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Serum α1-acid glycoprotein (AGP) concentration in non-symptomatic cats with feline coronavirus (FCoV) infection

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Previous studies have demonstrated that the concentration of α1-acid glycoprotein (AGP) transiently increases in asymptomatic cats infected with feline coronavirus (FCoV). In order to establish whether these fluctuations depend on the FCoV status, the serum concentration of AGP and anti-FCoV antibody titres and/or faecal shedding of FCoVs in clinically healthy cats from catteries with different levels of prevalence of FCoV infection were monitored over time. Serum AGP concentrations fluctuated over time in clinically healthy cats from the cattery with the highest prevalence of feline infectious peritonitis (FIP) and significantly increased just before an outbreak of FIP. Further studies are required to clarify whether the observed increase of AGP concentration is a consequence of the increased viral burden or a protective response against mutated viral strains. Nevertheless, the results of the present study suggest that AGP might be useful in monitoring FCoV–host interactions in FCoV-endemic catteries.

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Acute phase proteins (APP) are plasma proteins produced by hepatocytes, whose concentration increases (positive APP) or decreases (negative APP) during inflammation, under the stimulation of cytokines released from inflammatory sites (van Deventer et al 1990). The major feline APP is α1-acid glycoprotein (AGP) (Ceron et al 2005) which belongs to the lipocalin superfamily, a group of proteins able to bind and transport hydrophobic molecules (Lögdberg and Wester 2000). Previous studies have shown that in cats affected by feline infectious peritonitis (FIP), which is a lethal disease of cats caused by feline coronaviruses (FCoVs), the blood concentration of AGP increases (Duthie et al 1997). However, when cases of FIP are recorded in FCoV-endemic catteries, non-symptomatic cats living in the same cattery showed a transient increase in AGP concentration (Giordano et al 2004). This suggests that the appearance of pathogenic FCoV strains also elicits an inflammatory response in cats that do not develop clinical signs of disease. In the study cited above, only a small number of non-symptomatic cats were examined and the relationship between the fluctuation of AGP levels and the actual FCoV status of the cats was unclear (Giordano et al 2004).

The aim of the present study was to investigate whether the fluctuations of AGP levels in non-symptomatic FCoV-positive cats depend on their FCoV status. To this aim, we monitored over time the serum concentration of AGP and anti-FCoV antibody titres and/or faecal shedding of FCoVs of cats living in catteries with different levels of prevalence of FIP.

Materials and methods

Animals and study design

This study was performed on serum and faecal samples collected from cats grouped as follows:

(A) Specific pathogen-free (SPF) cats: This group included nine serum samples taken from

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three SPF cats (kindly provided by Prof Hans Lutz and Dr Marina Meli, Vetsuisse Faculty, University of Zurich). In order to evaluate possible fluctuations of AGP concentration not dependent on FCoV status, these three cats were sampled every 30 days.

(B) Asymptomatic FCoV-infected cats: 19 cats from three different catteries:
- Group B1 included approximately 40 Persian cats from a cattery where animals with specific problems (pregnancies, parturitions, infectious diseases, etc) were kept separated, while non-symptomatic cats shared the same environment. Sporadic cases of FIP (1–4 per year) had occurred in the cattery in past years, suggesting that the cattery was endemically infected with FCoV. Ten animals (four males and six females), aged 5–14 years, were randomly selected and sampled every 30 days over a 7 month period according to the protocol suggested by Addie and Jarrett (2001) in order to identify carriers and shedders.
- Group B2 included 10 homebred Persian cats living in a private household on which cases of FIP had never been recorded. Four cats (one male and three females, 4–11 years old) were randomly selected and sampled every 30 days over a 4 month period.
- Group B3 included five homebred domestic shorthair cats (one male and four females, aged 2–9 years) living in a private household in which only one case of FIP had occurred 4 years before this study. All these cats were sampled every 30 days over a 4 month period.

Approximately 3 ml of blood was taken from each cat on each occasion and placed in tubes without anticoagulant. Serum was obtained by centrifugation and stored at −30°C until analysis.

Faeces from SPF cats were collected when cats entered the study. Faecal samples were also collected from cats of cattery B1 at the same time their blood samples were taken. To avoid sampling errors, cats were kept in cages on the day of sampling and each sample was placed in a separate plastic envelope and transported to the laboratory. Cats from catteries B2 and B3 could freely move in their breeding facilities. Thus, it was not possible to monitor the faecal excretion of FCoVs in individual cats. For this reason, the FCoV status of these cats was evaluated only by measuring anti-FCoV titres in blood. As regards cattery B3, however, the referring veterinarian periodically checked by RT-PCR on faecal swabs many cats of the cattery, with only occasional positive results.

AGP measurement

AGP was measured using a commercially available radial immunodiffusion (SRID) kit (Tridelta Ltd, Maynooth, Kildare, Ireland) according to the manufacturer's instructions. Briefly, 5 µl of each serum sample or of standard solutions containing 5 mg/ml and 20 mg/ml of feline AGP was put in each well of a multiwell SRID plate. Plates were incubated at room temperature for 48 h and the diameter of precipitation rings was measured. Values obtained from the two standard solutions were used to design a standard curve. Values from the case samples were then plotted to extrapolate absolute AGP levels, expressed as mg/ml.

Serology

All cats included in the study were tested by the referring veterinarian for feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) using in-clinic Enzyme linked Immunosorbent Antibody (ELISA) test (Snap Combo, IDEXX Lab, Westbrook, USA) and were negative in all cases. The presence of anti-FCoV antibody titres was investigated using an indirect immunofluorescence test performed on 10 multiwell slides produced at the University of Zurich according to Osterhaus et al (1977). Briefly, slides were prepared by coating each well with 4.5×10^3 PD-5 cells, half of which were infected with swine transmissible gastroenteritis virus (TGEV), which serologically cross-reacts with FCoVs (Osterhaus et al 1977). After coating the wells, slides were fixed with acetone for stabilisation, and were then dried, frozen and stored at −30°C until use. Serology was performed by making twofold dilutions of each serum sample (1:25 to 1:400). Twenty microlitres of each dilution was applied to one well each and incubated (30 min, 37°C) in a humidified chamber. Slides were then washed three times with Phosphate Buffered Saline (PBS), dried, and 15 µl of fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti cat immunoglobulin (Nordic Immunological Laboratories, Tilburg, The Netherlands) was added to each well. After incubation (30 min, 37°C in a humidified chamber), the slides were washed, dried, cover-slipped with PBS and Kaiser's glycerin (1:3 ratio) and examined under a fluorescence microscope. Dilutions showing a distinct fluorescence signal in about one-half

of the cells were evaluated as positive, while reactions that did not show any positive signal or that were designated as dubious were judged as negative. Samples that were still positive at a 1:400 dilution were further diluted on a twofold basis until a negative result was obtained.

Faecal shedding of FCoV

The presence of FCoV RNA in faecal samples was investigated using Reverse transcriptase-polymerase chain reaction (RT-PCR). All the faecal samples were frozen at −80°C until analysis. After thawing, 1 g of each faecal sample was homogenised in 6 ml of saline solution and centrifuged (2400 × *g* for 45 min). The supernatants were removed and filtered using a pore size of 0.22 μm (Millipore, Billerica, USA). Three millilitres of each filtered supernatant was applied to a Centricon YM-30 column (Amersham Biosciences, Piscataway, USA) in order to concentrate viral particles to a final volume of 140 μl. Viral RNA was then extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Milan, Italy) and reverse-transcribed using a Ready-To-Go You-Prime First-Strand Beads Kit (Amersham Biosciences) with the FCoV specific primer p211 (20 pmol/ml) designed by Herrewegh et al (1995). The cDNAs, obtained as described above, were amplified by PCR using a reaction mix composed of 1 μl of cDNA, 1 × buffer, 2 mM $MgCl_2$, 0.2 mM Deoxyribonucleotide triphosphate (dNTP), 0.25 U of Taq polymerase (Eppendorf, Hamburg, Germany) and 1 μM of each primer (primers p211 and p205) (Herrewegh et al 1995). The thermal cycling protocol used was as follows: 94°C (2 min), 40 cycles of denaturation at 94°C (45 s), annealing at 62°C (40 s), elongation at 72°C (40 s) and a final elongation step at 72°C (10 min). In each assay, negative controls (reaction mix without cDNA) and positive controls (FCoV cDNA amplified from the vaccine Primucell FIP, Pfizer Animal Health, Exton, PA, USA) were included. Amplicons were then run on a 2% agarose gel and visualised using an ultraviolet transilluminator. Based on the presence or absence of amplicons, cats were classified as positive or negative for viral shedding.

Statistical analysis

Statistical analyses were carried out using specific software (Statistica for Windows, Statsoft Inc., Tulsa, USA). Data distribution was checked using Kolmogorov−Smirnov normality tests. Data from sequential samplings of groups A, B1, B2 and B3 were analysed using an analysis of variance (ANOVA) test for repeated measures followed by the Tukey's honest significant difference test or, in the case of non-normally distributed data, with the corresponding non-parametric Friedman's test.

The percentage of shedders in the sequential faecal samplings of cattery B1 were compared with each other using the Cochran's *Q* test.

Possible correlations between FCoV antibody titres and AGP concentration were investigated using the Spearman correlation test.

Results

Clinicopathological findings and cattery health status

Cats from groups A and B showed no clinical signs or laboratory changes consistent with FIP (anaemia, lymphopenia, increased globulins and/or α_2- γ-globulins) or with any other systemic inflammatory disease throughout the study period. Nevertheless, two episodes of FIP, confirmed by post-mortem examination, occurred in cattery B1, 2 weeks after sampling time point 5 (two animals affected) and 1 month after sampling time point 7 (one cat affected).

Faecal shedding of FCoVs in SPF cats (group A) and in cats from the cattery with endemic FCoV infection (group B1)

All faeces samples from the SPF cats were negative for FCoV genome.

All cats in group B1 yielded positive results in at least one sampling (Table 1). Specifically, five cats (cats 1, 4, 7, 8 and 9) were positive on each sampling, while the remaining five were negative in at least one sampling. The percentage of shedders varied among the samplings ($P = 0.021$). More than 80% of samples were positive in the first five samplings, whereas 50% of samples were positive at sampling 6. In the successive sampling 80% of samples were positive again (Fig 1).

FCoV antibody titres

All serum samples from SPF cats (group A) were negative for FCoV antibodies.

Non-symptomatic cats from cattery B1 had fluctuating FCoV antibody titres ranging from 0 to 1:3200, while values recorded in catteries B2 and B3, were always low, usually between 0 and 1:50, with rare peaks at 1:100 (Table 1).

Table 1. Results obtained during the study

Group	Cat no.	Sampling 1			Sampling 2			Sampling 3			Sampling 4			Sampling 5			Sampling 6			Sampling 7		
		F	T	A	F	T	A	F	T	A	F	T	A	F	T	A	F	T	A	F	T	A
A	1	−	0	0.24	−	0	0.24	−	0	0.22	−											
	2	−	0	0.23	−	0	0.21	−	0	0.25	−											
	3	−	0	0.25	−	0	0.25	−	0	0.21	−											
B1	1	+	1:50	0.40	+	1:400	0.31	+	1:100	0.42	+	1:200	0.27	+	1:3200	0.77	+	1:400	0.34	+	1:800	0.39
	2	+	1:100	0.39	+	1:800	0.34	−	1:25	0.40	+	1:200	0.40	+	1:400	0.39	−	1:400	0.26	+	1:3200	0.31
	3	+	1:100	0.39	+	1:1600	0.34	+	1:200	0.30	+	1:1600	0.37	−	1:800	0.28	−	1:800	0.30	-	1:400	0.31
	4	+	1:100	0.20	+	1:800	0.22	+	1:400	0.77	+	1:800	0.21	+	1:800	0.52	+	1:800	0.23	+	1:3200	0.25
	5	+	1:200	0.31	−	1:50	0.37	+	1:50	0.23	+	1:200	0.31	+	1:200	0.24	−	1:25	0.55	+	1:100	0.35
	6	+	1:100	0.30	−	0	0.28	+	1:50	0.39	+	1:25	0.22	+	1:400	0.22	−	1:100	0.39	-	1:200	0.25
	7	+	1:200	0.30	+	1:25	0.35	+	1:25	0.42	+	1:25	0.40	+	1:50	0.44	+	1:100	0.34	+	1:100	0.29
	8	+	1:50	0.34	+	1:200	0.37	+	1:50	0.37	+	1:50	0.37	+	1:200	0.88	+	1:200	0.30	+	1:400	0.38
	9	+	1:400	0.39	+	1:200	0.39	+	1:3200	0.31	+	1:50	0.35	+	1:400	0.52	+	1:50	0.44	+	1:50	0.41
	10	+	1:100	0.35	+	1:100	0.31	+	1:200	0.44	+	1:50	0.24	+	1:100	0.88	−	1:200	0.39	+	1:100	0.35
B2	1	nd	1:50	0.36	nd	1:50	0.32	nd	1:100	0.32	nd	1:50	0.35									
	2	nd	1:25	0.25	nd	1:25	0.24	nd	1:25	0.25	nd	0	0.30									
	3	nd	1:25	0.27	nd	1:50	0.34	nd	1:25	0.23	nd	0	0.31									
	4	nd	1:25	0.39	nd	0	0.25	nd	0	0.39	nd	1:50	0.32									
B3	1	nd	1:50	0.44	nd	1:25	0.52	nd	1:100	0.47	nd	1:50	0.44									
	2	nd	0	0.36	nd	1:25	0.31	nd	1:25	0.25	nd	1:50	0.24									
	3	nd	0	0.30	nd	0	0.35	nd	1:25	0.25	nd	0	0.28									
	4	nd	0	0.33	nd	0	0.31	nd	1:25	0.30	nd	0	0.38									
	5	nd	1:50	0.32	nd	1:50	0.41	nd	0	0.24	nd	1:25	0.35									

F = FCoV-PCR in faeces; T = antibody titre; A = AGP concentration (mg/ml), nd = not determined.

The values recorded in samples from cats of group B1 (median 1:200; interquartile range: 1:50−1:400) were significantly higher ($P = 0.006$) than that recorded in samples from cats of groups B2 (1:25; 0−1:50) and B3 (1:25; 0−1:50).

Although median values of antibody titres in group B1 were higher in samplings 5 and 7 compared to other samplings, no significant differences between the different samplings were detectable in this group ($P = 0.09$), mostly due to the high individual variability (Fig 2). Similarly, no significant differences between sequential samplings were observed in groups B2 ($P = 0.777$) and B3 ($P = 0.556$).

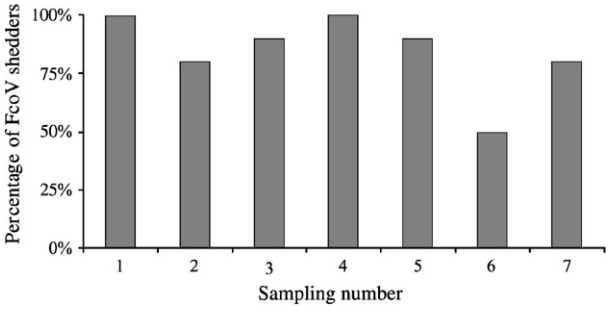

Fig 1. Cats from a cattery with endemic FCoV infection (group B1): percentage of shedders at the different samplings, as determined by RT-PCR on faecal samples.

Serum AGP levels

SPF cats had low serum AGP levels (Table 1) and did not show fluctuation of AGP concentration over different sampling times ($P = 0.717$). On the contrary, AGP concentrations in cats from group B1, B2 and B3 were variable between the different samplings (Fig 3). Specifically, the differences between sequential samplings were statistically significant in group B1 ($P = 0.008$), with higher values on sampling 5 (when AGP concentration increased, compared with values recorded on sampling 4, in six out of the 10 cats) but not in groups B2 ($P = 0.72$) and B3 ($P = 0.078$).

The AGP concentration in the blood of SPF cats (group A, median = 0.24; interquartile range 0.21−0.24) was significantly lower ($P = 0.003$) than the concentration in all samples obtained from groups B1 (0.35; 0.30−0.38), B2 (0.31; 0.25−0.34) and B3 (0.33; 0.28−0.38).

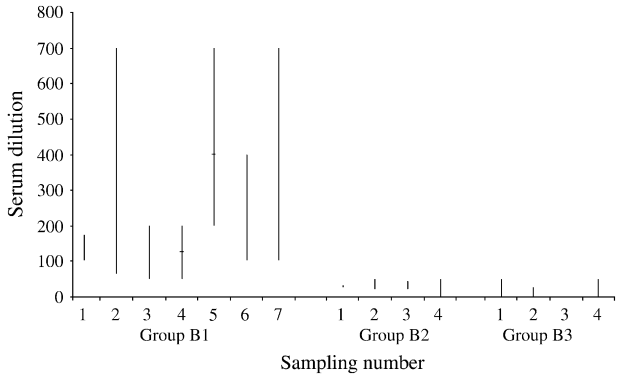

Fig 2. Distribution of FCoV antibody titres (median and I and III interquartile ranges) in sequential samplings from cats of groups B1 (high prevalence of FCoV, $n = 10$), B2 (low prevalence of FCoV, $n = 4$) and B3 (low prevalence of FCoV, $n = 5$).

Relationship between faecal shedding of FCoVs, FCoV antibody titres and AGP serum levels

FCoV antibody titres or serum AGP levels obtained on cattery B1 from cats which shed FCoVs in all the samples were not significantly different from those recorded in cats which resulted negative in at least one faecal sample. In all groups, serum AGP levels were not statistically correlated with FCoV antibody titres. Nevertheless, when the data from these two parameters were plotted together (Fig 4), it is evident that in group B1 both values show a tendency to increase at sampling 5, 2 weeks before the occurrence of an outbreak of FIP in the cattery.

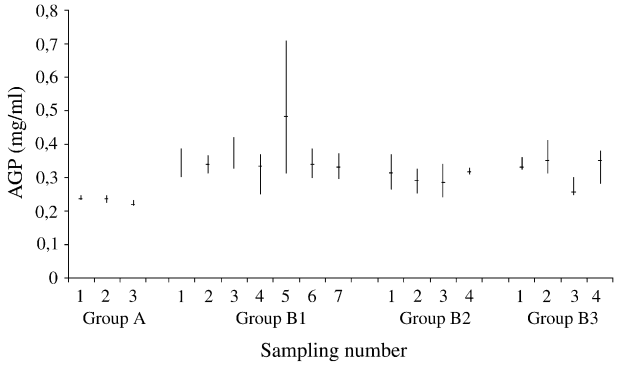

Fig 3. Distribution of serum AGP levels (median and I and III interquartile ranges) in sequential samplings from cats of groups A (SPF cats, $n = 3$), B1 (high prevalence of FCoV, $n = 10$), B2 (low prevalence of FCoV, $n = 4$) and B3 (low prevalence of FCoV, $n = 5$).

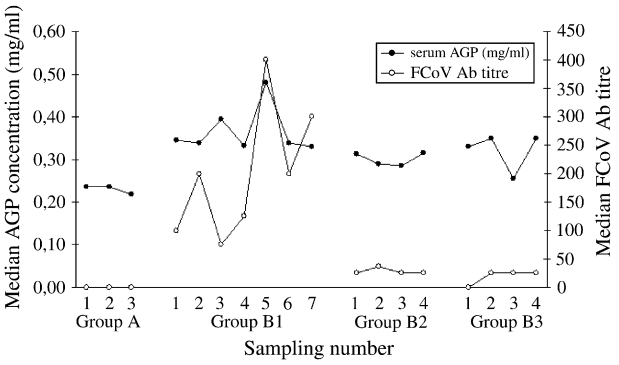

Fig 4. Median values of serum AGP concentration and FCoV antibody titres in the sequential samplings of the different groups.

Discussion

The aim of this study was to investigate the possible correlation between serum AGP levels and the FCoV status of non-symptomatic cats. The latter was investigated by evaluating anti-FCoV antibody titres and/or faecal shedding of FCoVs in SPF cats and in cats from catteries with different levels of FCoV infection. Unfortunately, FCoV shedding of individual cats could only be assessed in one cattery (group B1) but, taken together, our results from epidemiology, serology and/or faecal shedding allowed us to classify the catteries as 'high prevalence' (group B1) and 'low prevalence' (groups B2 and B3) of FCoV infection. The low number of FIP cases recorded in the past, the negative results of PCRs for FCoV genome performed by the referring veterinarian and the persistently low antibody titres recorded in our study, indicate a low rate of FCoV infection in groups B2 and B3, as, although seronegative shedders have been reported (Meli et al 2004), viral load usually correlates with the antibody titre (Gut et al 1999).

All cats from group B1 can be classified as FCoV shedders (Addie and Jarrett 2001) although only 50% of them were always positive for faecal shedding. This percentage differs from what was reported in the study cited above, which classified shedders as persistent and recurrent shedders (80% and 15%, of cats, respectively). This difference might depend on a different level of contamination of catteries by FCoVs, on a different genetic pattern of cats (Foley and Pedersen 1996), or on the differences in the PCR protocol, as we introduced a concentration step that might have increased the sensitivity of the method. It should be emphasised, however, that cats from the study of Addie and Jarrett (2001) were

housed individually, while in the current study cats shared a common environment. This does not allow us to rule out recurrent re-infection as a likely cause of the persistent positivity of faecal PCR.

The percentage of shedders decreased at sampling 6, which was performed 2 weeks after an episode of FIP. As all samples were processed with the same protocol and reagents, it is unlikely that this decrease represents a methodological problem. Based on our data, however, it is not possible to establish a direct cause–effect between this decrease and the occurrence of FIP in the cattery, as it is known that faecal viral shedding fluctuates in FCoV-endemic catteries even in the absence of clinical FIP cases (Addie and Jarrett 2001, Addie et al 2003, Meli et al 2004), according to the 'susceptible-infective-susceptible' (SIS) model of infection proposed by Foley et al (1997). According to this model, antibody titres also fluctuate in FCoV-endemic catteries, as was observed in the current study in group B1.

Serum AGP levels from SPF cats in this study were lower than those reported in control cats included in previous studies (Duthie et al 1997, Kajikawa et al 1999). These control sera, however, were collected from non-SPF cats, which are likely to have been exposed to several antigenic stimuli (Kristensen and Barsanti 1977). In contrast, non-symptomatic FCoV cats had AGP levels similar to those previously reported (Giordano et al 2004).

Although it cannot be excluded that increases of serum AGP concentration might have been triggered by non-specific inflammatory stimuli, no clinical signs consistent with inflammation were recorded during the study. Our results, therefore, confirm that serum AGP levels fluctuate over the time as previously reported (Giordano et al 2004). In addition, we demonstrated that these fluctuations were greater in the cattery with the higher prevalence of FCoV infection (group B1). Moreover, in group B1, AGP levels increased at sampling 5, 2 weeks before the occurrence of FIP cases in the cattery, but not at sampling 7, which was performed 1 month before the second episode of FIP. The difference in AGP response between samplings 5 and 7 might depend on the different time interval between the sampling and the occurrence of FIP cases.

Fluctuations of AGP levels and FCoV antibody titres were not statistically correlated. This is not surprising, as they are induced by different mechanisms. A more objective evaluation of the FCoVs' viral burden could have been obtained by quantitative RT-PCR on faeces or blood, which would also have allowed us to have more detailed information on a possible relationship between individual AGP levels, individual FCoV shedding and viraemia. Nevertheless, as it has been demonstrated that antibody titres correlate with viral load (Gut et al 1999), the increase of both antibody titres and serum AGP levels recorded at sampling 5 suggests that the occurrence of FIP cases is preceded, in non-symptomatic cats, by an increase of viral load and by an inflammatory response. However, it is unclear whether this response is evoked only by the higher viral load or if it is a specific and protective response against 'highly pathogenic' FCoVs. Sequencing of FCoVs isolated during this study may help to answer this question. However, the hypothesis that certain AGP responses might protect from the disease is exciting. AGP has important immunomodulatory functions (Bories et al 1990, Vasson et al 1994) and, in humans, changes in the AGP glycosylation pattern are associated with resistance or susceptibility to some viral diseases (Rabehi et al 1995). Interestingly, AGP isolated from cats with FIP is hyposialylated (Ceciliani et al 2004). It can, therefore, be postulated that all the cats in a cattery with endemic FCoV infection respond to increased viral burden by increasing the production of AGP but that only cats with hyposialylated AGP have persistently increased AGP levels and develop FIP.

In conclusion, serum AGP concentration fluctuated in clinically healthy cats living in a cattery with high prevalence of FCoV infection but not in SPF cats or in cats living in catteries with a low prevalence of FCoV infection. This probably represents a non-specific response to the increased viral burden, which, in turn, is associated with a high probability of FIP outbreaks. In the future, we will attempt to clarify whether the increase in serum AGP levels is a consequence of a higher viral load of non-mutated FCoVs or a protective response against mutated viral strains. In addition, the results of the present study suggest that AGP might be a useful tool to monitor FCoV–host interactions in FCoV-endemic catteries.

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