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A COMPARATIVE CLINICAL AND PARACLINICAL EVALUATION OF THE EFFECTS OF POLYSPECIFIC AVIAN IMMUNOGLOBULIN Y IN FIV+/- CATS

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Abstract

Feline Immunodeficiency Virus (FIV) infection represents a viral immunosuppressive condition that predominantly affects the immune system, having no curative therapeutic options. The present research focused on the biological and physical effect of the use of poly-specific avian immunoglobulin Y (IgY) on some immune molecules of FIV infected cats, especially on gamma-globulins and white blood cells, as well as on how this therapy can amend or improve the quality of life of infected cats. The polyspecific avian immunoglobulin Y was used to treat two groups of feline patients: 10 patients infected with FIV and 10 patients free of the disease. The patients were monitored via blood samples collected on days 0, 20 and 40. The samples were used for testing the biochemical profile, CBC, WBC and the plasma concentration of 10 cytokines: Fas, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p40 and RANTES. The study also attempted to establish the eventual side effects associated with the suppressed immune system of FIV infected cats.

***Key words**: Feline Immunodeficiency Virus (FIV), Immunomodulators, IgY, Cytokines.*

INTRODUCTION

FIV induces a chronic and latent infection in domestic cats (Erol & Pasa, 2013; Kahn, 2014) characterized by a polymorphous clinical manifestation (Bendinelli et al., 1995; Najafi et al., 2014), which can develop into a severe immunodeficiency in its final stage of evolution (Sykes, 2014; Najafi et al., 2014). The highest infection rate is in old, male stray cats that also manifest an aggressive behavior (Kahn, 2014; Gil et al., 2013). The main transmission route is through biting (Day, 2008; Mosallanejad et al., 2010a). FIV induces a disease similar to AIDS, characterized by the progressive depletion of CD4+ T helper cells (Shimojima et al., 2004; Hohdatsu et al., 2000). Additionally, it infects CD8+ and B cells, macrophages and dendritic cells, microglia and astrocytes (Day, 2008; Collado et al., 2012). Moreover, it is characterized by dysfunctionalities in the production of cytokines. Three distinct phases of disease evolution have been described: the primary or acute phase, the subclinical phase and the terminal phase (Sykes, 2014; Day, 2008). Of these, the terminal phase is frequently associated with overlapping infections (Sykes, 2014; Hartmann, 2015). The immune abnormalities secondary to FIV infection include: 1) the progressive reduction of the CD4:CD8 ratio; 2) the suppressed reaction of mononuclear blood cells to mitogens or specific antigens, with a reduced IL-2 production consequent the mitogen activation and an increased production of IL-1, IL-6 and TNF-α; 3) modified activity of macrophages; 4) modified lymphoid tissue; 5) polyclonal gammopathy (Day, 2008). The major clinical signs of FIV account for loss in weight, fever, muscular atrophy (Sykes, 2014; Shimojima et al., 2004), periodontal disease, respiratory infections, otitis (Sykes, 2014), enteritis, enlarged lymph nodes, opportunistic infections and neoplasms (Mosallanejad et al., 2010b; Kahn, 2014). Palliative therapy includes antimicrobials, pain management, nutritional

support (Greggs et al., 2011), fluids and teeth extraction (Gil et al., 2013). Immunomodulators are frequently used (Hartmann, 2015) and include interferon (IFN), staphylococcal protein A, *Serratia marcescens*, inactivated Parapoxovirus, Acemannan (de Mari et al., 2004). Antiviral chemotherapy is rarely indicated due to the lack of evidence for benefits of numerous antivirals, as well as due to the significant toxicity they inflict (Hartmann, 2015).

Avian Immunoglobulin Y (IgY) represents the main antibody obtained from the domestic hen (Gallus gallus domesticus) (Bentes et al., 2015; Gao et al., 2016) and is continously synthetised and transfered into the egg yolk (Dias da Silva & Tambourgi, 2010; Alustiza et al., 2016; Thomsen et al., 2016). IgY is the functional equivalent of mammalian IgG (Murai et al., 2016; Jiang et al., 2016b; Aranda-Uribea et al., 2017). Due to the fact that modern technologies allow IgY production at high and long lasting titers with relatively low costs of production (Jiang et al., 2016a; He et al., 2016), its use has been introduced in various medical fields: xenotransplant, diagnosis tools, passive immunization and alternatives to antibiotics (Ko & Ahn, 2007; Alustiza et al., 2016). IgY therapy has proven to be a successful alternative to traditional treatments for viral or bacterial pathologies (Alustiza et al., 2016; da Rocha et al., 2017), offering a passive protection against a broad spectrum of infections, as *Salmonella* spp., *Campylobacter jejuni* (Nasiri et al., 2016; Müller et al., 2015), *Escherichia coli* (Suartini et al., 2014; Kalantar et al., 2015), *Helicobacter pylori* (Müller et al., 2015), *Staphylococcus aureus* (Al-Edany, 2011; Nasiri et al., 2016), *Listeria monocytogenes, Pseudomonas* spp., Newcastle Disease virus, *Yersenia ruckeri, Edwardsiella tarda, Aeromonas salmonicida, Pseudomonas fluorescens* (Zorriehzahra et al., 2016), *and toward* Rotavirus strains or bovine coronavirus in mice, pigs and cattle (Dias da Silva & Tambourgi, 2010). The most important advantages of IgY use: 1) it is a natural compound; 2) no side effects reported; 3) not toxic for the environment; 4) it does not induce specific resistance by microorganisms; 5) it is highly specific and 6) it allows for targeted action against specific pathogens (Thu et al., 2017).

Objectives of the research. The present study continued the previous research in studying the effects of IgY in cats (Supeanu et al., 2015; Supeanu et al., 2016). The two previous studies targeted the clinical, haematological and biochemical evolution of feline patients after 10 days of IgY administration. The results of the two studies showed that chicken egg IgY administration in FIV infected felines had a positive impact on the general status of the organism and was free of adverse reactions. The positive results suggested the need for a larger study. Thus, a research protocol was initiated by using classic testing techniques as well as innovative ones, respectively the quantitative determination of 10 pro/anti-inflammatory interleukins by using the microarray method. The main objective was to identify the innermost effects avian IgY has on the feline organism. The side objectives were to identify clinical changes, variations in the haematological and biochemical parameters as well as establishing the way in which the cytokines concentration changes during and after IgY administration.

MATERIALS AND METHODS

Overview of study. A clinical-based, case-control study was conducted for non-stray cats that were brought for veterinary assistance in the Veterinary Medical Clinic of the Faculty of Veterinary Medicine in Bucharest and in private veterinary clinics, during august 2017-august 2018. Inclusion in the study was based on the written consent by the owners, a detailed anamnesis, the clinical inspection and a series of laboratory assays.

Potential risk factors assessed. All patients included in the study were of common breed (European short haired). A single patient was adopted from a different owner, the rest of the cats having a stray background. For this particular reason, no medical history of genetically transmissible pathologies existed. Moreover, the age of the patients was estimated at the time of adoption, allowing for significant errors especially in the case where they were adopted as adults. The body condition score was calculated at the time of the clinical

inspection, similar to the procedure applied in the study by Gil et al., 2013.

Patients included in the study. Inclusion in the study for the 20 cats was based on the evaluation of the FIV/FeLV status and on that for coronavirus. Quick diagnosis assays were used, and when the test results raised suspicions, further exploratory assays were performed in an authorised veterinary laboratory.

The following categories were not included in the study: FeLV positive cats, cats positive for coronavirus antigens, cats with severe clinical signs unable of self-sustaining basic functions, cats that had outside access due to the risk of not complying with the protocol, cats extremely reluctant to veterinary actions (the risk of self-injury and that for the owners and veterinarians, as well as the stress induced possibility of altering the final results).

Case and control recruitment. The triage of the patients was in accordance with their FIV status. Thus, 2 groups of patients were created:

- a case group: 10 cats infected with the FIV virus (FIV+);
- a control group: 10 cats not infected with the FIV virus (FIV-).

The product. The product used was highly purified polyvalent avian immunoglobulin Y in sterile water solution, in a concentration of 200 mg/100 mL of solution. The eggs from which the IgY was extracted were obtained from the hyperimmunization of laying hens, by using an immunogenic intramuscular administration of a mixture of several inactivated bacterial and fungal strains in accordance with a previously established vaccination protocol. The eggs and their derived products were periodically tested by the producer in order to verify the compliance with the expected IgY concentration.

For the clinical trial, a daily single dose of 10 mg IgY was used, respectively 4 mL oral solution, for 20 days. The administration was done directly in the oral cavity with the help of pipette or ad libitum by mixing it with a small quantity of the daily diet. It was expected that the time of the administration of the solution was the same in every day. Also, it was recommended that food should not be provided to the patients for at least one hour following the treatment.

Work protocol. The study spread over a period of 40 days, of which the first 20 days involved treating each patient with IgY. Three days were established for evaluating the patients:

- Day 0 (prior to the first IgY dose) - a detailed anamnesis was performed for each patient, alongside the first clinical assessment and the first blood sampling;
- Day 20 (last day of treatment) - the second clinical inspection and the second blood sampling;
- Day 40 - the third clinical evaluation and the last blood sampling.

The detailed anamnesis performed for all 20 cats included in the study is summarised in Table 1.

Table 1. Demographic and clinical data of cats

		Group				Total	
		FIV+		FIV-			
		Count	%	Count	%	Count	%
Gender	**Male**	9	90.0%	5	50.0%	14	70.0%
	Female	1	10.0%	5	50.0%	6	30.0%
Neutering	**No**	0	0.0%	1	10.0%	1	5.0%
	Yes	10	100.0%	9	90.0%	19	95.0%
Origin	**Stray**	10	100.0%	9	90.0%	19	95.0%
	Owner	0	0.0%	1	10.0%	1	5.0%
Deworming	**Occasionally**	5	50.0%	2	20.0%	7	35.0%
	Up-to-date	5	50.0%	8	80.0%	13	65.0%
Vaccinations	**No**	0	0.0%	2	20.0%	2	10.0%
	Occasionally	5	50.0%	1	10.0%	6	30.0%
	Up-to-date	5	50.0%	7	70.0%	12	60.0%

The clinical evaluation of the patients included in the study. The clinical evaluation was performed in days D0, D20 and D40 and was followed, during the same days, by sampling blood necessary for laboratory assays. D0 was considered as the standard for all

patients considering the following evaluations of the symptoms and paraclinical aspects (D20 and D40). It was taken into consideration that the study included both clinically health cats as well as those that had diverse symptomatology. A clinical scoring system was used for the evaluation of all included cats (Table 2). The system was adapted after one used in a similar study (Gil et al., 2013). 14 clinical parameters were scored using values from 0 to 2, where 0 was considered the absence of the respective symptom and 2 was noted as severe symptomatology. A total clinical score was calculated by adding individual scores for each clinical parameter previously multiplied by a factor attributed according to the relevance for the FIV clinical diagnosis.

Table 2. Clinical score used to evaluate the feline patients
(adapted from Gil el al., 2013)

Clinical parameter	Classification	Multiplication factor
Oral ulcers/ Gingivitis	0 - No evidence of oral lesions 1 - Mild to moderate oral lesions 2 - Severe oral gingivitis	1.9
Stomatitis/ Palatitis	0 - No evidence of stomatitis 1 - Mild to moderate hyperemia and stomatitis 2 - Severe hyperemia and caudal stomatitis	1.6
Ophthalmology abnormalities	0 - No evidence of ophthalmology changes 1 - Mild conjunctival hyperemia (mainly unilateral), mild keratitis 2 - Severe conjunctival hyperemia (mainly bilateral), active keratitis	0.7
Ocular discharge	0 - No evidence of ocular discharge 1 - Serous ocular discharge +2 – Muco-purulent ocular discharge	0.7
Lymphadenopathy	0 - No evidence of lymphadenopathy 1 - Mild localized lymphadenopathy 2 - Generalized lymphadenopathy	1.6
Nasal discharge	0 - No evidence of nasal discharge 1 - Serous nasal discharge 2 – Muco-purulent nasal discharge	1.1
Mucous membranes	0 - No evidence of pale mucous membranes 1 - Mild pale mucous membranes 2 – Severe pale mucous membranes	1.4
Dry coat/ Seborrhea	0 - Normal coat condition 1 - Dry coat and/or seborrhea	1.3
Body condition score	0 - Normal or fat: body condition score 4/6 to 6/6 1 – Mildly reduced body condition score 3/6 2 - Underweight animal with a body score of 1/6-2/6	1.8
Faecal appearence	0 - No evidence of diarrhea 1 - Clinical evidence of diarrhea	0.7
Concurrent diseases or Comorbidities	0 - No evidence of concurrent diseases 1 - Clinical evidence of concurrent disease 2 - Severe prostration/global weakness	2
Appetite for food and water	0 - Physiological appetite for food and water 1 - Low appetite for food and water	0.5
Social status	0 - The animal si socially active 1 - The animals tend to isolate itself	1.4
Body temperature	0 - Normal 1 - Fever	1.5

Laboratory testing. In order to establish the possible effects of poly-specific immunoglobulin Y on the organisms of feline patients, 3 blood samples from each patient were taken, as follows:

- the first blood sample was collected by using a Li-heparin recipient for the testing of the biochemical parameters;
- the second blood sample was collected by using an EDTA recipient for the testing of the haematologic parameters;

- the third blood sample was collected by using an EDTA recipient for the testing of the cytokines.

The following classes of paraclinical indicators were assessed: 5 biochemical parameters, 24 haematological parameters, included in the full blood-WBC count and 10 feline cytokines, determined through a microarray quantitative method; all the parameters are shown in Table 3.

Table 3. The parameters analyzed in the study

Parameter type	Analyzed biochemical parameter	Physiological interval (according to IDEXX, 2019a and IDEXX, 2019b)
Biochemical	ALT	12-130 U/L
	AST	0-48 U/L
	ALB	23-39 g/L
	UREE	5.7-12.9 mmol/L
	CREA	71-212 µmol/L
Haematological	RBC	5.0-10.0 *10^12/L
	HCT	30.0-45.0%
	HGB	9.0-15.1 g/dL
	MCV	41.0-58.0 fL
	MCH	12.0-20.0 pg
	MCHC	29.0-37.5 g/dL
	RDW	17.3-22.0%
	# RETIC	3.0-50.0 K/µL
	% RETIC	-
	WBC	5.50-19.50 *10^9/L
	# NEU	2.50-12.50 *10^9/L
	% NEU	-
	# LYM	0.40-6.80 *10^9/L
	% LYM	-
	# MONO	0.15-1.70 *10^9/L
	% MONO	-
	# EOS	0.10-0.79 *10^9/L
	% EOS	-
	# BASO	0.00-0.10 *10^9/L
	% BASO	-
	PLT	175-600 K/µL
	MPV	-
	PDW	-
	PCT	-
Cytokines	Fas (TNF RSF6 /Apo-1)	-
	IFNγ	-
	IL-1β (IL-1 F2)	-
	IL-2	-
	IL-4	-
	IL-5	-
	IL-8 (CXCL8)	-
	IL-10	-
	IL-12p40	-
	RANTES (CCL5)	-

The biochemical and haematological parameters were assessed in an authorized veterinary laboratory by using IDEXX equipment, shortly after the blood was collected.

Preparation and storage of the plasma samples for cytokines testing The blood samples that has been collected from the patients were processed in the same day for the extraction of 1 mL of plasma that was stored for future testing, using the following protocol: centrifugation of the blood sample (2500 rcf) for 10 minutes, extraction of 1 mL of plasma in the sterile laminar flow hood and the preservation in a sterile cryotube at -20°C until processing.

Plasma samples processing. After collecting 3 plasma samples from each patient, the samples were processed. For the quantitative determination of the 10 cytokines, the Abcam Feline Cytokine Antibody Array kit was used (10 targets, ab197414), in the immunology laboratory of the “Stefan S. Nicolau” Institute of Virology of the Romanian Academy.

Statistical analysis. The Linear Mixed model was used to analyse clinical scores and blood parameters. In these models, animals and days were included as subjects and repeated factors, respectively. The models evaluated the main effects of group (2 levels: FIV+ and FIV-), time (3 levels: 0, 20 and 40 days), and their interaction. Sidak adjustment was used for carrying out multiple comparisons. Diagnostic graphics were used to check assumptions and outliers. Log10 transformation was used to analyse AST but raw data are reported in the table. When the transformation did not improve the model, a nonparametric approach was chosen. Mann-Whitney tests were used to compare the groups at each time, while Friedman followed by Dunn’s multiple comparisons tests were used to compare the time effect for each group. Data were presented as mean and standard error (SE) or median (Mdn) and interquartile range (IQR). Moreover, the coefficient of variation (CV) was calculated.

Statistical analyses were performed with SPSS Statistics version 25 (IBM, SPSS Inc., Chicago, IL, USA) and GraphPad Prism, version 7.0 (GraphPad Software, San Diego, California USA). Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSIONS

Clinical score. A significant group effect was found in Total clinical score (P < 0.001). The estimated marginal mean of the score was higher in the FIV+ group (7.9 ± 1.1 and 2.5 ± 1.1 in FIV+ and FIV-, respectively; P < 0.001) and multiple comparisons showed significant differences at each observation day (P < 0.01; Figure 1). The total clinical scores did not change over time (P = 0.070 and P = 0.745 for Time and interaction effects, respectively).

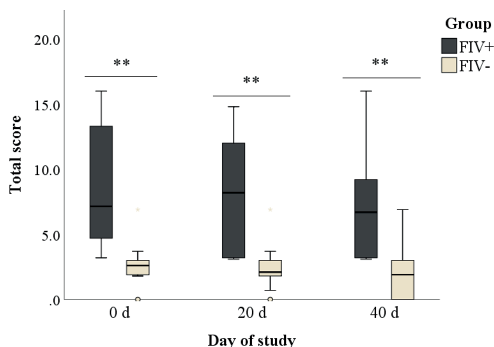

Figure 1. Changes in the total clinical score during the observation period in FIV+ and FIV- groups. **P < 0.01 (FIV+ vs FIV- in each day; Sidak correction)

Biochemical parameters (Table 4), blood count (Table 5), leukocyte formula and reticulocytes (Table 6). A significant effect of Group or Group x Time interaction was found for most of the parameters assessed. Multiple comparisons showed that RBC (P < 0.05), HCT (P < 0.01), HGB (P < 0.01) were higher in FIV- at days D0 and D20 while, in the same days, RDW (P < 0.01), and PLT (P < 0.05) were higher in FIV+ group. These parameters had similar values on day D40.

PCT had lower values in FIV- than FIV+ at D0 but no difference was found in the following days. Differences between FIV+ and FIV- were found only at D20 for ALT (P < 0.05), AST (log transformed data; P < 0.05), and Urea (P < 0.05): ALT and AST were higher in FIV- while Urea in FIV+ group.

Conversely, MCV and Eos% were higher in FIV- than FIV+ only at D40 (P < 0.05).

Lower values in FIV- than in FIV+ were found at all observation times for WBC (P < 0.05) while the opposite was always found for ALB (P < 0.01) and Lym% (P < 0.05).

As regard the changes over time, in FIV- group were found a significant increase at D40 compared to D0 and D20 for MPV (P < 0.01) as well as an increase at D40 compared to D20 for Baso% (P < 0.05). In both groups, a reduction was found at D40 compared to D20 for ALT (P < 0.05) while Ret% decreased at D20 compared to D0 only in FIV+ (P<0.01).

Finally, Crea, MCH, MCHC, PDW, Neut%, and Mono%, were not influenced neither by the group nor by time.

The coefficient of variation ranged from 0.075 for RDW to 1.333 for untransformed AST.

Table 4. Statistical variation of the serum concentration of the biochemical parameters

	Day of study	Group				*P value*		
		FIV+		FIV-		*Group*	*Time*	*Group x Time*
		Mean	SE	Mean	SE			
ALT (U/L)	**D0**	48.0a	7.3	64.0a	5.4	***0.035***	***0.048***	*0.593*
	D20	52.8a	8.0	80.6b	13.1			
	D40	42.2a	8.1	59.2a	3.9			
AST* (U/L)	**D0**	65.4a	25.2	162.2a	63.9	***0.002***	*0.457*	*0.469*
	D20	37.6a	8.1	174.4b	48.4			
	D40	35.6a	12.7	104.0a	38.7			
ALB (g/L)	**D0**	22.5a	2.4	33.3b	1.0	***0.005***	*0.341*	*0.082*
	D20	26.3a	3.1	32.4b	0.7			
	D40	24.4a	2.4	33.7b	1.9			
UREA (mmol/L)	**D0**	13.3a	3.9a	9.4a	0.6	*0.150*	*0.141*	***0.001***
	D20	16.3a	4.2	7.8b	0.5			
	D40	14.2a	4.5a	7.7a	0.3			
CREA (µmol/L)	**D0**	146.0a	24.3	136.0a	8.5	*0.695*	*0.564*	*0.140*
	D20	145.2a	25.8	147.2a	9.2			
	D40	151.5a	25.3	128.9a	7.6			

*analysis on log transformed data
Bold P values denote statistical significance at the 0.05 level.
Values followed by the same letter in each row do not differ significantly (P≤0.05; multiple comparisons with Sidak correction)

Table 5. Statistical variation of the haematological parameters

Parameter	Day of study	Group				*P value*		
		FIV+		FIV-		*Group*	*Time*	*Group x Time*
		Mean	SE	Mean	SE			
RBC	**D0**	7.05a	0.56	9.52b	0.73	***0.030***	*0.445*	*0.135*
	D20	7.08a	0.48	9.08b	0.36			
	D40	7.36a	0.66	8.13a	0.87			
HCT	**D0**	30.0a	2.1	41.8b	2.8	***0.005***	*0.558*	*0.247*
	D20	29.9a	1.4	40.1b	1.6			
	D40	31.0a	2.7	36.9a	3.8			
HGB	**D0**	11.8a	0.7	16.2b	0.9	***0.007***	*0.140*	*0.120*
	D20	11.6a	0.6	14.3b	0.4			
	D40	11.9a	0.7	13.4a	1.5			
MCV	**D0**	42.9a	1.4	44.4a	0.8	*0.188*	*0.709*	*0.086*
	D20	43.0a	1.6	44.3a	0.9			
	D40	42.2a	1.3	46.1b	1.4			
MCH	**D0**	17.2a	1.2	17.6a	1.3	*0.754*	*0.583*	*0.776*
	D20	16.9a	1.4	15.9a	0.7			
	D40	17.0a	1.4	16.4a	1.3			
MCHC	**D0**	36.9a	2.3	34.1a	0.3	*0.351*	*0.813*	*0.333*
	D20	35.5a	1.1	34.9a	0.8			
	D40	34.6a	1.0	34.9a	1.9			
RDW	**D0**	20.8a	0.3	19.5b	0.2	***0.001***	*0.502*	*0.370*
	D20	20.8a	0.3	18.7b	0.5			
	D40	20.6a	0.6	19.5a	0.4			
WBC	**D0**	14.96a	2.77	8.45b	1.11	***0.011***	*0.358*	*0.489*
	D20	16.19a	2.68	7.25b	1.03			
	D40	13.24a	2.32	7.05b	1.81			
PLT	**D0**	481a	75	296b	49	***0.017***	*0.093*	*0.265*
	D20	401a	52	180b	38			
	D40	370a	64	289a	55			
MPV	**D0**	9.8a	1.1	9.1a	1.0	*0.885*	***0.003****	*0.630*
	D20	9.6a	0.7	9.1a	0.8			
	D40	11.8a	1.6	12.5a	1.3			
PDW	**D0**	24.3a	1.0	24.2a	0.7	*0.302*	*0.527*	*0.338*
	D20	23.5a	0.7	24.3a	0.7			
	D40	23.7a	0.8	26.0a	1.5			
PCT	**D0**	0.48a	0.08	0.28b	0.06	*0.072*	*0.126*	*0.275*
	D20	0.38a	0.04	0.20a	0.05			
	D40	0.40a	0.07	0.35a	0.07			

*in FIV- group, significant increase at day 40 compared to days 0 and 20.
Bold P values denote statistical significance at the 0.05 level.
Values followed by the same letter in each row do not differ significantly ($P \leq 0.05$; multiple comparisons with Sidak correction)

Table 6. Statistical variation of the leukocyte formula and reticulocytes

Parameter	Day of study	Group				P value		
		FIV+		FIV-		Group	Time	Group x Time
		Mean	SE	Mean	SE			
% NEU	D0	48.7a	5.9	39.2a	3.4	*0.069*	*0.715*	*0.845*
	D20	49.8a	7.3	36.2a	3.3			
	D40	45.7a	5.9	35.9a	3.7			
% LYM	D0	27.29a	5.35	40.35b	4.46	***0.019***	*0.075*	***0.029***
	D20	23.32a	4.35	44.82b	4.09			
	D40	33.01a	4.35	39.11b	3.26			
% MONO	D0	15.76a	4.83	8.72a	1.60	*0.246*	*0.640*	*0.528*
	D20	11.77a	3.16	8.99a	2.70			
	D40	13.22a	3.54	9.46a	1.52			
% EOS	D0	7.76a	1.35	11.14a	1.09	*0.076*	*0.431*	*0.301*
	D20	7.95a	1.37	9.55a	1.11			
	D40	7.46a	1.44	14.78b	4.45			
% BASO	D0	0.44a	0.08	0.62a	0.06	*0.230*	***0.041****	*0.348*
	D20	0.47a	0.09	0.44a	0.08			
	D40	0.58a	0.11	0.75a	0.12			
% RETIC	D0	1.09a	0.21	0.68b	0.13	***0.038***	***0.005***[#]	*0.613*
	D20	0.68a	0.10	0.47a	0.09			
	D40	0.68a	0.11	0.43a	0.07			

*in FIV- group, significant increase at day 40 compared to day 20.
[#]in FIV+ group, significant decrease at day 20 compared to day 0.
Bold P values denote statistical significance at the 0.05 level.
Values followed by the same letter in each row do not differ significantly ($P \leq 0.05$; multiple comparisons with Sidak correction)

Cytokines (Table 7). The coefficients of variation of the cytokines were all >1.000, ranging from 1.077 for IL-1β to 2.463 for IL-8. No cytokines were influenced by the group while a significant time effect was found for IL-8 ($P<0.01$) in FIV+ and for IL-12 both in both groups ($P<0.05$). However, multiple comparisons only highlighted the reduction from D0 to D20 of IL-8 in FIV+.

Table 7. Statistical variation of the serum concentration of the 10 cytokines

Parameter	Day of study	Group FIV+/-				P value for group effect
		FIV+		FIV-		
		Mdn	IQR	Mdn	IQR	
Fas	D0	205.65a	(0.00, 1895.77)	175.88a	(0.00, 503.62)	*0.631*
	D20	100.41a	(0.00, 1039.59)	29.64a	(0.00, 217.27)	*0.529*
	D40	129.78°	(0.00, 1122.40)	173.43a	(43.51, 655.36)	*0.739*
P value for time effect		*0.192*		*0.514*		
IFN-ɣ	D0	0.00a	(0.00, 201.33)	0.00a	(0.00, 0.00)	*0.739*
	D20	0.00a	(0.00, 54.28)	0.00a	(0.00, 0.00)	*0.529*
	D40	0.00a	(0.00, 0.00)	0.00a	(0.00, 0.00)	*0.739*
P value for time effect		*0.562*		*0.444*		
IL-1β	D0	103.77a	(22.71, 173.31)	56.89a	(37.74, 104.38)	*0.393*
	D20	57.37a	(12.63, 93.58)	42.11a	(17.63, 83.95)	*0.796*
	D40	39.33a	(10.74, 103.27)	35.48a	(9.68, 51.25)	*0.529*
P value for time effect		*0.316*		*0.316*		
IL-2	D0	1047.60a	(0.00, 2493.43)	429.75a	(0.00, 1317.35)	*0.529*
	D20	722.90a	(0.00, 1863.55)	405.02a	(32.73, 562.62)	*0.739*
	D40	345.66a	(2.05, 2054.97)	141.54a	(14.82, 615.56)	*0.579*
P value for time effect		*1.000*		*0.830*		
IL-4	D0	7.49a	(0.00, 173.29)	0.00a	(0.00, 86.00)	*0.739*
	D20	0.00a	(0.00, 88.34)	11.29a	(0.00, 52.74)	*0.436*
	D40	0.00a	(0.00, 112.53)	33.06a	(14.63, 61.87)	*0.529*
P value for time effect		*0.901*		*0.616*		
IL-5	D0	141.48a	(90.37, 331.55)	142.60a	(83.63, 215.03)	*1.000*
	D20	88.27a	(55.27, 292.53)	128.65a	(120.98, 346.25)	*0.123*
	D40	92.34a	(49.66, 553.08)	159.44a	(98.28, 277.09)	*0.853*
P value for time effect		*0.316*		*0.223*		
IL-8	D0	15.92a	(10.73, 69.06)	13.74a	(8.38, 70.14)	*0.912*
	D20	8.38b	(1.09, 21.84)	12.08a	(3.19, 14.64)	*0.481*
	D40	10.20a	(4.02, 49.54)	24.24a	(10.43, 37.15)	*0.971*
P value for time effect		***0.008***		*0.710*		

IL-10	**D0**	3.19a	(0.00, 26.09)	5.93a	(0.00, 19.89)	*1.000*
	D20	0.00a	(0.00, 13.63)	0.84a	(0.00, 21.36)	*0.853*
	D40	1.04a	(0.00, 33.29)	0.00a	(0.00, 15.96)	*0.529*
P value for time effect		*0.071*		*1.000*		
IL-12p40	**D0**	53.83a	(0.00, 149.17)	17.20a	(3.66, 86.22)	*0.613*
	D20	2.54a	(0.00, 90.83)	6.27a	(0.00, 36.20)	*1.000*
	D40	3.04a	(0.00, 79.09)	8.87a	(0.00, 75.63)	*0.631*
P value for time effect		***0.025***		***0.023***		

Bold P values denote statistical significance at the 0.05 level.
For each parameter and group, values followed by the same letter in each column do not differ significantly ($P \leq 0.05$; Dunn's multiple comparisons test)

CONCLUSIONS

All feline patients tolerated well the IgY treatment. No case of rejecting the treatment was recorded, regardless if it was administered directly in the oral cavity or if it was mixed with the usual food.
No patient recorded a negative clinical picture in D40 comparative to D0. Several FIV+ patients showed improvement in the symptomatology during the IgY administration (D20). However, these clinical signs relapsed within 20 days from the end of the treatment and were consistent with the observations made in D0. In the case of the FIV- patients the same amelioration was noted for the clinical symptomatology, with the remark that the relapse did not occur after the treatment period ended, as was the case for the FIV+ patients.
When discussing the biochemical parameters, the IgY administration determined an increase of the serum concentration for most of them (ALT, AST, UREA), with the tendency of going over the upper physiological limit values. Twenty days after the completion of the IgY treatment, the values for the biochemical parameters' serum concentrations returned to the initial levels. No other observations could be made concerning the biochemical parameters, as they tend to follow the disease specific patterns for each case.
The haematological parameters also seemed to follow the disease's specific patterns, as there was only a slight improvement for most patients that were more notable close to D20, but not enough to claim that the IgY therapy had a decisive impact on their evolution. It is also very likely that they are influenced in a more significant manner by secondary infections and by other concurrent pathologies.
The 10 analyzed cytokines manifest either a pro-inflammatory influence (IFN-γ, IL-1β, IL-2, IL-5, IL-8, IL-12p40, RANTES) or an anti-inflammatory one (Fas, IL-4, IL-10). The observed IgY effect on the anti-inflammatory cytokines was a relatively inconsistent one. However, the IgY therapy induced a decrease of the pro-inflammatory cytokines levels during the time of the treatment, especially in the case of IL-8 IL-12p40. This effect was noticed strictly during the product use.
The present study recorded no side effects recorded to the administration of IgY.
The authors consider that it can reasonably conclude that IgY administration in FIV+ and FIV-feline patients has a positive impact on the general functions of the feline organism, without side effects, thus confirming the observations recorded in the two studies aforementioned. Additionally, the study proves that the IgY administration has an anti-inflammatory effect that was highlighted by the drop in the values of pro-inflammatory cytokines, a particularly important effect in relation with the immune impairment registered in this patients.
The final conclusion of the study is that avian immunoglobulin therapies could be usually used as adjuvant to support the immune functions in FIV infected patients that have no allergies for eggs.

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Conflict of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Compliance with ethical standards
All of the procedures used in the clinical trial were compliant with the provisions of the European regulations for the welfare and

protection of animals used for scientific purposes.

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