



## Relationship between rate of infection and markers of inflammation/immunity in Holy Birman cats with feline coronavirus



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### ABSTRACT

The aim of this study was to assess whether Holy Birman cats (HB) have a peculiar immune profile and a higher rate of infection by feline coronaviruses (FCoV). Leucocyte and lymphocyte subsets, antibody titers,  $\alpha_1$ -acid glycoprotein (AGP), globulin fractions, IL-4, IL-12 and IFN- $\gamma$  in blood and fecal FCoV excretion were determined in HB (n = 75) and in cats from other breeds (n = 94). Significantly higher CD4/CD8 ratio, IFN- $\gamma$  concentration and IL12/IL4 ratio and significantly lower IL-4 concentration and proportion of shedders were found in HB than in other breeds. No other differences were found. In conclusion, this study did not provide evidence of peculiar immune profiles in HB, except for a prevalent Th1 profile, that may explain why in our caseload the rate of shedders was lower in HB than in other breeds.

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### 1. Introduction

Feline infectious peritonitis (FIP) is a lethal disease caused by the feline coronavirus (FCoV). FCoV is widely diffused in feline populations, in which they usually infect enterocytes and replicate only at the intestinal level. The FCoV genome is highly unstable and replication errors frequently occur. As a consequence, every cat sheds a population of heterogeneous “quasispecies” (Gunn-Moore et al., 1999), characterized by a high adaptability (Domingo et al., 1996): various studies have shown that FCoV genetic heterogeneity correlates with the spread of the infection in the population and with the severity of clinical forms (Battilani et al., 2003; Kiss et al., 2000). The latter occur when the enteric biotype (feline enteric coronavirus or FECV) mutates into strains able to replicate within monocytes, thus inducing FIP (Vennema et al., 1998). However, the pathogenesis of the disease in the presence of mutations is not completely understood. Currently, two theories about the role of mutated FCoVs exist: the “internal mutation theory” and the “circulating virulent/avirulent theory” (Barker et al., 2013). According to this latter theory, both virulent and avirulent biotypes may circulate in a cattery and the disease occurs when mutated viruses infect susceptible cats (Brown et al., 2009). In addition, Dewerchin et al. (2005) showed that the ability of the host monocyte determined the amount of virus replication, no matter whether the virus was virulent or avirulent.

The development of FIP in FCoV-infected cats depends also on the immune responses of the cat: a weak cell mediated (Th1) immunity, eventually associated with a strong humoral (Th2) immunity characterized by an overproduction of antibodies that induce a type III hypersensitivity reaction, leads to the appearance of clinical forms, while a strong cell-mediated immunity seems to be involved in resistance to FIP (Pedersen, 1987). As further support of the protective role of immunity, FIP occurs frequently in young and old cats, that have a less efficient immune system (Pedersen, 2009). Moreover, the comparison of results from FCoV-infected, clinically healthy cats and from cats with FIP showed some differences regarding indicators of innate or cell-mediated immunity, such as lymphocyte subsets, that decrease in blood and lymph nodes in FIP cats but not in FCoV-positive cats (Kipar et al., 1999, 2001; Paltrinieri et al., 2003),  $\alpha_1$ -acid glycoprotein (AGP), that increases and is hyposialylated in FIP cats (Cecilian et al., 2004; Duthie et al., 1997), possibly depressing neutrophil functions (Rossi et al., 2013) and transiently increases and is hypersialylated in FCoV-positive cats (Giordano et al., 2004; Paltrinieri et al., 2007), and proinflammatory cytokines or interferon- $\gamma$  (IFN- $\gamma$ ) whose expression and serum concentration decreases in FIP cats and increases in FCoV-positive cats (Gelain et al., 2006; Giordano and Paltrinieri, 2009; Gunn-Moore et al., 1998; Kiss et al., 2004).

Moreover, following incubation with protein N peptides derived from the sequence of FCoVs from healthy cats or from cats with FIP, feline blood cells respectively increase or decrease the production of IFN- $\gamma$  (Rossi et al., 2011).

From this description it is clear that the immune response plays a role in FCoV–host interaction. Genetic factors can influence the efficiency of immune responses, and a genetic predisposition to

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develop FIP has been demonstrated (Foley and Pedersen, 1996). Moreover, epidemiological studies suggested different individual responses in cats from the same cattery (Addie et al., 2003): the large majority of cats are recurrent shedders, likely due to continuous re-infection by environmental FCoV, which, in turn, depends on fluctuation of their level of immunity (Foley et al., 1997) while some cats persistently shed FCoV and some others have only a transient episode of shedding (Addie et al., 2003). Both these latter categories are in some way resistant to FIP. Finally, FIP has been reported to occur more frequently in some breeds, and especially in Holy Birman cats (Pesteanu-Somogyi et al., 2006). The reason for this higher frequency of FIP is not clear, but a particular feature of the immune response against FCoV has been hypothesized, also based on genomic analyses that identified some candidate genes, coding for molecules involved in immune responses, potentially associated with resistance/susceptibility to FIP (Golovko et al., 2013).

The aim of the present study is to assess whether clinically healthy Holy Birman cats have some peculiarity regarding the indicators of innate or cellular immunity listed above, as well as regarding the Th1-Th2 cytokine profiles, which could explain their predisposition to develop FIP. Moreover, we would like to assess whether the innate/Th1 profiles of Holy Birman cats are associated with a higher rate of infection by FCOVs.

## 2. Material and methods

### 2.1. Animals and study design

Blood and/or feces were initially collected from 169 cats (of different breeds, including Holy Birman cats) in 23 breeding catteries or colonies. Only cats that were clinically healthy were sampled. Moreover, only cats older than 1 year were sampled to avoid the influence of age-related changes. Additional exclusion criteria were the administration of vaccines or of anti-inflammatory treatments before sampling or the presence of metabolic diseases potentially influencing the immune status (i.e. endocrinopathies, neoplastic diseases, etc.).

All the cats were sampled during routine wellness visits under informed consent of the owners. Therefore, approval from our university ethical committee is not required for diagnostic or monitoring purposes.

On the occasion of each sampling the owners were requested to fill in a questionnaire regarding the characteristics of the cattery (e.g. number of cats per cattery, type of housing, presence of isolation facilities, frequency of participation in cat shows, frequency of non specific infectious diseases or of respiratory or gastrointestinal signs). In this questionnaire, the owners were asked about the frequency of routine periodical testing for FCOVs (serology or PCR in feces), if any, about the frequency of positive results, about the number of cases of FIP recorded and confirmed by post-mortem tests in the cattery or in kittens sold to other owners in the past 5 years.

Moreover, the owners were requested to refer to our institution any suspected case of FIP, to confirm the diagnosis through post-mortem tests and to complete the information provided by the owners about the prevalence of FIP in each cattery. Results of blood tests from cats with FIP, however, were not included in this study. Finally, the owners were interviewed one year after the end of the sampling period to obtain information on the health status of cats enrolled in the study.

### 2.2. Blood sampling and pre-processing

Blood (approximately 2 mL) was collected from the cephalic vein and transferred in part in tubes containing EDTA and in part into plain tubes (Venoject, Terumo Italia Srl, Rome, Italy). Just after sampling, routine hematology was performed with an automated laser

counter (Sysmex ST-2000iV, Kobe, Japan) on blood collected in EDTA, followed by microscopic analysis of blood smears to confirm the differential leukocyte count, and flow cytometry performed as described below.

Serum was obtained by centrifugation ( $1,100 \times g$ , 8 min) of blood collected in plain tubes. After collection and separation of serum, a basic panel of biochemical tests focused on assessing the health status of each cat and to confirm the absence of metabolic diseases potentially interfering with the immune status, was performed on an automated biochemistry analyzer (Cobas Mira, Roche Diagnostic, Basel, Switzerland). This panel included creatinine, glucose, total protein, urea, alkaline phosphatase,  $\gamma$ -glutamyl transferase, aspartate aminotransferase, alanine aminotransferase, cholesterol, triglycerides, calcium, phosphate. Then the remaining serum was aliquoted, transferred to Eppendorf tubes and frozen at  $-20^\circ\text{C}$  for a maximum of 6 months to perform serology, AGP measurement, serum protein electrophoresis, and cytokine analysis as described below.

When the amount of whole blood or serum was not enough to perform the whole panel of tests, only some of the parameters regarding innate or specific immunity was assessed. Additionally, sera that were grossly lipemic or hemolytic were not processed. Therefore, the number of samples used for the different tests described below was often different from that of sampled cats. The actual number of samples used for each test is reported in the results section.

### 2.3. Flow cytometry

Five hundred  $\mu\text{L}$  of anticoagulated blood were lysed by incubation (10 min at room temperature) with 7 mL of the lysing solution provided by the manufacturer of the flow cytometer (Beckton Dickinson, San Jose, CA, USA). Tubes were then centrifuged at  $4^\circ\text{C}$  ( $500 \times g$ , 10 min) and the pellet was washed using 7 mL of PBS (Dulbecco's phosphate buffered saline, Sigma Aldrich, St. Louis, MO, USA). Centrifugation and washing was repeated twice, then the pellet was resuspended in 500  $\mu\text{L}$  of RPMI 1640 medium (Sigma Aldrich) added with 5% fetal bovine serum (FBS, Sigma Aldrich) and 0.2% sodium azide (Merck, Darmstadt, Germany). The concentration of cells was adjusted to 10,000/ $\mu\text{L}$  to avoid imbalance of the amount of antigens and antibodies.

Immunophenotyping by flow cytometry was performed on aliquots of 50  $\mu\text{L}$  of the cell suspension to identify lymphocyte subpopulations as previously described (Paltrinieri et al., 2003) using the following panel of antibodies for feline surface antigen: 2.5  $\mu\text{L}$  of mouse anti feline CD4 (specific for T helper cells, clone MCA1350, Serotec, Oxford, UK), 1  $\mu\text{L}$  of mouse anti feline CD8 alpha/beta (specific for T cytotoxic cells, clone MCA1347G, Serotec, Oxford, UK), 50  $\mu\text{L}$  of mouse anti feline CD5 (specific for T cells, clone MCA2038S, Serotec, Oxford, UK), and 1  $\mu\text{L}$  of mouse anti canine CD21, specific for B cells, that cross reacts with feline species (clone MCA1781R, Serotec). After 20 minutes of incubation at  $4^\circ\text{C}$ , the cell suspensions were washed twice as explained above. These non-conjugated monoclonal antibodies were detected by indirect labeling with 50  $\mu\text{L}$  fluorescein isothiocyanate (FITC)-conjugated rabbit anti mouse IgG (STAR9B, Serotec). After 20 minutes of incubation at  $4^\circ\text{C}$ , cell suspension was washed twice again and resuspended.

Data acquisition was performed with a flow cytometer FACSCalibur (Becton Dickinson) and Cell Quest software (Becton Dickinson). Based on morphologic scattergram, a gate corresponding to lymphocytes was drawn, and 10,000 events were collected from the gated region. An appropriate negative control (isotype-matched antibody) was used for each test antibody. The CD4+/CD8+ ratio was then calculated as an expression of the equilibrium of Th1/Th2 immunity.

## 2.4. Serology

The presence of anti-FCoV antibodies was assessed as described in a previous study (Paltrinieri et al., 2007) using an indirect immunofluorescence test performed on 10 multiwell slides produced at the University of Zurich according to Osterhaus et al. (1977).

Serology for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) was performed using commercially available ELISA kits (IDEXX SNAP® FIV/FeLV Combo Plus Test, IDEXX Laboratories, Inc, Westbrook, MA, USA) following the manufacturer's instruction.

## 2.5. Serum protein electrophoresis

Total serum proteins were measured using the automated analyzer Cobas Mira and a commercial kit (Real Time Diagnostic System, Viterbo, Italy) based on the biuret method.

Serum protein electrophoresis was performed on agarose gel using the automated analyzer Hydrasis (Sebia Italia Srl, Bagno a Ripoli, Florence, Italy) and the specific manufacturer's reagents (Hydragel 15  $\beta$ 1– $\beta$ 2, Sebia Italia Srl) following the procedure described in another study (Paltrinieri et al., 2014). Total serum proteins and the percentage of electrophoretic fractions were used to calculate absolute protein concentrations (g/L) for each electrophoretic fraction. The serum concentration of the two most important electrophoretic fractions associated with inflammation/immunity (namely  $\alpha$ <sub>2</sub>- and  $\gamma$ -globulins), the serum concentration of serum albumin, that during inflammation works as a negative acute phase protein (Paltrinieri, 2008), and the A/G ratio were then included in this study.

## 2.6. Measurement of alpha-1-acid glycoprotein (AGP) in serum

Feline AGP was measured using a radial immunodiffusion (SRID) kit (Tridelta Development Ltd, Maynooth, Kildare, Ireland) following the manufacturer's instructions. Values from the case samples were then plotted to extrapolate absolute AGP levels, expressed as mg/mL.

## 2.7. Measurement of cytokines

Serum concentration of IL-4, IL-12 and IFN $\gamma$  was determined using specific ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's recommendations.

Briefly, after the overnight incubation at room temperature with the specific capture antibodies and the addition of the blocking solution, 100  $\mu$ L of each serum sample or standard were added to each well and plates were then incubated with biotinylated monoclonal antibodies specific for IL-4, IL-12, and INF- $\gamma$ , respectively. After washing, streptavidin-HRP was added. The concentration of each cytokine was determined using a standard curve generated by 2-fold serial dilutions of the standards. The absorbance was measured by an automated plate reader (Labsystems Multiskan MS, DASIT, Cornaredo, Italy) using 450 nm as the primary wavelength.

All the cytokine measurements were performed in duplicate, and the mean values of the two readings were used to statistically analyze the results.

## 2.8. RT-PCR on feces

Frozen fecal swabs were thawed and submerged in 200  $\mu$ L of PBS with pH 7.2  $\pm$  0.2 (Dulbecco's phosphate buffered saline, Sigma Aldrich) within a 1.5 mL tube and were incubated at 40 °C for 5 minutes, then the swabs were inverted and centrifuged (5000 g  $\times$  4 mins). Thus swabs were discarded and the supernatant were transferred into 0.5 mL sterile tubes and incubated at 95 °C for 5 minutes. Reverse-transcription and amplification were done as described in a previous study (Gelain et al.,

2006) using the forward and reverse primers FCoV1128f and FCoV1229r (Gut et al., 1999) that allow RT and amplification in a single step in a single tube. Based on the presence or absence of amplicons, cats were classified as positive or negative.

## 2.9. Statistical analysis

Statistical analysis was performed in an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it software (Analyse-it Software Ltd, Leeds, UK).

The proportion of seropositive and/or PCR positive cats recorded in Holy Birmans was compared with that of other breeds using a Pearson's chi square analysis.

The comparison of continuous values recorded in Holy Birman cats and in cats of other breeds was done using a Kruskal–Wallis test. In order to assess possible differences in the prevalence of infection, based on the information reported by the owners and on the results of serology and PCR recorded within this study, catteries were arbitrarily classified as follows:

- Type L: Low rate of positivity: < 25% of cats were seropositive or PCR-positive.
- Type M: Medium rate of positivity: 25–50% of cats were seropositive or PCR-positive.
- Type H: High rate of positivity: 50–75% of cats were seropositive or PCR-positive.
- Type V: Very high rate of positivity: > 75% of cats were seropositive or PCR-positive.

## 3. Results

### 3.1. Characteristics of the study population

The characteristics of the catteries included in the study are reported in Table 1. Fourteen catteries were composed of Holy Birman cats (cats included in this study: n = 75) and 9 were composed of cats (cats included in this study: n = 94) from other breeds such as Chartreux (n = 6); Domestic Shorthair (DSH, n = 24); Maine Coon (n = 6); Norwegian Forest (n = 15); Persian (n = 4); Siberian (n = 39). The age distribution of Holy Birman cats and of cats of other breeds were respectively 1–10 years (median = 4 years) and 1–14 years (median = 4 years).

Most of the catteries had similar management. The main differences were related to the number of cats per cattery but in most cases cats were kept in house with the owners, kept in isolation in the case of diseases or of other pathophysiological conditions (e.g. estrus, pregnancy/lactation) and frequently brought to cat shows. Cats were grouped in order to avoid unwanted matings, that were mostly done using external males, or to prevent aggressive behavior. The general health status of the catteries was good (no specific recurrent problems were identified) or moderately altered (i.e. characterized by recurrent non specific and/or transient signs, mostly affecting the upper respiratory or the gastrointestinal system), except for one of the two shelters that had frequent episodes of severe illness. Also in this cattery, however, no diseases were recorded in the day of samplings.

Results of routine hematology and clinical chemistry of sampled cats (data not shown) were always within reference intervals of the species or of specific breeds (Kaneko et al., 1997; Paltrinieri et al., 2014; Rizzi et al., 2010). Moreover, all the cats were seronegative for FIV and FeLV.

As regard the FCoV status (Table 1), 9 catteries of Holy Birman cats were classified as type V, 2 as type H, 2 as type M and only one as type L. However, also the large majority of catteries of breeds other than Birmans were classified as type H (n = 1) or V (n = 6),

**Table 1**  
Characteristics of the catteries included in the study and results of anti-FCoV serology and of PCR on feces recorded in this study.

Cat	Number of cats		Management	Health status	Periodical testing <sup>a,††</sup>	FIP Cases <sup>a</sup>	Serology/PCR <sup>c</sup>			FCoV status	
	Total	Sampled <sup>†</sup>					Serology		PCR <sup>‡</sup>		Serology or PCR <sup>‡</sup>
							Titre <sup>†††</sup>	Rate <sup>‡</sup>			
1 (B)	7	6 (2–10; 5.5)	h/ng/is/r/i	Mod (URD)	(1/y) Neg	1 (K)	0 (0–50)	0/6 (0%)	1/6 (16.7%)	1/6 (16.7%)	L
2 (S)	19	13 (1–8; 3)	h/g/is/f/ie	Mod (GID)	(3/y) 30%	3 (K)	25 (0–200)	2/13 (15.4%)	2/13 (15.4%)	4/13 (30.8%)	M
3 (B)	6	6 (1–10; 6)	h/ng/ni/r/ie	Good	(1/y) Neg	0	25 (0–100)	3/6 (50.0%)	0/6 (0.0%)	3/6 (50.0%)	M
4 (B)	3	3 (1–5; 2)	h/ng/ls/r/n	Good	None	0	100 (50–400)	2/3 (66.7%)	2/3 (66.7%)	2/3 (66.7%)	H
5 (B)	2	2 (2–4; 3)	h/ng/ni/r/n	Good	(1/y) 50%	0	150 (100–200)	2/2 (100.0%)	0/2 (0.0%)	2/2 (100.0%)	V
6 (B)	2	2 (3–3; 3)	h/ng/ni/r/n	Good	None	0	300 (200–400)	2/2 (100.0%)	ND	2/2 (100.0%)	V
7 (B)	2	2 (1–5; 3)	h/ng/ni/r/i	Good	None	0	450 (100–800)	2/2 (100.0%)	1/2 (50.0%)	2/2 (100.0%)	V
8 (S)	10	10 (1–7; 3.5)	h/g/is/f/ie	Mod (GID)	(1/y) >50%	0 (K)	50 (0–200)	3/10 (30.0%)	2/10 (20.0%)	4/10 (40.0%)	M
9 (B)	12	10 (1–9; 2.5)	h/g/is/f/ie	Mod (URD)	(1/y) 30%	2 <sup>b</sup> (K)	200 (100–800)	10/10 (100.0%)	4/10 (40.0%)	10/10 (100.0%)	V
10 (D)	>50	4 (1–14; 5)	c/g/is/n/nm	Bad (URD, GID, p)	none	0	63 (25–400)	2/4 (50.0%)	2/4 (50.0%)	3/4 (75.0%)	H
11a (S)	12	11 (1–10; 3)	h/g/is/f/ie	Good	(2/y) 50%	2 (K)	200 (100–400)	10/11 (90.9%)	8/11 (72.7%)	11/11 (100.0%)	V
11b (C)	6	6 (2–8; 6)					200 (50–400)	5/6 (83.3%)	3/6 (50.0%)	5/6 (83.3%)	V
12 (D)	>50	20 (1–12; 6)	c/g/is/n/nm	Mod (URD)	None	5 <sup>b</sup>	200 (0–800)	18/20 (90.0%)	6/20 (30.0%)	19/20 (95.0%)	V
13 (N)	16	15 (1–12; 5)	h/g/is/f/ie	Mod (URD)	(3/y) 70%	3 <sup>b</sup> (K)	200 (100–800)	13/15 (86.7%)	9/15 (60.0%)	13/15 (86.7%)	V
14 (MC)	10	6 (2–6; 4)	h/ng/ni/r/e	Good	(rare) 40%	1 <sup>b</sup>	200 (25–800)	5/6 (83.3%)	4/6 (66.7%)	5/6 (83.3%)	V
15 (S)	7	5 (2–8; 4)	h/ng/is/f/ie	Good	(1/y) 10%	0 (K)	200 (100–200)	5/5 (100.0%)	4/5 (80.0%)	5/5 (100.0%)	V
16 (B)	15	11 (1–7; 3)	h/g/is/f/ie	Mod (URD)	(1/y) 50%	0	50 (25–1600)	5/11 (45.5%)	1/11 (9.1%)	6/11 (54.5%)	H
17 (B)	7	7 (1–7; 4)	h/ng/ni/f/ie	Good	None	0 (K)	25 (25–200)	2/7 (28.6%)	3/7 (42.9%)	3/7 (42.9%)	M
18 (B)	7	7 (2–7; 2)	h/ng/ni/f/ie	Good	None	0	400 (200–1600)	7/7 (100.0%)	4/7 (57.1%)	7/7 (100.0%)	V
19 (B)	4	3 (3–8; 5)	h/g/ni/f/ie	Good	(1/y) 50%	0	400 (200–800)	3/3 (100.0%)	1/3 (33.3%)	3/3 (100.0%)	V
20 (B)	15	7 (2–8; 5)	h/g/is/f/ie	Good	(3/y) >50%	0	200 (100–800)	7/7 (100.0%)	3/7 (42.9%)	7/7 (100.0%)	V
21 (B)	7	2 (5–6; 5.5)	h/ng/is/f/ie	Good	(1/y) 30%	0 (K)	450 (100–800)	2/2 (100.0%)	2/2 (100.0%)	2/2 (100.0%)	V
22 (B)	10	7 (1–6; 4)	h/g/is/f/ie	Good	(1/y) 60%	2 (K)	200 (100–800)	5/7 (71.4%)	1/7 (14.3%)	6/7 (85.7%)	V
23 (P)	5	4 (2–6; 4)	h/ng/is/f/ie	Good	None	2 <sup>b</sup> (K)	38 (25–200)	1/4 (25.0%)	4/4 (100.0%)	4/4 (100.0%)	V

Cat: Cattery number and breed: B = Holy Birman; C = certosin; D = Domestic Shorthair; MC = Maine Coon; N = Norwegian forest cat; P = Persian; S = Siberian.

Number of cats = †age range and median age are reported in brackets.

Management: c = cages; e = external matings; f = frequent participation to cat shows; g = groups; h = house; i = internal matings; ie = internal and external matings; is = isolation facilities; n = no participation to cat shows; ng = no groups; ni = no isolation facilities; nm = no matings; r = rare participation to cat shows.

Health status: GID = gastrointestinal diseases; Mod = moderate; p = outbreaks of parvovirus; URD = upper respiratory disease.

Periodical testing: ††frequency of testing is reported in brackets (y = year), followed by the approximate rate of positive results.

FIP Cases: K = cases of FIP reported by the owners of kittens sold out from the cattery.

Serology/PCR = †††median titre and, in brackets, minimum and maximum titre; ‡number of positive/number of sampled cats and, between parenthesis, percentage of positive cats.

FCoV status: L = Type L cattery, i.e. cattery with a low rate of positivity (<25% of cats were seropositive or PCR positive); M = Type M cattery, i.e. cattery with medium rate of positivity (25–50% of cats were seropositive or PCR positive); H = Type H cattery, i.e. cattery with a high rate of positivity (50–75% of cats were seropositive or PCR positive); V = Type V cattery, i.e. cattery with very high rate of positivity (>75% of cats were seropositive or PCR positive).

<sup>a</sup> Information reported by the breeders.

<sup>b</sup> Including the cases recorded in this study.

<sup>c</sup> Results recorded within this study (Serology or PCR from cats with FIP are not included in this table, which refers only to clinically healthy cats).



**Table 2**  
Results recorded in Birman cats and in cats from other breeds.

	Birman	Other breeds
WBC × 10 <sup>3</sup> /μL <sup>a</sup>	10.2 ± 3.9 (9.3)	11.8 ± 5.4 (10.3)
Neutrophils × 10 <sup>3</sup> /μL <sup>a</sup>	6.0 ± 3.3 (5.1)	7.0 ± 3.8 (6.0)
Lymphocytes × 10 <sup>3</sup> /μL <sup>a</sup>	3.5 ± 1.6 (3.3)	3.6 ± 3.0 (2.9)*
Monocytes × 10 <sup>3</sup> /μL <sup>a</sup>	0.2 ± 0.1 (0.2)	0.4 ± 0.6 (0.3)*
Total proteins (g/dL) <sup>b</sup>	7.9 ± 1.1 (7.6)	7.2 ± 1.2 (7.1)**
Albumin (g/dL) <sup>b</sup>	4.5 ± 0.9 (4.6)	4.0 ± 1.3 (4.2)*
α <sub>1</sub> -globulin (g/dL) <sup>b</sup>	0.2 ± 0.1 (0.2)	0.2 ± 0.1 (0.2)
α <sub>2</sub> -globulin (g/dL) <sup>b</sup>	0.8 ± 0.3 (0.9)	0.8 ± 0.3 (0.9)
β <sub>1</sub> -globulin (g/dL) <sup>b</sup>	0.5 ± 0.1 (0.5)	0.5 ± 0.3 (0.5)*
β <sub>2</sub> -globulin (g/dL) <sup>b</sup>	0.4 ± 0.2 (0.4)	0.3 ± 0.3 (0.3)***
γ-globulin (g/dL) <sup>b</sup>	1.3 ± 1.0 (1.1)	1.1 ± 0.7 (1.0)
A/G ratio <sup>b</sup>	1.5 ± 0.5 (1.5)	1.5 ± 0.6 (1.4)
AGP (mg/ml) <sup>c</sup>	0.47 ± 0.33 (0.39)	0.46 ± 0.21 (0.38)
CD5 × 10 <sup>3</sup> /μL <sup>d</sup>	2.24 ± 1.09 (2.10)	1.93 ± 1.20 (1.63)*
CD4 × 10 <sup>3</sup> /μL <sup>d</sup>	1.34 ± 0.67 (1.29)	0.96 ± 0.78 (0.77)***
CD8 × 10 <sup>3</sup> /μL <sup>d</sup>	0.78 ± 0.43 (0.69)	0.76 ± 0.52 (0.66)
CD21 × 10 <sup>3</sup> /μL <sup>d</sup>	0.85 ± 0.83 (0.69)	0.89 ± 0.82 (0.67)
CD4/CD8 ratio <sup>d</sup>	2.02 ± 1.60 (1.73)	1.43 ± 0.99 (1.22)***
IL12 pg/mL <sup>e</sup>	303.4 ± 168.7 (240.6)	283.1 ± 140.2 (231.9)
IL4 pg/mL <sup>e</sup>	36.7 ± 28.1 (31.2)	58.8 ± 56.3 (38.5)**
IL12/4 ratio <sup>e</sup>	10.1 ± 6.5 (7.3)	6.9 ± 4.4 (5.5)***
IFN-γpg/mL <sup>f</sup>	115.7 ± 65.1 (99.0)	103.3 ± 82.7 (76.5)*

<sup>a</sup> Birman n = 71; Other breeds n = 87.

<sup>b</sup> Birman n = 68; Other breeds n = 89.

<sup>c</sup> Birman n = 75; Other breeds n = 93.

<sup>d</sup> Birman n = 71; Other breeds n = 80.

<sup>e</sup> Birman n = 62; Other breeds n = 81.

<sup>f</sup> Birman n = 62; Other breeds n = 80.

Eosinophils and basophils are not reported since they are not the main effectors of innate or specific immunity.

\* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001.

except for 2 catteries of Siberian cats that were classified as type M. In almost all the catteries, the rate of positivity recorded in this study was similar to that recorded in the past by the owners. With rare exceptions, FIP occurred frequently in catteries (or in kittens sold out from catteries) of type H or V, independently of the breed. During the study period, cases of FIP were recorded either in Holy Birman cats (cattery 9) or in cats of other breeds (catteries 12, 13,

14, 23). In total, 5 cases of FIP were recorded in catteries of Holy Birman and 16 in catteries of other breeds.

The proportion of cats with positive PCR was significantly lower (P < 0.05) in Holy Birman cats (23/73 cats tested; 31.5%) than in cats of other breeds (44/94; 46.8%). Conversely, no significant differences were found between the proportion of seropositive cats in Holy Birman (52/75, 69.3%) and in cats of other breeds (64/94; 68.1%) and between the proportion of cats with at least one positive test (e.g. PCR or serology) in Holy Birman cats (55/75; 73.3%) and in cats of other breeds (73/94; 77.7%). Similarly, antibody titers did not significantly differ in Holy Birman (median 1:200; min-max 0–1:1600) compared with cats of other breeds (1:100; 0–1:800). There were no apparent associations between median antibody titers or number of seropositive or PCR positive animals and the occurrence of cases of FIP.

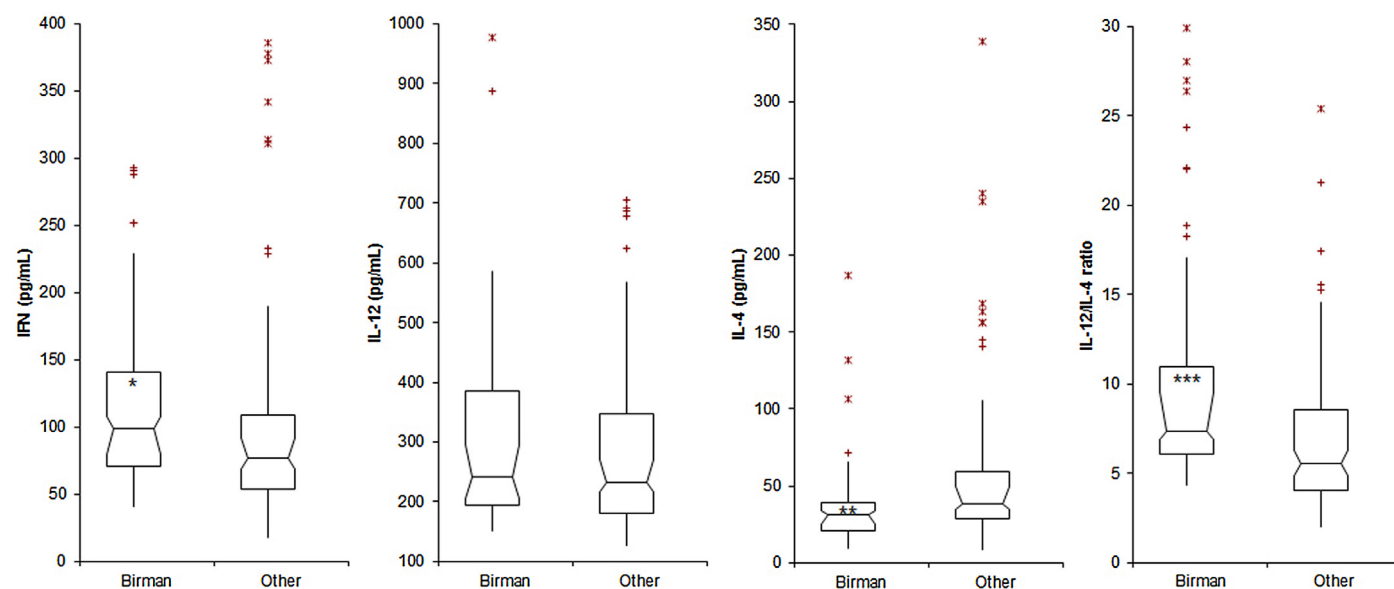
### 3.2. Markers of inflammation and innate immunity

Data regarding leukograms, electrophoretic fractions and AGP concentration are reported in Table 2.

The only significant difference regarded lymphocyte and monocyte counts that were lower in Holy Birman than in the whole groups of cats of other breeds. Results recorded in different catteries of Holy Birman cats or in individual cats (data not shown) showed that the highest WBC, neutrophil and monocyte values were found in 3 type V catteries (18, 20 and 21), while the lowest AGP concentration was found in cattery 1 (type L). Three Holy Birman cats from type H or V catteries had AGP concentrations (0.85, 1.71 and 2.08 mg/mL, respectively) higher than the upper limit of the reference interval (0.56 mg/mL) in spite of normal leukogram and electrophoretograms. Conversely, lymphocyte counts and the concentration of globulin fractions were variable and apparently not associated with the rate of positivity or with the health status.

### 3.3. Markers of specific immunity

Data regarding lymphocyte subsets and cytokine concentrations are also reported in Table 2, and the distribution of data regarding cytokines is summarized in Fig. 1. This comparison



**Fig. 1.** Graphical representation of the distribution of data regarding the concentration of IFN-γ, IL-12, IL-4 and IL12/IL-4 ratio. Boxes indicate the I-III percentiles, whiskers indicate the extend to further observation within the I quartile minus 1.5\*IQR or to further observation within the III quartile plus 1.5\*IQR, the horizontal line indicates the median value. The symbols “+” and “\*\*\*” indicates the near and far outliers, respectively. The bolded asterisks within the boxes indicate significant differences between Holy Birman cats and cats of other breeds (\*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001).

revealed several differences although in both groups a wide individual variability was observed. Compared with other breeds, Holy Birman cats had higher T cell counts, mostly due to a higher number of CD4+ cells and consequently a higher CD4/CD8 ratio, a significantly higher concentration of IFN- $\gamma$ , and a lower concentration of IL-4. Therefore the IL12/4 ratio was significantly higher in Holy Birman cats compared with other breeds, in spite of the lack of differences in the concentration of IL-12.

The comparison of results recorded in different catteries of Holy Birman cats (data not shown) did not show evident differences, with rare exceptions not associated with the rate of positivity.

#### 4. Discussion and conclusion

This study was focused on the analysis of blood parameters associated with inflammation and immunity in Holy Birman cats, as an attempt to explain the high susceptibility of this breed to feline infectious peritonitis (FIP) (Golovko et al., 2013; Pesteanu-Somogyi et al., 2006).

The inclusion criteria were established in order to include in the study only clinically healthy cats without laboratory changes consistent with diseases potentially affecting immune responses.

All catteries had similar management, apart from some minor changes, mostly related to the number of cats per cattery (that was higher in the two groups of DSH) or to the participation to cat shows and mating programs (that were less frequent in small catteries). The health status of the catteries was good in general or characterized by recurrent episodes of non specific infectious diseases. Only one rescue shelter of DSH had a history of frequent outbreaks of infectious diseases, likely related to the larger size of the group associated with a higher environmental circulation of infectious agents. Nevertheless, all the catteries had a history of FCoV positivity or of FIP cases, although the rate of positivity was different. The rate of positivity was assessed using both serology and PCR since this approach provides a more complete overview of the “FCoV status” than serology or PCR alone (Addie and Jarret, 2001; Addie et al., 2003) because seropositive cats may not shed FCoVs and, vice versa, seronegative cats, that do not have local immune defenses, may host the virus in the gastrointestinal tract, according to the susceptible-infected-susceptible (SIS) model (Foley et al., 1997). Therefore, differently from experimental studies on which the infection status of each cat can be precisely known, the actual presence of infection of each cat in field studies can only be estimated. However, the combined approach is the best method to actually know the overall rate of infection in a cattery. Although the proportion of positive cats and the level of positivity was different in the different catteries, this survey confirmed that the level of environmental infection by FCoVs in multicat environments is generally high, as reported in the literature (Addie et al., 2003; Pedersen, 2009). However, no evident association between the median titer and the number of seropositive cats was found. Additional information about the level of infection of single cats or of Birman cats compared with other breeds may have been drawn by a quantitative analysis of fecal shedding through a quantitative PCR or by a semi-quantification of PCR products used in previous studies (Gelain et al., 2006). Unfortunately, none of these two approaches has been followed in this study.

There were no significant differences regarding the rate of seropositivity between Holy Birman cats and cats of other breeds or between the different catteries, in some case due to the high individual variability within each cattery. No association was found between the rate of positivity (serology or PCR) and the number of cats, the general health status or the management of the cattery (e.g. participation to cat shows, grouping of cats). However, a statistical comparison among groups of catteries with different management was not possible since most of the catteries had similar characteristics, and the number of observations was therefore

unbalanced. Conversely, with a few exceptions (cattery 1 and 23), cases of FIP occurred mainly in catteries with more than 10 cats, supporting the association between cattery size and probability of FIP (Kass and Dent, 1995). More importantly, the prevalence of positive cats, the magnitude of antibody titers and the frequency of FIP were not higher in Holy Birman cats than in the other breeds. The magnitude of antibody titers recorded in this study was similar to that recorded in a previous study (Bell et al., 2006a). In a similar study the antibody titer of Holy Birman cats was higher compared with some other feline breeds (some of which, e.g. Persians, were included also in the current study) but not with others (e.g. Abyssinian, Russian Blue, etc.) (Bell et al., 2006b) and therefore it is not surprising that in the present study, on which all the other breeds were merged into a single group, no significant differences were found. However, in this study results from the different “non-Birman” breeds were not analyzed separately, except in the tables describing the study population, since the focus of the study was to specifically analyze the indicators of innate or specific immunity in Holy Birman cats, that have been reported to be more predisposed to FIP (Pesteanu-Somogyi et al., 2006). Our epidemiological results seem to contrast with this latter study, that, however, was based on a retrospective analysis of medical records at one teaching hospital and hence may have been biased by the referral hospital population. In addition, in the study cited above, the data referred to a longer period of time (16 years), while our results, although generated with a larger caseload and therefore potentially more adequate to reflect the general population of Holy Birman cats, were focused on the 5 years preceding the sampling. Interestingly, despite the fact that the prevalence of FIP or of seropositivity was similar to that of other breeds, the proportion of shedders was lower in Holy Birman cats than in other breeds. This may suggest that Birman cats are mostly recurrent shedders on which intestinal viruses stimulate an immune response that rapidly clear FCoVs from the intestine (Addie et al., 2003).

The second step of the study was focused on the evaluation of parameters associated with innate and acquired immunity. This evaluation did not find evidence for a particular activation of innate immunity, since electrophoretograms and leukograms were always within the reference intervals of the general feline population or of Holy Birman cats (Paltrinieri et al., 2014; Rizzi et al., 2010) and the few differences between Holy Birmans and other breeds were probably non-specific. Also AGP values were within the reference intervals, except for individual cats from type H or V catteries that had high serum concentration of AGP in the absence of other changes consistent with inflammation. The lack of electrophoretic changes in animals with high AGP values is not surprising since on one hand the baseline concentration of AGP is so low that even in the presence of strong increases the serum concentration remains low and on the other hand due to its acidic nature, AGP does not bind the stains routinely used in electrophoretic systems (Keren, 2003). However, the lack of changes in inflammatory markers despite the increase of serum AGP may depend on the kinetics of this protein, which increases earlier than other inflammatory markers (Paltrinieri, 2008). Additionally, the possibility that increases of AGP may be related in some way to early changes during the development of FIP can be excluded since all the cats included in this study were still alive and did not develop clinical signs potentially consistent with FIP during the year after sampling.

Therefore, the high AGP values may be interpreted as a transient stimulation of the innate immune response. This never occurred in cats of other breeds, despite the likelihood that exposure to circulating microorganisms was probably similar. This suggests that individual Holy Birman cats may have a more intense AGP response: this response may be induced by FCoVs as already hypothesized (Paltrinieri et al., 2007, 2008). However, this hypothesis may not be proven since the temporal association between FCoV

fecal shedding and the presence of high AGP values was not assessed in this study. Unfortunately, it was not possible to assess whether these changes were associated with peculiarities of the glycosylation pattern of AGP, as demonstrated in previous studies (Ceciliani et al., 2004; Paltrinieri et al., 2007), since the procedure of AGP purification and of assessment of the glycosylation pattern requires high volumes of serum (Ceciliani et al., 2004; Rossi et al., 2013).

Results regarding cell-mediated immunity were quite surprising since a significantly higher CD4 count and CD4/CD8 ratio, a higher concentration of IFN- $\gamma$  and a higher IL-12/IL-4 ratio was found in Holy Birman cats, suggesting that cell-mediated immunity is even more efficient in these cats than in other breeds, independently on the rate of positivity, on the number of cases of FIP or on the general health status. However, it should be stressed that, although both suggestive of a strong cell mediated immunity, the cytokine profile and the CD4/CD8 ratio recorded in this study cannot be considered to be definitive proof of a stronger Th1 response. Definitive information on the efficiency of cell mediated immunity of Holy Birman cats compared with other breeds can be obtained by *in vitro* studies focused on cytokine production on isolated blood cells or by experimental studies on which the actual state of infection can be monitored without the external variables typical of field studies such as the current one. This may be particularly important since our results seem to contrast with what was reported by Pesteanu-Somogyi et al. (2006). In their study, these authors found that Birman cats are predisposed to develop FIP, while the lymphocyte and cytokine profile found in the present study should theoretically protect FCoV infected cats from the development of FIP (Pedersen, 2009). However, this may explain why the catteries of Holy Birman cats included in our study did not have a higher prevalence of FIP compared with other breeds. However, this Th1 profile might theoretically be one of the factors that confer resistance to permanent colonization of the intestinal tract, thus explaining why most cats were seropositive sometimes with medium-high titers (and therefore likely recently infected) but PCR negative (i.e. not infected at the time of sampling). Further studies are needed to assess whether this possibly high re-infection rate plays a role in increasing the probability of mutation of FCoVs, thus predisposing infected animals to develop FIP.

In conclusion, this study did not find evidence of an altered pattern of innate/cell mediated immunity in Holy Birman cats except for an occasional moderate increase of serum AGP in a few cats. On the contrary, the lymphocyte and the cytokine profile of Holy Birman cats were consistent with an efficient Th1 immunity. This Th1 profile is theoretically protective against FIP and it may explain why in our caseload, Holy Birman cats had a lower rate of shedders and did not show a higher rate of seropositivity or a higher prevalence of FIP cases than that recorded in other breeds.

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