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Flow cytometric detection of alpha-1-acid glycoprotein on feline circulating leucocytes

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Objective To assess whether alpha-1-acid glycoprotein (AGP) can be detected on the membrane of feline circulating leucocytes.

Design The presence of AGP on circulating leucocytes was investigated in both clinically healthy cats and cats with different diseases. A group of feline coronavirus (FCoV)-positive cats, comprising cats with feline infectious peritonitis (FIP) and cats not affected by FIP but seropositive for FCoV, were included in this study because the serum concentration of AGP increases during FCoV infection.

Procedure Flow cytometry (using an anti-feline AGP antibody), serum protein electrophoresis, routine haematology and measurement of the serum AGP concentration were performed using blood samples from 32 healthy cats (19 FCoV-seropositive), 13 cats with FIP and 12 with other diseases (6 FCoV-seropositive). The proportion of cats with AGP-positive leucocytes in the different groups (e.g. controls vs sick; FIP vs other diseases, etc.) or in cats with different intensities of inflammatory response was compared using a Chi-square test.

Results AGP-positive leucocytes were found in 23% of cats. Compared with controls, the proportion of patients with positive granulocytes and monocytes was higher among sick cats (especially cats with diseases other than FIP) and cats with high serum AGP concentration, but not in cats with leucocytosis or that were FCoV-seropositive.

Conclusion AGP-positive leucocytes can be found in feline blood, especially during inflammation. Conversely, no association between AGP-positive leucocytes and FIP was found. Further studies are needed to elucidate the mechanism responsible for this finding and its diagnostic role in cats with inflammation.

Keywords alpha-1-acid glycoprotein; cats; leucocytes; feline coronavirus; feline infectious peritonitis

Abbreviations AGP, alpha-1-acid glycoprotein; APP, acute-phase protein; EDTA, ethylenediamine tetraacetic acid; FCoV, feline coronavirus; FCS, fetal calf serum; FIP, feline infectious peritonitis; FITC, fluorescein; FIV, feline immunodeficiency virus; FSC, forward scatter; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SRID, single radial immunodiffusion; SSC, side scatter

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Alpha-1-acid glycoprotein (AGP) is the major feline acute-phase protein (APP). It has both a high affinity for small hydrophobic molecules and immunomodulatory properties.[1,2] In humans, the rate of AGP sialylation seems to protect from viral infections.[3,4] The serum concentration of feline AGP increases in several pathophysiological conditions and it is used as a biomarker of inflammation for many inflammatory/infectious diseases, together with other APPs such as serum amyloid A.[2,5] The most prominent increases have been recorded in cats with feline infectious peritonitis (FIP), a lethal disease caused by the feline coronavirus (FCoV), on which AGP is hyposialylated.[2,6,7] Because of these huge increases, AGP is considered the most powerful biomarker of FIP, especially when the pre-test probability of this disease is high.[2,6] Transient increases in both the serum concentration and the rate of sialylation of AGP have also been recorded in clinically healthy cats living in FCoV-endemic environments.[8–10]

In human and bovine leucocytes, the expression of messenger RNA (mRNA) coding for AGP and of AGP itself on the membrane or in intracellular granules, has been reported in both blood[11–15] and tissues,[16] but the significance of this is debated. The presence of AGP on the leucocytes of cats has never been investigated.

The aim of this study was to investigate by flow cytometry the presence of AGP on circulating leucocytes in healthy FCoV-seropositive and FCoV-seronegative cats and in cats affected by different diseases, including FIP. This information would increase our knowledge about AGP and its possible diagnostic role. If the presence of AGP-positive leucocytes is clearly associated with specific pathological conditions, then detection of AGP-bearing leucocytes would be an additional ancillary test to support a clinical diagnosis of inflammatory disease.

Material and methods

Animals and sampling

This study was carried out using 57 blood samples collected from a group of 32 clinically healthy cats and a group of 25 cats that had clinical signs and laboratory changes consistent with FIP (n = 13), inflammation (n = 7: 4 intestinal, 1 ocular, 1 respiratory, 1 abscess), trauma (n = 3), lymphoma (n = 1) or feline immunodeficiency virus (FIV) infection (n = 1). Cats with clinical signs did not receive any treatment before being included in this study.

Cats were grouped according to physical examination and the results of diagnostic imaging and biochemical, haematological and serological tests performed by the referring veterinarian, as well as by follow-up results, including postmortem examination in the case of death. Cats that were classified as clinically healthy had no clinical signs or laboratory abnormalities. Among the sick cats, the diagnosis of FIP was based on necropsy and histology and immunohistochemistry for FCoV antigens; cats that died with a clinical suspicion of FIP not confirmed by immunohistochemistry were not included in the study. The diagnosis of FIP was excluded if necropsy results were inconsistent with FIP, including negative immunohistochemistry for

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FCoV, if there was improvement in clinical condition after appropriate therapies or there was a lack of signs consistent with FIP during a 1-year follow-up.

A further group included cats that either had FIP or were seropositive for FCoV. Hereafter, the term 'seropositive' will be used to specifically indicate cats without FIP but with circulating anti-FCoV antibodies, and the term 'FCoV-positive' will be used to more generally indicate seropositive cats and cats with FIP.

All the cats were privately owned and sampled during routine diagnostic screening and informed consent was given by the owners. Blood was collected from the jugular vein and divided into two aliquots. One aliquot was put in a plain tube to obtain serum by centrifugation, which was then frozen and analysed within 2 months, and the other aliquot was placed in an EDTA-coated tube for haematology and flow cytometry, which were performed within 24 h of sampling.

Complete cell blood count

A complete cell blood count of 55 blood samples was performed using an impedance counter (Hemat 8, SEAC, Calenzano, Firenze, Italy). The differential leucocyte count was determined microscopically on blood smears stained with May Grünwald-Giemsa. Haematology was not performed for the remaining 2 samples in which AGP was measured by both single radial immunodiffusion (SRID) and flow cytometry.

Serum protein electrophoresis

Cellulose acetate electrophoresis of 55 samples was performed using automated equipment (Genio, Interlab Srl, Rome, Italy). Electrophoresis was not performed in the remaining 5 samples in which AGP was measured by both SRID and flow cytometry. Strips were run in a buffer solution (16 min, 140 V), stained with Ponceau Red, destained and diaphanised in the solution provided by the manufacturer (Genio, Interlab Srl). Electropherograms were generated by the densitometer included in the instrument.

AGP measurement

AGP was measured using SRID kit (Tridelta, Maynooth, Kildare, Ireland) as follows: 5 µL of each sample or of standard solutions containing 0.5 mg/mL and 2.0 mg/mL of AGP, respectively, were put in each well of a multiwell SRID plate. After incubation (48 h at room temperature), the diameter of the precipitation rings was measured. Values obtained from the standard solutions were used to design a reference curve. Values from the case samples were then plotted to extrapolate the AGP concentrations.

Serology

Anti-FCoV serology was performed for 50 cats (7 cats with FIP were not tested, but were included in the FCoV-positive group because intralesional virus was immunohistochemically detected at necropsy) using an indirect immunofluorescence test performed on multiwell slides produced at the University of Zurich.[17] Briefly, each well was coated with 4.5×10^3 PD-5 cells, half of which were infected with swine transmissible gastroenteritis virus, which serologically cross-reacts with FCoV. Slides were then fixed with acetone, dried, frozen and stored at –30°C until use. Serum samples were diluted on a 2-fold basis (1:25 to the first dilution that provided a negative result) and 20 µL of each dilution was applied to the wells. After incubation (30 min, 37°C in a moist chamber), the slides were washed three times with phosphate-buffered saline (PBS) and dried. Next, 15 µL of fluorescein (FITC)-conjugated rabbit-anti-cat immunoglobulin (Nordic Immunological Laboratories, Tilburg, The Netherlands) was added to each well. After incubation (30 min, 37°C), the slides were washed, dried, cover-slipped with PBS and Kaiser's glycerin (1:3 v/v) and examined using a fluorescence microscope. Dilutions showing a distinct fluorescence signal in approximately half of the cells were classified as positive.

Flow cytometry

Anticoagulated blood (500 µL) was incubated (10 min at room temperature) with 7 mL of a lysis solution containing 8% ammonium chloride to lyse the red blood cells. Tubes were then centrifuged at 7°C (500*g*, 10 min) and the pellet washed with 7 mL of PBS. Centrifugation and washing were repeated twice and the pellet was resuspended in 500 µL of RPMI containing fetal calf serum (FCS) (5%) and sodium azide (0.2%). Cells were then counted as before and diluted to 10,000 cells/µL in RPMI containing FCS and sodium azide.

Next, 50 µL of polyclonal anti-feline AGP antibody (dilution: 1:100) raised in sheep (kindly provided by Professor David Eckersall, University of Glasgow, UK) were added to the cell suspension. As a negative control, 50 µL of cell suspension were added with 50 µL of sheep normal serum diluted 1:100. After incubation (20 min in the dark), cells were washed twice with RPMI/FCS/sodium azide and centrifuged (500*g*, 10 min, 7°C). As a secondary antibody, 50 µL of FITC-conjugated donkey anti-sheep IgG (STAR88F, Serotec, Oxford, UK) was used. In order to identify any possible non-specific binding of the secondary antibody, 50 µL of the cell suspension was incubated with the secondary antibody alone.

Leucocyte subpopulations were identified with monoclonal antibodies specific for the following antigens: CD3 (T cells, clone CF54A, VMRD, Pullmann, USA, 50 µL, 1:50), CD11b (expressed on many cell lines, but with the highest expression in neutrophils; clone CA163E10, Serotec, 50 µL 1:16) and CD14 (monocytes, clone TUK4, PE-conjugated, Serotec, 25 µL, 1:25). As a secondary antibody for unconjugated primary antibodies, 50 µL of FITC-conjugated anti-mouse IgG (STAR9B, Serotec) were used. After incubation, the cells were washed, resuspended in PBS and acquired with a FACScalibur flow cytometer equipped with Cell Quest software (Becton Dickinson, San Jose, CA, USA). Data from a minimum of 10,000 events were collected.

Leucocyte subpopulations were identified by a back-gating technique in which cells were displayed as fluorescence 1 (FL1) or fluorescence 2 (FL2) versus side scatter (SSC), and a gate was placed around positive cells for each lineage-restricted cell marker. This positive population was then identified in the forward scatter (FSC) versus SSC plot, in which a second gate was drawn. This second gate on the scatterplot was used to display cells on the AGP histogram.

Statistical analysis

Statistical analyses were performed in an Excel spreadsheet with the Analyse-it set of macroinstructions (Analyse-it Software, Leeds, UK). For each cell population (neutrophils, lymphocytes, monocytes), Pearson's Chi-square test was used to compare the proportion of positive cats in the comparisons. Results were considered significant at $P < 0.05$.

Results

Clinicopathological changes

Clinically healthy cats occasionally had laboratory abnormalities (Table 1). In most cases, however, these changes were minimal or moderate, except for the evident lymphocytosis found in two cats ($7.40–10.7 \times 10^9$ lymphocytes/μL; reference interval $1.5–7.0 \times 10^9$/μL). Nineteen cats from this group were seropositive for FCoV (titres 1:100–1:800).

All the cats with FIP were affected by the wet form and had laboratory changes consistent with FIP (lymphopenia, increased α_2- and/or γ-globulin) except for two cats that had AGP values within the reference range (0.40 and 0.52 mg/mL, respectively). Five of the six cats with FIP for which serology was performed were FCoV-seropositive (titres 1:800–1:3200). For further statistical comparisons, all the cats with FIP were included in the FCoV-positive group because they all had intralesional FCoV detected at necropsy.

Cats with inflammatory processes other than FIP had mild neutrophilia or neutropenia, moderate to severe lymphopenia, increased concentrations of α_2- or γ-globulin or increased AGP (0.63–0.68 mg/mL). Three of these cats were also seropositive for FCoV (titres 1:100–1:1,600).

Lymphocytosis or lymphopenia was found in the cats with trauma. One cat with lymphocytosis also had monocytosis, increased AGP concentration and was FCoV-seropositive (titre 1:100). One of the lymphopenic cats also had an increased AGP concentration (0.73 mg/mL).

The cat with lymphoma had lymphocytosis, increased AGP concentration (0.66 mg/mL) and was FCoV-seropositive (titre 1:800), and the cat with FIV had increased α_2-globulin and AGP (0.95 mg/mL) concentrations and was FCoV-seropositive (titre 1:100).

Presence of AGP on blood leucocytes

In approximately 23% (13/57) of cats, AGP was detectable by flow cytometry in at least one leucocyte population. Specifically, AGP was more frequently detected in granulocytes than in lymphocytes and monocytes. When present, the positive signal was clearly detectable, although it was weak in some cases (Figure 1).

Less than 10% of clinically healthy cats had AGP-positive leucocytes (Table 2). The percentage of cats with AGP-positive neutrophils was higher in the sick (36.0%) than in the healthy cats. The percentage of cats with FIP and AGP-positive neutrophils (30.8%) was not significantly higher (P = 0.08) than in the controls (6.2%). By contrast, the percentage of cats with positive neutrophils (41.2%) and monocytes (33.3%) was significantly higher in cats with diseases other than FIP than in the controls.

No significant difference in the proportion of cats with AGP-positive leucocytes was found between FCoV-positive and FCoV-negative cats (Table 3). Among the FCoV-positive cats, however, the proportion of cats expressing AGP on neutrophils was higher in sick cats (41.2%) affected either by FIP (30.8%) or by other diseases (66.6%), than in the controls (5.2%), and the percentage of cats with AGP-positive

Table 1. Summary of the frequency of clinicopathological changes recorded in groups A (healthy cats) and B (sick cats) and in the subgroups of sick cats

	Neutrophils		Lymphocytes		Monocytes	α_2-globulin	γ-globulin	AGP	'FCoV-positive'
	$>12.5 \times 10^9$/L	$<3.0 \times 10^9$/L	$>7.0 \times 10^9$/L	$<1.5 \times 10^9$/L	$>0.9 \times 10^3$/μL	>11 g/L	>22 g/L	>0.56 g/L	
Clinically healthy	1/32	0/32	4/32	2/32	1/32	1/32	0/32	1/32	19/32
Sick	6/23	2/23	2/23	10/23	2/23	11/20	6/20	17/25	19/25
FIP	4/11	0/11	0/11	10/11	1/11	7/10	5/10	11/13	13/13[a]
Inflammation	2/7	2/7	0/7	3/7	0/7	3/5	1/5	2/7	3/7
Trauma	0/3	0/3	1/3	2/3	1/3	0/3	0/3	2/3	1/3
Lymphoma	0/1	0/1	1/1	0/1	0/1	0/1	0/1	1/1	1/1
FIV	0/1	0/1	0/1	0/1	0/1	1/1	0/1	1/1	1/1

[a]All the cats with FIP were included in the 'FCoV-positive' group because intralesional FCoVs were detected at necropsy. Six of these cats were also tested by immunofluorescence and five had an antibody titre higher than 1:100 (positive threshold of the laboratory).

AGP, alpha-1-acid glycoprotein; FCoV, feline coronavirus; FIP, feline infectious peritonitis; FIV, feline immunodeficiency virus.

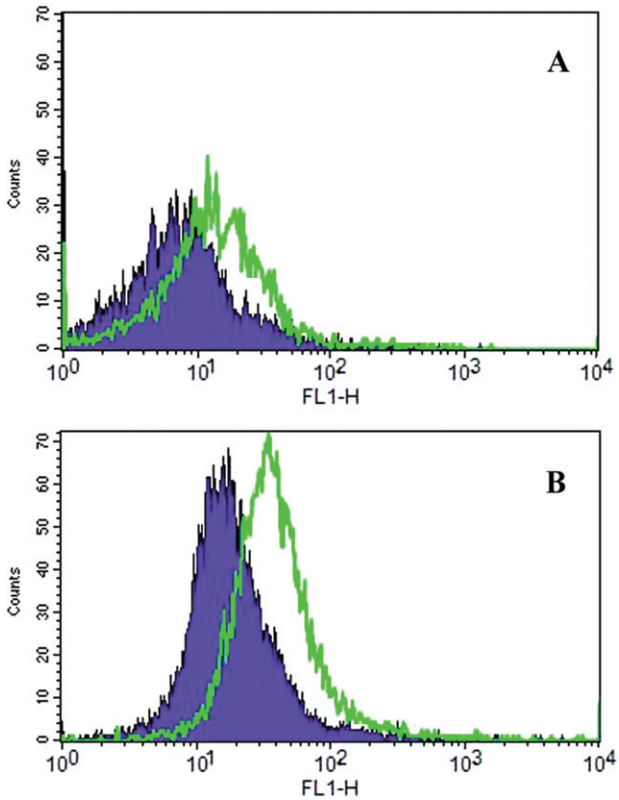

Figure 1. Flow cytometric evaluation of alpha-1-acid glycoprotein (green line) on gated circulating lymphocytes (A) and monocytes (B) compared with negative control (violet area).

monocytes was higher in sick cats (26.3%), although this difference was significant only for cats with other diseases (50.0%) and not for cats with FIP (15.4%).

The proportion of cats with AGP-positive neutrophils and monocytes was significantly higher among the cats with increased AGP (38.9% and 27.8%, respectively) than in the group with normal AGP (12.8% and 7.7%, respectively). No significant differences in the proportion of cats with AGP-positive leucocytes were found between cats with normal or increased leucocyte counts (Table 4).

Discussion

In humans, a protein composed of AGP and a hydrophobic transmembrane peptide has been identified[11,12] and it is thought that the production and/or the release of this protein from activated leucocytes[11,18] could be responsible for the increased concentration of circulating AGP recorded in serum during inflammation or neoplasia.[11] Release of intracytoplasmic AGP from human and bovine granulocytes has also been reported.[14,15] In contrast, in vitro studies have demonstrated an uptake of soluble AGP by T cells,[19] neutrophils and especially monocytes or macrophages,[20,21] on which AGP-binding depends on both the dose and the glycosylation pattern of AGP.[20] It has thus been postulated that excess AGP in serum is cleared by leucocytes via endocytosis,[13] as possibly occurs in myeloid neoplasms,[22] or by binding with membrane receptors such as the ligand of sialic acid called 'immunoglobulin-like lectin 5'.[23]

The results of the present study demonstrate that feline AGP on the surface of circulating leucocytes can be detected by flow cytometry, although less than 10% of clinically healthy cats had positive cells in their blood. The antibody used in this study can recognise both purified feline AGP by Western blotting and AGP exposed on tissue cells by immunohistochemistry[24–26] and does not cross-react with other proteins in feline serum or effusions.[24,25] The high frequency of negative results likely reflects the low number of cats actually exposing AGP on leucocytes rather than poor specificity of the antibody. Further, the amount of AGP on circulating leucocytes is probably low, because it was detected by flow cytometry and not by preliminary tests based on immunocytochemistry (data not shown). A more accurate method of investigating the type of AGP-positive cell would have been a double-staining technique using an anti-AGP antibody and antibodies against leucocyte markers. Unfortunately, both the anti-feline AGP antibody and the anti-leucocyte antibodies used in the present study were not conjugated, thus only an indirect double-staining technique could have been performed, increasing the risk of losing cells during the washing procedures and having high background fluorescence because of non-specific binding.

Based on our results, it is not possible to understand why some clinically healthy cats had AGP-positive leucocytes in their blood. In some cases, these cats had slight laboratory changes, interpretable as either normal individual variability compared with the reference intervals[27] or as the effect of transient pathophysiological processes such as fear (lymphocytosis) or stress (neutrophilia and/or lymphopenia).[28] Therefore, we cannot exclude that stress or fear is associated with the presence of AGP-positive leucocytes.

AGP-positive neutrophils and/or monocytes are detected in cats with increased serum AGP concentrations, but not with leucocytosis, suggesting a direct relationship with inflammation rather than with a high number of cells potentially able to bind AGP. This hypothesis is supported by the fact that all the sick cats, which were very heterogeneous in term of disease type, had inflammatory changes such as increased AGP and/or globulin fractions. Moreover, the inflammatory conditions included in this study, excepting FIV and lymphoma, had an acute clinical course. The activation of the acute-phase reaction seems to be the key event associated with the presence of AGP-positive leucocytes, especially neutrophils and monocytes, which are involved in innate immunity.

Our study did not focus on the mechanism responsible for the presence of AGP on leucocyte membranes. Tests that focus on understanding whether the presence of AGP on leucocytes of sick cats depends on cell activation, as postulated in humans and bovines,[11,14,15] or on increased serum AGP were not performed in this study. A preliminary polymerase chain reaction (PCR) test failed to amplify mRNA coding for AGP in leucocytes (data not shown). The negative result, however, does not exclude the possibility that AGP is synthesised in myeloid precursors and stored in neutrophil granules, as it in humans,[14] and released after inflammatory stimuli.[14,15] This latter hypothesis, however, cannot be tested with the present study design because samples were collected from healthy cats or from cats with spontaneous diseases, and bone marrow aspiration from these cats would have represented an unnecessarily painful and stressful procedure.

Table 2. Summary of AGP-positivity on different leucocyte populations (number of positive/total number of cats) as a function of health status

	Neutrophils	Lymphocytes	Monocytes	At least one
Clinically healthy	3/32	2/32	2/32	3/32
Sick cats	9/25*	5/25	6/25	10/25*
FIP	4/13**	3/13	2/13	4/13
Other diseases	5/12*	2/12	4/12*	6/12*
Total	12/57	7/57	8/57	13/57

*P < 0.05 vs clinically healthy cats; **P < 0.05 vs clinically healthy FCoV-positive cats (see Table 3).
AGP, alpha-1-acid glycoprotein; FCoV, feline coronavirus; FIP, feline infectious peritonitis.

Table 3. Summary of AGP-positivity on different leucocyte populations (number of positive/total number of cats) as a function of FCoV status

FCoV status		Neutrophils	Lymphocytes	Monocytes	At least one
Positive	Clinically healthy	1/19	1/19	0/19	1/19
	Sick	8/19*	5/19	5/19*	9/19*
	FIP	4/13*	3/13	2/13	4/13
	Other diseases	4/6*	2/6	3/6*	5/6*
	Total	9/38	6/38	5/38	10/38
Negative	Clinically healthy	2/13	1/13	2/13	2/13
	Other disease	1/6	0/6	1/6	1/6
	Total	3/19	1/19	3/19	3/19

*P < 0.05 vs clinically healthy FCoV-positive cats.
AGP, alpha-1-acid glycoprotein; FCoV, feline coronavirus.

Table 4. Summary of AGP-positivity on different leucocyte populations (number of positive/total number of cats) as a function of the serum AGP level and leucogram abnormalities

	Neutrophils	Lymphocytes	Monocytes	At least one
Normal AGP	5/39	3/39	3/39	5/39
High AGP	7/18*	4/18	5/18*	8/18*
Normal neutrophils	9/48	4/48	7/48	10/48
Neutrophilia	1/7	1/7	0/7	1/7
Normal lymphocytes	9/49	4/49	6/49	9/49
Lymphocytosis	1/6	1/6	1/6	2/6
Normal monocytes	9/52	5/52	7/52	10/52
Monocytosis	1/3	0/3	0/3	1/3

*P < 0.05 vs normal alpha-1-acid glycoprotein (AGP).

However, this aspect merits further investigation in animals with chronic inflammation (e.g. non-effusive FIP, characterised by a stronger activation of the immune system) and by monitoring the follow-up to assess whether the course of the disease and/or the response to therapies is associated with changes in the number of AGP-positive leucocytes.

Finally, it seems that FCoV infection does not influence the presence of AGP on leucocytes. In this study, FCoV status was estimated by serology, which correlates with the viral burden determined by PCR testing of faeces[29] and suggests the presence of active FCoV–host interactions potentially able to activate an acute-phase reaction. FCoV-seropositive cats were found among both clinically healthy and sick cats, as previously reported,[30] but the proportion of cats with AGP-positive leucocytes did not significantly differ according to FCoV status. Serum AGP concentration, AGP sialylation and the leucocyte responses of clinically healthy, FCoV-positive cats differ from those of cats with FIP[7,8,31] and the finding of a different number of cats with AGP-positive leucocytes would have supported the hypothesis that AGP-bearing leucocytes might influence the FCoV–host interaction, potentially because

of the immunomodulatory properties of AGP.[20] However, this hypothesis is not supported by the results of this study, because the proportion of cats with AGP on leucocytes increased in cats with FIP or diseases other than FIP. The possible role of AGP in the resistance or susceptibility to FIP hypothesised in previous studies,[32] if any, does not involve AGP-bearing leucocytes.

In conclusion, this is the first study to document the presence of AGP on the membrane of circulating feline leucocytes. Specifically, this study demonstrated that AGP can be occasionally found on the leucocytes of clinically healthy cats. The proportion of cats with AGP-positive neutrophils and monocytes increased among the sick animals, most likely associated with the presence of inflammation rather than with the presence of FCoV or leucocytosis. From a clinical perspective, this result suggests that the presence of AGP-positive leucocytes could be considered a marker of inflammation. Other markers of inflammation, including the APP concentration in serum,[32] are currently available in routine practice and most of these tests are more accessible and less expensive that flow cytometry. Nevertheless, when the measurement of other serum markers of inflammation provides equivocal results, the detection of AGP-expressing leucocytes by flow cytometry could be an ancillary test to support a clinical suspicion of inflammation. This study did not focus on the mechanism responsible for the presence of AGP on leucocytes membranes, but these results are encouraging of future studies of these mechanisms and the events that induce an increased presence of AGP-positive leucocytes in cats with inflammation. Additionally, further studies should evaluate the diagnostic and/or prognostic role of the presence of AGP-positive leucocytes.

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