Serum Amyloid A levels and α2- and Gamma globulins on Serum Protein Electrophoresis in cats exposed to and infected with Leishmania infantum

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Research

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Abstract

Background: Although dogs are the main reservoir host of *Leishmania infantum*, recent investigations indicate a role for cats in its epidemiology. Feline leishmaniosis (FeL) remains poorly characterised, partly due to the lack of diagnostic tools. This study aimed to compare Serum Amyloid A (SAA) levels and serum protein electrophoresis (SPE) profiles (specifically, α2- and gamma globulins) in cats naturally exposed to or infected by *L. infantum* from southern Italy with those of healthy controls and of cats with neoplastic or inflammatory conditions from non-endemic areas.

Methods: Serum or plasma samples from four cohorts of cats were analysed for SAA and by SPE, i.e G1: healthy controls from *Leishmania*-non-endemic regions of Switzerland, G2: cats pre-diagnosed with neoplastic or inflammatory conditions from the University of Cambridge's sample archive, G3: *L. infantum* seropositive, qPCR-negative cats from southern Italy, G4: *L. infantum* seropositive and qPCR-positive cats from southern Italy. SAA data was assessed for normality using the Kolmogorov-Smirnoff and Shapiro-Wilk normality tests, then compared using a homogeneity of variance test for non-parametric data; the Kruskall-Wallis test, followed by Dunn's multiple comparison test were used to compare SAA serum levels between groups. The Fisher's Exact test was used to assess statistically significant differences in the numbers of animals displaying elevated gamma globulins and increased α2-globulins between groups.

Results: Overall, 68 samples were analysed (G1 *n*=16, G2 *n*=20, G3 *n*=20, G4 *n*=12). Cats previously exposed to and/or infected with *L. infantum*, as well as cats suffering from neoplastic and inflammatory conditions showed significantly higher SAA levels (median values G1=0.00 (0.00-0.00) mg/L, G2=0.85 (0.00-49.55) mg/L, G3=0.00 (0.00-4.53) mg/L, G4= 0.00 (0.00-7.5) mg/L), and higher percentages of cats with increased α2-globulins (G1=20.0% ±10.3, G2=80.0% ±8.9, G3=70.0% ±10.2, G4=75.0% ±12.5) and gamma globulins (G1=0.0% ±0, G2=65.0% ±10.7, G3=50.0% ±11.2, G4=58.3% ±14.2) than healthy control cats. For all three markers, there was no significant difference between G2, G3 and G4.

Conclusions: This study indicates that, whilst levels of gamma and α2-globulins and SAA are significantly elevated in cats infected by *L. infantum,* they cannot be used to differentiate between *L. infantum* infection or exposure and neoplastic or inflammatory conditions. Nevertheless, these indicators might assist monitoring of ongoing FeL if further studies indicate reduction during or following successful treatment.

Background

Zoonotic visceral leishmaniosis (VL) is a neglected tropical disease caused by *Leishmania infantum*, which is transmitted by *Phlebotomus* spp. sand flies in the Old World (Maroli et al., 2013). The disease is distributed worldwide and endemic in many regions including the Mediterranean basin (WHO 2018). Dogs are the main reservoir hosts of *L. infantum*, thus representing the major focus of veterinary research for this parasite (Baneth et al., 2008). Canine leishmaniosis is endemic in Italy, where rate of infection increased from hyper-endemic southern and central areas towards northern regions (Mendoza-Rorldan et

al., 2020). The epidemiological role of other animal species as alternative reservoir hosts of *L. infantum* has long been neglected. Nevertheless, recent epidemiological investigations in other species have pointed towards a likely implication of domestic and wild felids in parasite circulation (Pennisi et al., 2015; Soares et al., 2016; Iatta et al. 2019, 2020). In particular, endemic foci of feline leishmaniosis (FeL) have recently been evidenced in Southern Italy, and the overall prevalence of feline infection in these areas is likely underestimated (Pennisi et al., 2012; Otranto et al., 2017).

FeL is often asymptomatic or subclinical (Solano-Gallego et al., 2007, Maia et al., 2010, Nasereddin et al., 2008), with clinical cases mainly characterised by cutaneous lesions, with crusty or nodular dermatitis being most common, and lymphadenomegaly (Pennisi et al., 2015, Brianti et al., 2019) being scantly reported. Typical diagnostic samples include skin (lesions), lymph node, bone marrow and blood (Pennisi et al., 2013). Diagnostic tests for the direct detection of the parasite include microscopic observation through cytology, histology and immunohistochemistry and culture of the promastigote forms on specific media, on tissue samples including skin, bone marrow and lymph node. Molecular detection methods include real time-PCR (q-PCR) and conventional PCR (c-PCR) (e.g. using blood, skin samples, and conjunctival swabs) as well as detection of serum antibodies against the protozoa by indirect tools such as immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA).

When present, clinical signs are nonspecific and alterations in haematological, biochemical and urinary profiles, typically found in dogs with leishmaniosis (Paltrinieri et al., 2016), may support suspicion of FeL. Due to the frequently asymptomatic nature of the disease, a straightforward measure of infection progression and response to treatment would facilitate clinical management of FeL. Measurement of acute phase proteins (APPs), including C-reactive protein (CRP), haptoglobin, and serum amyloid A (SAA) is increasingly being used in the diagnosis, monitoring and prognosis of a range of veterinary inflammatory conditions (Martínez-Subiela et al., 2001, Eckersall and Bell, 2010) including *L. infantum* infection in dogs (Martínez-Subiela et al., 2002b; Paltrinieri et al., 2016).

The acute phase response is an early, non-specific defence mechanism characterised by the release of proinflammatory cytokines which stimulate an increase in serum APPs, in response to infection, inflammation, tissue injury, neoplasia and other processes (Cerón et al., 2005). APPs including CRP, haptoglobin and ceruloplasmin are significantly increased in dogs naturally infected by *L. infantum*, irrespective of the presence of clinical signs (Martínez-Subiela et al., 2002b). SAA is considered a less sensitive indicator of canine leishmaniosis (CanL) compared to other APPs (Martínez-Subiela et al., 2002a), however it is a major indicator of feline inflammatory conditions, including trauma, post-operative inflammation and sepsis (Kajikawa et al., 1999, Sasaki et al., 2003, Tamamoto et al., 2008). SAA is substantially increased in cats with clinical signs of vector-borne infections such as *Hepatozoon felis* and *Babesia vogeli*, compared to uninfected and infected asymptomatic cats (Vilhena et al., 2017). Recently, protein-related laboratory abnormalities were reported in tigers naturally infected by *L. infantum* (Cavalera et al., 2020). Information on serum APP levels in cats infected with vector-borne pathogens remains limited (Vilhena et al., 2017), however these findings may suggest a useful diagnostic and prognostic role for SAA.

Serum protein electrophoresis (SPE) provides a straightforward measure of serum proteins in fractions. The α2 fraction on SPE contains a number of APPs, thus its quantification may indicate an inflammatory process (Taylor et al., 2010); however, the levels of α2-globulins on SPE in FeL have, thus far, not been investigated. SPE is commonly used to identify and characterise gammopathy (pathologically increased serum gamma globulins, the most significant of which are immunoglobulins) in dogs infected by *L. infantum* (Solano-Gallego et al., 2009; Paltrinieri et al., 2016; Maia et al., 2018). Gammopathy is normally present in CanL as well as most FeL cases investigated to date (Pennisi et al., 2013). The aim of this study is to evaluate and compare the serum SAA levels and SPE profiles, specifically α2 and gamma globulins, in cats from a VL-endemic area naturally exposed to or infected by *L. infantum*, with healthy cats from a VL non-endemic area and in cats from a VL non-endemic area with neoplastic or inflammatory conditions.

Materials And Methods

Aim, animal enrolment and diagnostic procedures

This study aimed to compare SAA levels and percentages of animals showing elevated α2 and gamma globulin peaks on SPE in cats naturally exposed to or infected by *L. infantum* from southern Italy, healthy controls from a VL non-endemic area and cats with neoplastic or inflammatory conditions from a VL non-endemic area. A total of 68 serum or plasma samples were obtained from four groups of cats:

G1

16 healthy control cats from *Leishmania*-non-endemic regions of Switzerland (north of the Alps). The samples originated from the archive at the Clinical Laboratory or the Institute of Parasitology, Vetsuisse Faculty, University of Zurich (VetLab Zurich and IPZ, respectively), and included healthy stray cats recruited into a spay/neuter programme (n = 5), healthy blood donors (n = 6) and healthy laboratory cats (n = 5). All cats were recorded as clinically healthy following a thorough clinical examination by an accredited veterinary surgeon. Routine haematology and biochemistry analyses were performed with samples from blood donors, and animals that had travelled abroad were excluded. Cats from the spay and neuter programme were recruited only from north of the Alps, a *Leishmania*-non-endemic area. Laboratory animals are born and kept in controlled experimental units, with no contact to Phlebotominae. G1 included 3 plasma and 13 serum samples;

G2

20 cats pre-diagnosed with neoplastic or inflammatory conditions selected from the sample archive at the Queen's Veterinary School Hospital of the University of Cambridge;

G3

20 cats from southern Italy (i.e., Aeolian Islands, Sicily and Apulia regions) testing seropositive by IFAT but qPCR-negative for *L. infantum*;

G4

12 cats from southern Italy (Aeolian Islands, Sicily) testing seropositive by IFAT and qPCR-positive for *L. infantum*.

Detection of antibodies against *L. infantum* was performed by IFAT as described by Otranto et al. (2009) using a cut-off value of 1:40 (the screening of these samples was performed before the validation of a new IFAT cut-off value of 1:80 by Iatta and colleagues in 2020). Blood and conjunctival swabs from the enrolled cats were subjected to DNA extraction, using the DNeasy Blood & Tissue Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The detection of a *L. infantum* kinetoplast DNA minicircle fragment (120 bp) was achieved by real time-PCR (qPCR), using primers, probes and protocol described elsewhere (Francino et al., 2006).

Serum protein electrophoresis and SAA

Serum protein electrophoresis was performed on alkaline buffered agarose gels using the HYDRAGEL PROTEIN(E) K20 (SEBIA, Lisses, France) according to the manufacturer's instructions, using 10 µl of serum. Gels were analysed using the EPSON PERFECTION V700 PHOTO imaging densitometer, using the SEBIA Phoresis Rel 8.6.3 software. The concentration of protein in individual fractions is the product of the sample total protein and the percentage density of the fraction. Total protein was measured using the biuret method on an automated analyser (Olympus AU480; Beckman Coulter Inc., Fullerton, USA). Numbers of cats whose plasma/sera displayed increased α -2 and gamma peaks on SPE were counted for each group according to reference intervals calculated by Taylor and colleagues (2010); the latter study used a method of agarose gel electrophoresis comparable to ours, based on a sample of 77 healthy British cats. Clonality of increased gamma peaks was assessed, with monoclonal gammopathies defined as showing a narrow γ peak, with a base width similar to the albumin peak or with peak height exceeding four times the peak width (Taylor et al., 2010). SAA concentrations were determined using a turbidimetric immunoassay LZ-SAA (Eiken Chemical Co., Tokyo, Japan), with analysis on an automated analyser (Olympus AU480; Beckman Coulter Inc., Fullerton, USA).

The Fisher's Exact test was used to assess statistically significant differences in the numbers of animals showing increased gamma globulins and α2-globulins between groups. Standard errors for the proportions (SEp) were calculated as:

$$\mathrm{SEp} = \sqrt{p(1-p)/n}.$$

Distributions of SAA values within each group were tested for normality using the Kolmogorov-Smirnoff and Shapiro-Wilk normality tests. Distributions were then compared using a homogeneity of variance test for non-parametric data; no significant differences between the four distributions were recorded. Since the data were non-normally distributed, a non-parametric test, the Kruskall-Wallis test, followed by Dunn's multiple comparison test were used to compare SAA serum levels between groups. Statistical analyses

were performed using the IBM SPSS statistics 25 with significance set at P<0.05. No further statistical adjustments were made for multiple comparisons.

Results

A total of 68 samples were analysed; one G1 (healthy control) sample did not undergo SPE, and one G4 (positive for *L. infantum* by both IFAT and qPCR) sample did not undergo SAA analysis as insufficient sample was available (Table 1).

The proportion of cats showing increased α2- and gamma globulins on SPE was significantly higher in cats from groups G2, G3 and G4 (G2 = 80.0% ±8.9 and 65.0% ±10.7, G3 = 70.0%±10.2 and 50.0% ±11.2, G4 = 75.0% ±12.5 and 58.3% ±14.2) compared to healthy controls (G1 = 20.0% ±10.3 and 0% ±0). There was no statistically significant difference in the proportion of animals showing increased gamma globulins or increased α2 - globulins between groups G2, G3 and G4 (Fig. 1). In all cases gammopathy was polyclonal. SAA was significantly higher in each pathological group (median values were G2 = 0.85 (0.0-49.55) mg/L, G3 = 0.0 (0.0-4.53) mg/L and G4 = 0.0 (0.0-7.5) mg/L) compared to healthy controls (G1 = 0.00 (0.0–0.00) mg/L), but no significant difference in SAA between G2, G3 and G4 was detected (Fig. 1).

Table 1
Number of sampled cats, SPE and SAA results per sampled group.

Variable	GROUP 1 Healthy Controls	GROUP 2 Neoplastic or inflammatory disease	GROUP 3 *L. infantum* seropositive qPCR-negative	GROUP 4 *L. infantum* seropositive qPCR positive
Total number	16 (15 for SPE)	20	20	12 (11 for SAA)
SPE Results [a]				
Gamma globulins increased	0 (0 ± 0)	13 (65.0 ± 10.7)	10 (50.0 ± 11.2)	7 (58.3 ± 14.2)
α2 peak increased	3 (20.0 ± 10.3)	16 (80.0 ± 8.9)	14 (70.0 ± 10.2)	9 (75.0 ± 12.5)
SAA results (mg/L) [b]	0.00 (0.00–0.00)	0.85 (0.00-49.55)	0.00 (0.00-4.53)	0.00 (0.00-7.5)

[a] Data presented as: number (percentage ± standard error on the percentage (SE_p))

[b] Data presented as median (25th -75th percentile)

Discussion

This study evaluated gamma globulins and acute phase proteins, specifically α2-globulin concentration on SPE and SAA concentration, in cats infected by and exposed to *L. infantum*. The results suggest that elevated gamma globulins are a significant feature of *Leishmania* infection in cats, as reported in dogs (Solano-Gallego et al., 2009). Furthermore, hyperglobulinaemia has been recorded in most clinical cases of FeL (Pennisi et al., 2013, 2015, Brianti et al., 2019). SAA concentration and the percentage of animals showing elevated α2-globulins were significantly higher in infected and exposed cats, suggesting that the elevation of these markers, indicating an acute phase response, is a significant feature of FeL. This is the first study evaluating SAA in FeL, however there is evidence that symptomatic cases of two other feline vector borne diseases, *Hepatozoon felis* and *Babesia vogeli*, are also associated with significantly elevated SAA (Vilhena et al., 2017). Elevation in α2-globulins is a recognised feature of infection in dogs (Paltrinieri et al., 2016), but is not well documented in FeL.

There was no significant difference in SAA serum concentration, or percentage of animals showing elevated gamma or α2-globulins between either group of animals infected by or exposed to *L. infantum* (G3 and G4) and animals affected by neoplastic or inflammatory disease (G2). This suggests that SAA, gamma and α2-globulins are not specific markers of FeL, but this does not preclude their role as part of a diagnostic plan, and in monitoring and assessing prognosis of disease.

The absence of a significant difference in the three investigated markers between seropositive but qPCR-negative (G3) and seropositive and qPCR-positive cats (G4) suggests that elevated gamma and α2-globulins and SAA may be a significant feature of both active infection and parasite exposure. It is also possible that G3 cats had a very low parasitaemia that went undetected through qPCR; indeed, in dogs, apart from tissue lesions, biological samples with the best sensitivity for molecular parasite detection are bone marrow or lymph node, skin, conjunctiva, buffy coat, and whole peripheral blood, in descending order of sensitivity (Paltrinieri et al., 2010, Maia et al., 2008, Gradoni et al., 2000). Thus, false negatives using conjunctival swabs and blood in this study may have occurred.

Potentially, G3 animals may indeed be free of parasite or have very low parasite levels, but their immune response against *L. infantum* relies on high gamma globulins and/or APP levels (instigated either by low level *L. infantum* parasitaemia or other factors). Thus, possibly, the markers examined here are not useful to assess disease progression but may be useful in investigating the immune response in FeL. A significant progressive decrease in α2-globulin serum concentration has been reported in dogs during treatment with meglumine antimoniate followed by allopurinol (Carreira et al., 2017). Similarly, a decrease of some APPs, particularly haptoglobin, has been shown during treatment in dogs with leishmaniosis (Sasanelli et al. 2007, Martinez-Subiela et al. 2011), highlighting the possible importance of APP measurement in monitoring response to treatment also in cats infected by *L. infantum*.

A limitation of our study is that the 'healthy control' animals (G1) and the neoplastic and inflammatory disease group (G2) were not tested to rule out *L. infantum* infection; however, these cats were from VL-non-endemic areas of Switzerland (G1) and the UK (G2), and included laboratory animals bred and kept in a controlled environment as well as blood donor cats. Furthermore, G3 and G4 animals were not tested

for concurrent infectious and inflammatory disease, such as Feline Infectious Peritonitis, sepsis, gingivitis or other vector borne diseases, which would all induce an acute phase response and elevated gamma globulins.

Despite its limitations, this study suggests a potentially important role for assessing SAA, gamma and α2-globulins in the monitoring of infection and the characterisation of the immune response to FeL. Though only few laboratory parameters were investigated here in a limited number of cats, these markers may be useful in suspected *L. infantum* infection, thus further studies on a large feline population are required to better clarify their role in the diagnosis of FeL.

Conclusions

This study suggests that elevated SAA, gamma and α2-globulins are significant features of infection and exposure to *L. infantum* in cats. These markers are non-specific for infection and did not differentiate infected from exposed animals. Our findings suggest a possible role for these markers in characterisation of the immune response in FeL. This study has some limitations but demonstrates that these markers deserve further investigation with larger sample sizes adequately controlled for concurrent infectious and inflammatory disease.

Abbreviations

FeL, Feline Leishmaniosis; APP, Acute Phase Protein; SAA, Serum Amyloid A; CRP, C-Reactive Protein; SPE, Serum Protein Electrophoresis; q-PCR, quantitative (real-time) PCR; ELISA, Enzyme-Linked Immunosorbent Assay; IFAT, Immuno-Fluorescence Antibody Testing; kDNA, kinetoplast-DNA; PCR, Polymerase Chain Reaction; FIP, Feline Infectious Peritonitis; VL, visceral leishmaniosis.

Declarations

Ethics approval and consent to participate

All procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides. All samples used were surplus diagnostic samples and were not specifically collected for this study.

For healthy control cats from Switzerland, the objective and design of the study were approved for owned, shelter and stray cats by the owners, shelter administrator and animal welfare organisers of the neutering campaigns, respectively.

Written consent is obtained from all owners of patients at the Queen's Veterinary School Hospital of the University of Cambridge, permitting surplus diagnostic samples to be used for research. All samples are archived according to the Department of Veterinary Medicine animal welfare guidelines.

Cats from VL-endemic area were sampled under the framework of a field study approved by the Italian Ministry of Health (authorization no. 0006088-10/03/2015-DGSAF-COD_UO-P) and the cats' owners provided signed informed consent before sampling.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GS carried out SPE and SAA measurements on the samples and analysed and interpreted the data. GS was the main contributor in writing the manuscript, with advice and guidance from CC, DO and RI. JA, MS and EB provided the samples together with advice and information. GS, JA, EB and DO contributed to study design, with guidance from all other authors. All authors read and approved the final manuscript.

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Author's information

This study was conducted as a final year elective project by GS, during her veterinary studies at the University of Cambridge. Since qualification, GS worked in first opinion large animal practice in the United Kingdom before moving to her country of origin, Switzerland, where she is currently completing her military service as a Veterinary Officer.

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Figures

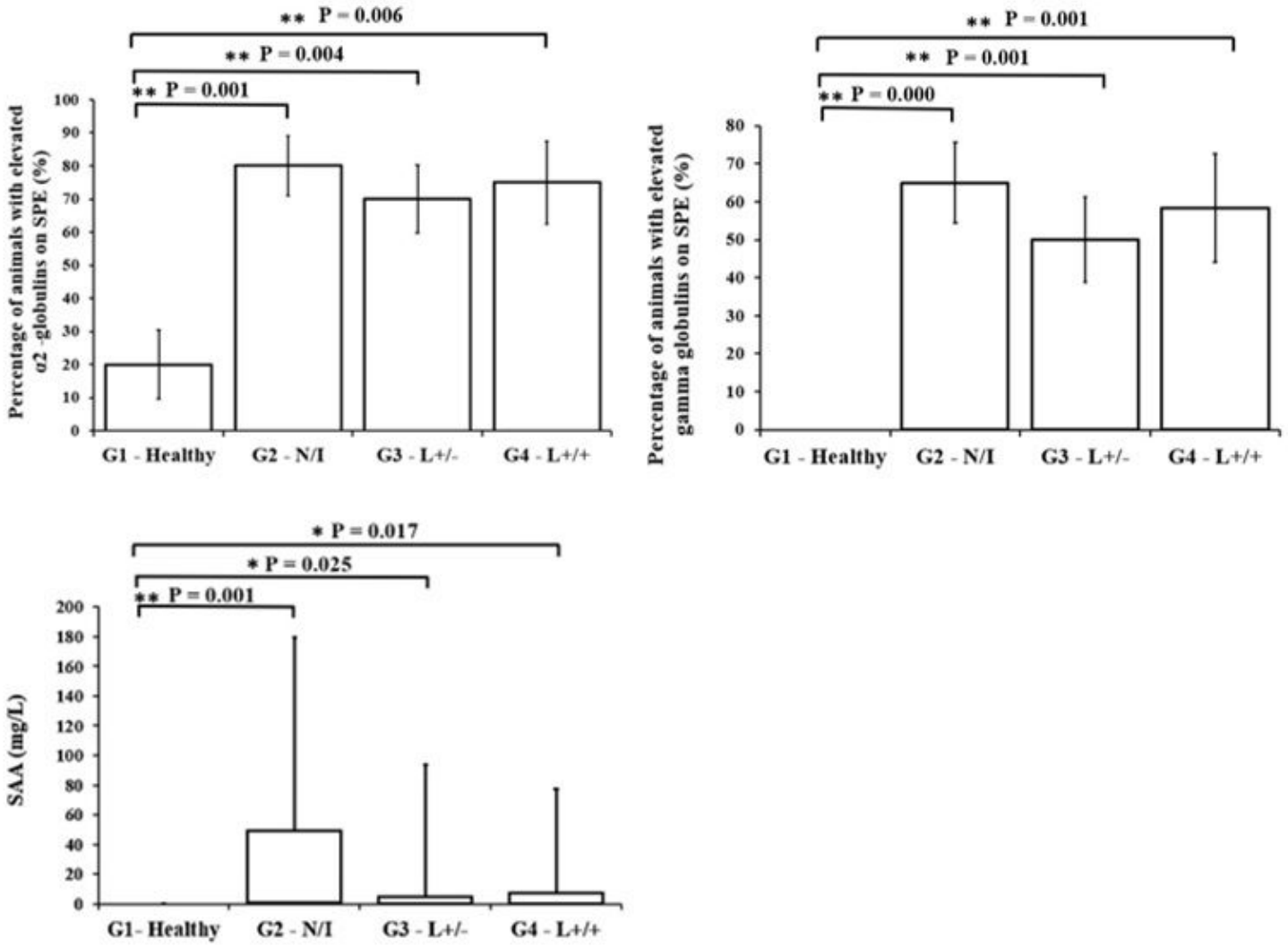

Figure 1

Proportions of cats showing increased α2-globulins (top left) and increased gamma globulins (top right) on SPE, and median, interquartile range and maximum of serum amyloid A (SAA) concentrations (bottom left) in healthy control cats (G1, n=16 (15 for SPE)), cats affected by neoplastic or inflammatory disease (G2, n=20), cats seropositive and qPCR-negative for L. infantum (G3, n=20) and cats seropositive and qPCR-positive for L. infantum (G4, n=12 (11 for SAA)).