest
587 that viral clearance from the blood and even the intestine might not prevent recurrent
588 viremia and possibly even the development of FIP, since persistently infected,
589 healthy, non-viremic FECV carriers were found to bear virus in tissue macrophages,
590 i.e. in sinus macrophages in mesenteric lymph nodes and in pulmonary intravascular
591 macrophages (PIM).[100] This indicates that macrophages in the intestine take up the
592 virus from enterocytes and carry it to the regional lymph nodes and eventually in the
593 blood. Viral RNA was also detected in the liver where the virus most likely infects
594 Kupffer cells (KC).[100,121] Both PIM and KC phagocytose particles from the blood, but
595 could also replicate and release virus into the circulation or transmit it to monocytes.
596 If the mutation and transformation of FECV to FIPV can take place in these
597 macrophages, this could result in FIP at any time post initial infection.[133]
598
599 *Activation of FIPV-infected monocytes*
600 The morphological hallmark and initiating lesion of FIP is a granulomatous phlebitis
601 and periphlebitis that is mediated by highly activated monocytes, most likely during a
602 phase of high-level monocyte-associated viremia with substantial viral
603 replication.[84,97,145] Studies on natural cases have shown that the phlebitis develops
604 through direct interaction between monocytes and activated endothelial cells. The
605 monocytes strongly express cytokines, such as TNF-α and IL-1β, and adhesion
606 molecules, such as CD18, that allow their interaction with activated endothelial cells,
607 and express enzymes, such as matrix metalloproteinase-9, which dissolve the
608 vascular basement membrane at sites of monocyte emigration; the endothelial cells
609 appear systemically activated and the restrictive distribution of vascular lesinos, i.e.
610 veins and in selected organs, is likely a consequence of selective responsiveness of

611 the endothelium.[97] The observed simultaneous, generalised activation of both
612 vascular endothelial cells and macrophages in hemolymphatic tissues could be
613 mediated by activated monocytes alone, provided they release sufficient amounts of
614 cytokines.[97,99] The latter appears likely, considering also that cats with FIP show
615 increased VEGF transcription in (virus infected) monocytes and increased serum
616 VEGF levels.[155] Furthermore, peritoneal exudate cells (PEC) of cats with FIP exhibit
617 high TNF-α mRNA levels and were previously shown to release IL-1β and IL-6, and
618 even alveolar macrophages collected by bronchoalveolar lavage from FIP cats show
619 significant upregulation of TNF-α, GM-CSF, granulocyte (G)-CSF, IL-6 and other B
620 cell differentiation factors, all suggesting strong generalised monocyte/macrophage
621 activation in response to FIPV.[59,60,150,152,153]
622 What ultimately triggers the fulminant monocyte activation in infected cats is not yet
623 known. However, FIPV infection of the monocytes is apparently an essential
624 prerequisite, which was recently shown *in vitro* in isolated feline monocytes and
625 macrophages. FIPV rapidly induced activation of the p38 mitogen-activated protein
626 kinase (MAPK), which directly regulates the expression of proinflammatory cytokines
627 via phosphorylation of a range of signaling molecules, in PBMC, likely early during
628 entry and, though less intensely, between 6 and 12 hpi, when virus is being
629 produced.[38,104,137] This was associated with the induction of TNF-α and IL-1β, but not
630 IL-6 production, as demonstrated in the PBMC supernatant at 24 hpi.[137] VEGF
631 transcription was shown to be significantly upregulated at 48 hpi in isolated feline
632 monocytes and alveolar macrophages, and feline alveolar macrophages showed
633 increased TNF-α production at 48 and 72 hpi, all exclusively in association with viral
634 replication.[155] A similar increase was seen for G-CSF and GM-CSF transcription at
635 72 hpi; however, upregulation only became significant and TNF-α and VEGF levels

636 were further increased, when cells were inoculated with virus in combination with an
637 antibody against the FCoV S protein that is known to induce ADE.[147,152,153,155]
638
639 *The immune system in FIP and FCoV infection*
640 With the aim to identify the role of the immune system in the pathogenesis of FIP, the
641 blood cytokine transcription (IL-4, IL-6, IL-10, IL-12 p40, IL-18, IFN-γ, TNF-α) was
642 monitored in experimentally infected animals. A first study reported an initial mild
643 increase in IL-6 and IFN-γ transcription in PBMC that correlated with transient
644 pyrexia, followed by a drop of all other examined cytokines and IFN-γ, possibly as a
645 consequence of the lymphopenia that developed simultaneously.[63] A second study
646 demonstrated TNF-α upregulation during the development of FIP.[103] Another group
647 then screened serum VEGF levels by ELISA and found an increase in association
648 with body effusions.[155] In natural FIP cases, at the time of death, the blood showed
649 very high interindividual variation in cytokine mRNA levels; however, IFN-γ mRNA
650 was generally scarce or absent.[53] Blood IFN-γ levels of FIP cats were then found to
651 be similar to those of healthy carriers, but high IFN-γ concentrations were present in
652 effusions.[56] These were considered a likely consequence of the observed IFN-γ
653 transcription within lesions.[16] Believed to be released by the T cells in the lesions,
654 IFN-γ could be responsible for macrophage attraction and local activation, which
655 would also enhance Fc receptor expression on their surface and thereby virus uptake
656 and replication.[16] In light of the above results, the generally variable pathological
657 changes and in particular the apparent multiphasic nature of the disease, it is
658 possible that the fulminant monocyte activation, which is essential for the
659 development of FIP vasculitis, does only occur as brief bouts, followed by a phase in
660 which self-sustained granulomatous lesions develop. Such cytokine peaks might be

661 missed when PBMC or whole blood are sampled on a regular basis or immediately
662 prior to death.
663 FCoV infection of cats, regardless of the development of FIP, initiates a humoral
664 immune response, as evident by the development of antibody titres and,
665 morphologically, the formation of secondary follicles in lymphatic tissues, and by the
666 presence of FCoV-specific antibodies within plasma cells both in lymphatic tissues
667 and in older FIP lesions.[41,92,94,96,99,164] Some cats can eliminate the infection and then
668 become reinfected, either with a different or the same virus strain, but tend not to
669 develop FIP as a consequence of this.[4,5,49] It is assumed that protective immunity is
670 mainly cell mediated, and there is evidence that the development of high anti-S titers
671 and a high S:M antibody ratio leads to virus clearance.[60,131] However, in many
672 animals, the antibodies that are formed, and in clinical FIP often with very high titres,
673 are not able to eliminate the virus and/or the infected cells. In contrast, experimental
674 studies provided evidence that ADE plays a role in FIP.[122,127,170] FIPV ADE is based
675 on the enhanced uptake of virus and anti-S antibody by macrophages/monocytes via
676 the Fc receptor and can occur *in vivo* when immunized cats are infected with FIPV of
677 the same serotype.[29,113,151] It has however only been seen with some highly virulent
678 FIPV and appears to be of less relevance in the natural disease.[2,131] While ADE
679 could explain the rapid spread of infection in the monocyte/macrophage population, it
680 would not explain why the infected cells are not eliminated by the immune system.
681 The latter can be explained by recent *in vitro* studies. In the presence of specific
682 antibodies, FCoV infected isolated feline monocytes rapidly internalize the viral
683 glycoprotein that is expressed on the surface as antigen-antibody complexes.[39,40]
684 The internalization is mediated by the S and M proteins and, interestingly, does occur
685 with both FIPV and FECV. FIPV also inhibits the complement mediated lysis of

686 infected cells, even if they express viral antigen on their surface.[30] This process is
687 independent of the accessory 3 and 7 proteins, but the underlying mechanism has
688 not yet been identified.[30] Nonetheless, the internalization of FCoV proteins by
689 infected cells may play a role in the pathogenesis of FIP, since the lack of an immune
690 response that eliminates infected cells will allow more pronounced virus production
691 and/or a quiescent infection state.[39] This phenomenon could also allow the virus to
692 persist in tissue macrophages.[100]
693 As mentioned, there are marked differences between the composition and activity of
694 the hemolymphatic tissues in FCoV-infected cats with and without FIP. Whereas
695 healthy carriers exhibit distinct lymphoid hyperplasia with lymphocyte proliferation,
696 FIP cats show depletion which is morphologically reflected in the often markedly
697 depleted, though mainly secondary, lymphatic follicles, the depletion of T cell zones,
698 and in particular the generally marked thymus atrophy.[36,94,96] This is mainly a
699 consequence of lymphocyte apoptosis, and TNF-α expression by lymphocytes, in
700 particular in lymphatic tissues with FIP lesions, might be the underlying
701 mechanism.[33,66,96] The findings are matched by the marked blood lymphopenia
702 observed in the terminal stage of FIP and a persistent drop in circulating CD4+ and
703 CD8+ T cell numbers as well as an increased rate of apoptosis in PBMC in the
704 course of the disease.[33,36,150] Among PBMC, mainly CD8+ cells were shown to be
705 affected by apoptosis, and PEC and PBMC of cats with FIP seem to produce the
706 necessary TNF-α to mediate this.[150] An *in vivo* study on experimentally infected cats
707 confirms this finding, as it demonstrated increased TNF-α transcription in PBMC of
708 cats that developed FIP, whereas animals that remained healthy had low TNF-α and
709 high IFN-γ mRNA levels.[103] High IFN-γ transcription, together with an increase in IL-
710 1β, was also seen in the blood of naturally infected healthy carriers regardless of

711 antibody titres and virus shedding, prior to the occurrence of FIP cases in the
712 catteries. This was considered a consequence of an increase in CD8+ T cells and
713 coincided with the release of acute phase proteins, indicating that cytokine
714 production by PBMC contributes to the protection of FCoV infected cats against
715 FIP.[53]
716 Despite the obvious functional differences in the T and B cell component, FCoV
717 infection is associated with proliferation and activation of monocytes/macrophages
718 and their precursors in hemolymphatic tissues of infected cats and, to a higher
719 degree, cats with FIP.[96] In an attempt to identify the underlying mechanism,
720 hemolymphatic tissues of FCoV infected cats with and without FIP were assessed for
721 the transcription of cytokines that mediate macrophage activation.[99] SPF cats served
722 to establish constitutive transcription levels, which highlighted the general flaw of any
723 such "global expression" study; the high variability in individual transcription levels
724 even in gender and age matched SPF animals, let alone a group as heterogeneous
725 as naturally infected cats with FIP with regard to age, gender, disease type and
726 stage.[53,99] Nonetheless, some relevant differences in the cytokine transcription were
727 identified. Naturally infected healthy cats exhibited significantly higher IL-10
728 transcription levels in the spleen and lower IL-6, G- and M-CSF levels in mesenteric
729 lymph nodes than cats with FIP, whereas FIP was associated with significantly lower
730 IL-12 p40 mRNA levels in lymphatic tissues.[99] A similar trend of reduced IL-12 p40
731 transcription was found in lesioned vs. virus-free mesenteric lymph nodes of cats with
732 FIP and in brains with FIP lesions.[33,51] This indicates that an effective immune
733 response together with an IL-10 mediated limitation of macrophage activation and
734 increased cellular cytotoxicity allows infected cats to limit the viral infection and
735 remain healthy, whereas a lack of IL-12 inhibits an effective immune response and

736 allows monocyte/macrophage activation and ultimately FIP, probably as a
737 consequence of impaired T cell-mediated macrophage activation.[99] The fact that
738 cytokines produced by macrophages and known to activate these and induce their
739 proliferation (such as G-, M- and GM-CSF, IL-6, TNF-α) were not or not significantly
740 upregulated in these tissues despite the presence of abundant activated
741 macrophages was considered as further evidence of a systemic effect, i.e. the
742 release of the relevant cytokines by infected, activated monocytes.[99]
743 Several attempts have been made to match the pathogenesis of FIP with known
744 immunopathogenic mechanisms. For example, FIP has long been considered an
745 immune complex-mediated type III hypersensitivity disease, since fibrinogen and C3
746 was demonstrated cell free and viral antigen, IgG and complement within leukocytes
747 in vascular and focal granulomatous-necrotizing lesions. Also, cats with FIP exhibit
748 FCoV-specific immune complexes in blood and glomerula and show high γ-globulin
749 and C3 serum levels.[85,86,117,122,169,170] However, circulating FCoV-specific immune
750 complexes are not only found in diseased animals, but can also be detected, at least
751 transiently, in infected cats that remain healthy.[92,109] Also, the typical FIP vasculitis
752 does not show features of immune complex vasculitis.[97] While this does not confirm a
753 type III reaction as the essential pathogenic mechanism, it does not exclude its
754 contribution to the disease, for example in its acceleration (see above). Other authors
755 have considered a type IV hypersensitivity reaction as the basis for the development
756 of the granulomatous lesions, due to the dominance of CD4+ cells in the otherwise
757 macrophage dominated lesions.[16,118] In any case, the available data provide definite
758 evidence that the immunity to FIP is cell mediated and it has been postulated that it
759 requires viral persistence.[131] Furthermore, there is evidence from both experimental

760 and natural infections that an effective early T cell response to FCoV is essential for
761 the prevention of FIP, since it appears to ensure the limitation of viral replication.[36,119]
762 There has been a lot of speculation why cats develop the dry, the wet or a mixed
763 form of FIP, and it is widely agreed that a strong humoral together with a very weak
764 cellular immunity leads to the former, with lesser effusion with increasing cellular
765 immunity.[131] However, a recent study provided evidence that also the effusions are
766 initiated by FIPV-infected monocytes/macrophages, since the latter have been shown
767 to produce VEGF.[155] VEGF is a very strong mediator of vascular permeability and
768 does cause hyperpermeabilty also of feline vascular endothelial cells.[43,155] Also,
769 serum VEGF levels were seen to correlate with the quantity of body effusions.[155]
770 These findings do not necessarily contradict the existing theories, but are very much
771 in line with all other data that render the infected, activated monocyte the key
772 mediator of the disease.
773 Several studies have provided data that indicate individual differences in
774 susceptibility to FCoV infection in general and to the effect of FIPV on monocytes.
775 Molecular studies found evidence that individual cats can be entirely resistant to
776 FCoV infection and that the monocytes of some cats can be completely resistant to
777 FCoV, or to FECV alone, while the monocytes of some cats are more prone to
778 productive FCoV infection than those of others.[4,5,38,157] Finally, there appear to be
779 differences in FIPV serotype I internalization in individual cats, suggesting that its
780 receptor is expressed differentially between cats.[160] At present, the knowledge is still
781 lacking as to the reason for the individual differences and the key event that blocks
782 the capability of monocytes to inhibit virus production.
783
784 **Diagnosis of FIP**

785 The post mortem diagnosis of FIP relies on a combination of gross and histological
786 examination, in combination with the demonstration of viral antigen in the lesions.[57] In
787 contrast, the non-invasive ante-mortem diagnosis of FIP still remains a challenge,
788 especially in the dry form of the disease.[71,95,131] A combination of indirect and/or
789 direct virus detection with evaluation of blood hematological and chemical parameter
790 as well as medical history and clinical symptoms, the so-called FIP algorithm, is so
791 far the best predictor of disease.[6]
792
793 *Host blood parameters*
794 Common blood alterations include lymphopenia, mild to moderate regenerative
795 anemia, hyperproteinemia, and hypergammaglobulinemia. Other laboratory
796 parameters, such as liver enzymes, bilirubin, urea and creatinine might be helpful,
797 but high values merely reflect organ damage, which is most likely a consequence of
798 FIP lesions.[6]
799 More than the single parameters, the albumin to globulin ratio (A:G ratio) has a high
800 diagnostic value, and at values above 0.8, FIP is extremely unlikely.[6,70] A more
801 recent retrospective study evaluating the A:G ratio showed a very poor positive
802 predictive value (PPV) even for a cut-off value of 0.6. However, the negative
803 predictive value (NPV) was 100% and 99% for an A:G ratio of <0.8 and <0.6%,
804 respectively.[88]
805 Recent studies have focused on the diagnostic value of an acute phase protein,
806 alpha-1 acid glycoprotein (AGP). Serum levels are highly elevated in cats with FIP (>
807 3 mg/ml), but are also high in other inflammatory conditions or neoplastic diseases,
808 such as lymphoma.[25,31,141] Furthermore, AGP levels may also rise in asymptomatic
809 FCoV carriers, especially from households with endemic infection.[54] However, when

810 interpreted alongside pre-tests, i.e. epidemiological factors, clinical information and
811 FCoV serology, moderate AGP increases are useful discrimination parameters when
812 the probability of FIP is high, whereas with low FIP probability, only very high AGP
813 levels support the diagnosis of FIP.[120] A recent retrospective study found complete
814 concordance between AGP levels and immunohistology in challenging diagnostic
815 cases.[54]
816
817 *Analysis of effusions*
818 The presence of effusions facilitates the diagnosis, since tests on effusions have a
819 higher diagnostic value than blood tests.[6,70] FIP effusions typically have a very high
820 protein content (>35 g/l), but a low cellularity (<5000 nucleated cells/ml) with a
821 dominance of macrophages and neutrophils. When sufficient cells are present, the
822 demonstration of viral antigen in macrophages confirms the diagnosis with a very
823 high PPV.[22,70,71]
824 The Rivalta test, commonly used to differentiate between FIP effusions and effusions
825 due to other diseases, is not very specific. The high protein content, including fibrin
826 and inflammatory mediators, in FIP effusions normally induces a positive reaction.
827 However, a recent study on a large cohort of cats with effusions has shown that,
828 while it has a high NPV, this test has a lower sensitivity, specificity, and PPV than
829 previously reported.[47,71]
830 A:G ratio can also be measured in effusions with high PPV if the ratio is <0.4, and
831 with high NPV if the ratio is >0.8.[142] Also, very good correlation exists between AGP
832 values in effusions and serum.[14] The demonstration of FCoV-specific antibodies in
833 the effusions is only meaningful when the titre is high (≥ 1:1600), whereas the
834 absence of antibodies has a good NPV.[70]

835

836 *Indirect virus detection: serology*

837 Serology, based on the detection of FCoV antibody titres by a range of methods
838 (immunofluorescence, ELISA, rapid immunomigration) is widely used also
839 commercially to assist in the diagnosis of FIP and for quarantine purposes. The tests
840 are applied to blood and effusions, and apparent false negative results are a known
841 problem. A recent study addressed this issue and showed a correlation of lower
842 antibody levels in samples containing higher amounts of virus, as shown by qRT-
843 PCR.[110] It was hypothesized that these false negative results were due to antibody
844 binding to virus in the sample instead of the virus in the serological tests.[110]
845 Also, a high percentage of healthy FCoV carriers are antibody-positive, of which only
846 a small percentage develops FIP.[1,146] Very high titres (≥ 1:1600) in combination with
847 pre-tests that suggest FIP indicate an increased likelihood of FIP, unless obtained
848 from animals in an endemic environment, such as multiple-cat households.[70,130] It
849 should also be noted that different methodological approaches and even different
850 laboratories might yield different results from the same sample, depending on the
851 antigen used.[71]
852 Serology is generally considered a useful tool for the screening and management of
853 catteries and quarantine purposes, in particular since antibody titres are correlated to
854 shedding intensity and frequency.[41]
855 The detection of circulating antigen-antibody complexes, for example by a
856 competitive ELISA on serum, was shown to have a PPV of 67% and a NPV was
857 84%.[70,85] However, healthy FCoV carriers can also show circulating immune
858 complexes.[109]

859

860 *Direct virus detection*

861 Immunohistology has been used for two decades to detect FCoV antigen in lesions
862 and has a very high PPV.[22,92,118,156] It is therefore considered the gold standard and
863 by many diagnostic pathologists an essential component of the definite diagnosis of
864 FIP, in particular in histologically inconclusive cases.[41,57,131] For the definite ante
865 mortem diagnosis, optimally, surgical biopsies of granulomatous lesions are used
866 (Fig. 7), while random Tru-cut biopsies or fine needle aspirates are often not
867 helpful.[55] The demonstration of FCoV antigen in macrophages in effusions, as
868 mentioned above, is an alternative, non-invasive tool for the *intra vitam* diagnosis of
869 FIP. A positive result is highly predictive of FIP, whereas a negative result does not
870 exclude FIP.[22,70] The latter is due to both the low cellularity of the effusions and the
871 relatively low sensitivity of the method, which can only detect heavily virus laden
872 cells. In the authors' experience, based on the parallel staining of cytological
873 preparations and formalin-fixed, paraffin embedded cell pellets prepared from the
874 effusions (minimum 1 ml), more reliable results can be obtained from the
875 concentrated cell preparations in the pellets (Fig. 8).

876 RT-PCR, especially real-time RT-PCR, is a sensitive method to detect virus RNA in
877 different samples, such as feces, blood, effusions, and tissues of FCoV-infected cats
878 and those with FIP, however, these cannot differentiate between the
879 pathotypes.[45,65,77,102] The detection of FCoV RNA in the feces is mainly used for
880 management purposes in catteries, i.e. to determine the kinetics of viral shedding.[4,41]
881 FCoV is known to spread systemically with infection, regardless of the development
882 or presence of clinical signs of FIP; therefore, diagnostic tests that identify viremia
883 can only be used to support other tests towards a diagnosis of FIP in cats with

884 relevant clinical features.[41,62,109] The detection of virus in effusions, however, has
885 proven to have a high PPV, but a negative result does not exclude FIP.[52,159]
886 A recent study identified two alternative amino acid differences in the putative fusion
887 peptide of the FIPV S protein in FECV and FIPV and confirmed that together these
888 two substitutions distinguish FIPV from FECV in >95% of cases.[28] Although it cannot
889 be excluded, due to the quasispecies nature of FCoV, that other mutation patterns
890 could lead to disease, so far, this is the most promising potential diagnostic tool that
891 involves the direct virus detection.[11,42,64,102] Nevertheless, as these alterations are not
892 present in the virus shed by cats with clinical FIP, any routinely employed diagnostic
893 test based on this result will need to be sufficiently sensitive. Also, as a commercial
894 test, a protocol would be preferable that could directly detect these mutations without
895 the need of a further, time-consuming sequencing step.
896 A recent publication takes previous attempts to correlate FIPV replication in
897 monocytes with FIP further and suggests a new methodological approach for the
898 robust simultaneous detection of virus replication and viral load, by a real time PCR
899 based on primer-probe energy transfer.[24,84,145] Rather than a tool to identify infected
900 cats, the authors proposed this method for the identification of persistent shedders
901 and thereby the potential sources of emerging FIP variants. Since the test was also
902 able to reliably detect virus replication in FIP effusions, it might be useful for the
903 confirmation of FIP at least in the wet form, provided the results are confirmed on
904 larger case cohorts.[84]
905

906 With increasing knowledge of the pathophysiological mechanisms that drive the
907 virus-host interaction in FCoV infection and with the constant improvement of
908 molecular techniques, there is reasonable hope that in the near future, the diagnostic

909 tools for the diagnosis of FIP can be refined to specifically detect FIPV and to
910 integrate the assessment of more host response parameters tailored to FIPV.
911
912

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919
920

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1419 **Figure Legends**

1420

1421 **Fig. 1**. Gross FIP lesions. a. Cat with wet FIP, exhibiting a serofibrinous and
1422 granulomatous serositis and granulomatous lesions in the liver (arrows). b-f. Cats
1423 with dry FIP. b. Enlarged mesenteric lymph node with granulomatous inflammation.
1424 c. Jejunum with multiple granulomas in the serosa. d. Jejunum with small subserosal
1425 granulomatous lesions that follow the veins (phlebitis and/or periphlebitis; arrow). e.
1426 Kidney with granulomatous phlebitis and periphlebitis of a Vena capsularis. f. Cat
1427 with dry FIP and multifocal granulomatous phlebitis and periphlebitis of a cortical
1428 leptomeningeal vein (arrow). All lesions were confirmed by subsequent histological
1429 examination and immunohistological demonstration of FCoV antigen within the
1430 lesions.

1431

1432 **Fig. 2**. Distribution of FIP lesions in cats that had undergone a thorough post mortem
1433 and histological examination for the diagnosis of FIP, including the histological
1434 examination of all major organs and tissues and confirmation of the disease by the
1435 immunohistological demonstration of FCoV antigen. N=77 ([1]N = 54; [2]N = 86).

1436

1437 **Fig. 3**. Natural FIP case with thoracic effusion and fibrinous and granulomatous
1438 pleuritis. Pleura with inflammatory processes of variable duration. a) Overview with
1439 dense basal layer of plasma cells (black arrowheads), overlain by layer of granulation
1440 tissue with new vessels (arrows) and embedded macrophage dominated infiltrates
1441 (white arrowheads) and surface layer of fibrin (*) with embedded inflammatory cells.
1442 HE stain; Bar = 50µm. b) In another area, the basal plasma cell layer (black
1443 arrowhead) is overlain by a loose granulation tissue with new vessels (arrows) and

1444 occasional fibroblasts (white arrowheads). HE stain; Bar = 20μm. c) Granuloma in the
1445 granulation tissue. FCoV antigen is present within several macrophages in the
1446 granuloma. Horseradish peroxidase method (mouse anti-CoV, clone FCoV3-70,
1447 Meyer's hematoxylin counterstain.[92] Bar = 20μm.
1448
1449 **Fig. 4**. Natural FIP case with thoracic effusion and with fibrinous and granulomatous
1450 pleuritis (see Fig. 3). Diaphragm, exhibiting a chronic plasma cell dominated (black
1451 arrowheads) diffuse pleuritis with new vessel formation (arrows) consistent with
1452 granulation tissue formation. Towards the surface, an infiltrate of macrophages and
1453 neutrophils is observed (white arrowheads). Occasional macrophages are found that
1454 express FCoV antigen (inset). HE stain and horseradish peroxidase method (mouse
1455 anti-CoV, clone FCV3-70, Meyer's hematoxylin counterstain (inset) .[92] Bars = 20μm
1456 (inset: Bar = 10μm).
1457
1458 **Fig. 5**. Natural FIP case, dry form. Kidney, cortex with multiple FIP lesions. a) Stellate
1459 vein (SV) with fibrinoid necrosis (arrows) and granulomatous vasculitis, partly
1460 occluded by leukocytes (arrowhead). HE stain; Bar = 50μm. b) Closer view of a),
1461 highlighting the necrosis of the vessel wall (arrows) and the infiltrate, dominated by
1462 often degenerate macrophages. There are also focal plasma cell aggregates
1463 (arrowhead) immediately outside the vascular wall. HE stain; Bar = 20μm. c) Closer
1464 view of b). FCoV antigen is present within monocytes in the vascular lumen
1465 (arrowheads) and in the vasculitis (arrow). Horseradish peroxidase method (mouse
1466 anti-CoV, clone FCoV3-70, Meyer's hematoxylin counterstain.[92] Bar = 10μm. d)
1467 Stellate vein distant from the vein in a)-c), exhibiting two focal perivascular plasma

1468 cell accumulations and activated endothelial cells (arrowheads). Bar = HE stain;
1469 20µm.
1470
1471 **Fig. 6**. SPF cat euthanized without clinical signs and post mortem changes
1472 consistent with FIP. The animal had been housed for 30 weeks with animals dying
1473 from FIP and had shown a clinical episode of CNS symptoms and ocular changes
1474 consistent with FIP. Brain, medulla oblongata. a) Mononuclear perivascular infiltrates
1475 (arrows) in the white matter and diffuse infiltrates in the leptomeninx (*). HE stain; Bar
1476 = 50µm. b) The infiltrate is dominated by CD45R-positive B cells. Avidin biotin
1477 complex peroxidase method (rat anti-mouse CD45R, clone B220/Ly5,
1478 Papanicolaou's hematoxylin counterstain.[92] Bar = 50µm. c) The perivascular infiltrate
1479 (arrow) as well as the leptomeningeal infiltrate contains plasma cells with FCoV-
1480 specific antibodies. Peroxidase anti-peroxidase method (DF-2 FIPV suspension,
1481 followed by mouse anti FCoV (clone FCoV3-70), Papanicolaou's hematoxylin
1482 counterstain.[92] Bar = 10µm.
1483
1484 **Fig. 7**. Natural FIP case, dry form. Mesenteric lymph node biopsy. a) Granulomatous
1485 lesions are present in the serosa (*) and occasionally within the lymph node in
1486 association with the cortical sinuses (arrow). HE stain; Bar = 50µm. b) Viral antigen is
1487 expressed by macrophages in the serosal lesions and in parenchymal lesions (inset:
1488 higher magnification of *). Horseradish peroxidase method (mouse anti-CoV, clone
1489 FCoV3-70, Meyer's hematoxylin counterstain.[92] Bar = 50µm. Inset: Bar = 10µm.
1490
1491 **Fig. 8**. Natural FIP case, wet form with abundant abdominal effusion. a) Cytological
1492 specimen (smear) from the effusion, comprised of macrophages/mesothelial cells

1493 (arrow) and neutrophils (arrowhead). May-Grünwald-Giemsa stain. b) Macrophages
1494 in the smear express viral antigen. c) Macrophages in a formalin fixed and paraffin
1495 embedded cell pellet express abundant (arrow) to small amounts (arrowhead) of viral
1496 antigen. Horseradish peroxidase method (mouse anti-CoV, clone FCoV3-70, Meyer's
1497 hematoxylin counterstain.[92] Bars = 10µm.