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Antigen-blocking phenomenon in dogs affected by *Dirofilaria immitis*: frequency, reversion, associated immune response and co-positivity with other vector-borne pathogens

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ABSTRACT

Detection of *Dirofilaria immitis* antigens is a cornerstone for diagnosis of heartworm disease (HWD). However, no antigen detected (NAD) results may occur secondary to various biological phenomena, including antigen-blocking (AB). Serum heat treatment has been proposed as a strategy to dissociate circulating immune complex (CIC) and reverse AB. This study aimed to: (i) determine the frequency of NAD results; (ii) evaluate the efficacy of heat treatment in reversing NAD results; (iii) investigate potential associations between AB, immune response and seropositivity to other vector-borne pathogens (VBPs). Blood and serum samples were collected from 101 *D. immitis*-infected dogs from Sardinia (n = 73) and Sicily (n = 28), as well as from 7 healthy dogs serving as controls. *Dirofilaria immitis* microfilariae and antigens were detected using Knott's test and IDEXX SNAP Leish 4Dx®, respectively. Seropositivity with *Anaplasma* spp., *Ehrlichia* spp. and *Leishmania infantum*, was evaluated simultaneously. *Dirofilaria immitis* antigen-negative serum samples underwent heat treatment. Serum concentrations of interferon gamma (IFN-γ), interleukin 12 (IL-12), and interleukin 4 (IL-4) were quantified via enzyme-linked immunosorbent assays (ELISA) across three groups: antigen-blocked dogs (AB, n = 8), SNAP-positive/Knott-positive dogs (DC, n = 20), and healthy non-infected dogs (C, n = 7). Initial antigen negativity was observed in 11 out of 101 samples (10.9%), with heat treatment successfully reversing NAD in 8 of the 11 cases (72.7%) yielding an overall confirmed AB frequency of 7.9% (8/101). Interleukin analysis revealed significantly higher IL-4 levels in the AB group [median 53.71 pg/mL (Q1–Q3: 35.94–71.00); $P < 0.001$] and significantly higher IL-12 levels in the DC group (49.85 ± 14.56 pg/mL; $P = 0.001$). Overall VBPs seropositivity was significantly higher in the AB group (4/8, 50.0%) than in the DC group (2/20, 10.0%; $P = 0.0384$). Specifically, *L. infantum* seropositivity was detected in 37.5% (3/8) of AB dogs compared to 5.0% (1/20) of DC dogs. Antigen blocking emerged as a relevant cause of NAD results in canine HWD, occurring in approximately 8% of infected dogs in this study. Heat treatment successfully restored antigen detection in most NAD samples. The association between antigen blocking and increased IL-4 concentrations, potentially amplified by co-positivity with other VBPs, suggests that a Th2-biased immune response may contribute to CIC formation and subsequent antigen masking.

1. Introduction

Heartworm disease (HWD), caused by the filarial nematode *Dirofilaria immitis,* is a chronic disease affecting the cardiopulmonary system of domestic and wild carnivores, as well as humans, and therefore represents a zoonotic threat. The spreading endemicity of HWD across Europe highlights the need for reliable diagnostic and therapeutic management of infected animals (Genchi et al., 2011; Morchón et al., 2022; Napoli et al., 2023). In veterinary clinical practice, the combined detection of circulating microfilariae (mfs) via Knott's test, and

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D. immitis antigens constitutes the gold standard for diagnosis. To this end, commercial rapid enzyme-linked immunosorbent assays (ELISA) devices are widely utilized to detect antigens released predominantly by the adult female reproductive tract in serum, plasma, or whole blood samples (Henry et al., 2018). However, no antigen detected (NAD) results remain a critical diagnostic challenge in infected dogs. Undetectable antigenemia despite active infection may stem from few adult parasites, infections involving adult parasites of only one sex, the use of macrocyclic lactones, delayed antigenemia (i.e., delayed appearance of detectable circulating antigens relative to the onset of microfilaremia) or the presence of antigen-blocking phenomenon (Tonelli, 1989; Rishniw et al., 2012; Velasquez et al., 2014; Drake et al., 2015). Antigen blocking occurs when host anti-*Dirofilaria* antibodies are present in excess relative to circulating antigens, binding free parasite antigens into circulating immune complexes (CIC). These bound antigens are sterically masked from recognition by capture antibodies in immunoassay devices, leading to false-negative outcomes. Furthermore, hypergammaglobulinemia, often triggered by co-infections with other vector-borne pathogens (VBPs) such as *Leishmania infantum*, may exacerbate antigen masking. Indeed, the sympatric distribution of *L. infantum* and *D. immitis* in some European areas renders this phenomenon particularly relevant from both diagnostic and clinical perspectives (Little et al., 2018; Ciucă et al., 2016). In canine HWD, the immune response tends to be dynamic and is influenced by the stage of infection. It has been demonstrated that patent infections (with circulating mfs) are predominantly associated with a T-helper 2-type (Th2) immune response, characterized by increased antibody production, whereas occult (amicrofilaremic) infections are associated with a predominant T-helper 1-type (Th1) response, which appears to contribute to the elimination or control of circulating mfs (Morchón et al., 2007; Oleaga et al., 2009). Cytokine profiles reflect these functional pathways: Th1 responses drive cell-mediated immunity via interleukin−12 (IL-12) and interferon-gamma (IFN-γ), while Th2 responses drive humoral immunity via interleukin−4 (IL-4) and interleukin−10 (IL-10) (Mosmann et al., 1986).

Previous studies have documented a considerable frequency of NAD results in naturally *D. immitis*-infected dogs. Specifically, from 6.0% to 38.7% of dogs that tested positive for *D. immitis* via the Knott's test were antigen-negative, highlighting a significant diagnostic discrepancy (Magi et al., 2012; Alho et al., 2014; Vieira et al., 2014; Nonnis et al., 2025). To address this diagnostic limitation, serum heat treatment has been proposed as a method to heat-dissociate CIC, thereby releasing free antigen for immune detection (Little et al., 2018). Nevertheless, while heat treatment improves diagnostic sensitivity, it may compromise specificity by exposing cross-reactive epitopes shared with other parasite species, such as *Dirofilaria repens* or *Angiostrongylus vasorum*, *Acanthocheilonema reconditum* or *Spirocerca lupi* (Gillis et al., 1984; Schnyder and Deplazes, 2012; Aroch et al., 2015; Ciucă et al., 2016; Venco et al., 2017).

Therefore, the present study was designed to: (i) assess the frequency of NAD results in a cohort of dogs naturally infected with *D. immitis*; (ii) expand current knowledge on the efficacy of heat treatment in reversing NAD status; (iii) investigate potential associations between antigen blocking and circulating concentrations of IFN-γ, IL-12, IL-4, as well as seropositivity to other VBPs (i.e., *Anaplasma* spp., *Ehrlichia* spp. and *L. infantum*).

2. Material and methods

2.1. Study population

The study was conducted using 101 blood and serum samples from naturally *D. immitis*-infected dogs collected during previous studies conducted in Sardinia (n = 73) and Sicily (n = 28) (Brianti et al., 2022; Nonnis et al., 2025). At original sampling, whole blood has been collected into both anticoagulant (K_3-EDTA) and serum tubes and transported to the laboratory within a few hours for processing and storage. Additionally, blood and serum samples were collected from seven clinically healthy uninfected dogs to establish a negative control group (C group) for baseline interleukin and protein profiling.

2.2. Modified Knott's test

Modified Knott's test was performed on all EDTA blood samples, according to the methodology described by Genchi et al. (2021). The microfilariae found were identified to species level based on their morphology and morphometry (Magnis et al., 2013), and their number expressed as microfilariae per millilitre of blood (mfs/mL).

2.3. Antigen testing and heat treatment

Serum samples were screened for *D. immitis* antigens and for antibodies against *Anaplasma* spp., *Ehrlichia* spp., and *L. infantum* using the rapid SNAP Leish 4Dx® (IDEXX Laboratories, Inc., Westbrook, USA) according to the manufacturer's instructions. Samples testing negative for *D. immitis* antigens were aliquoted (~100 μL) and stored frozen until heat treatment. Heat treatment was performed as described by Weil et al. (1985). Briefly, the thawed serum aliquot was mixed with an equal volume of EDTA and heated at 100 °C for 5 min in a dry heat block. The sample was then centrifuged at 16,000 relative centrifugal force (RCF) for 5 min, and the resulting supernatant was tested, in parallel with the non–heat-treated aliquot, for *D. immitis* antigen using the same rapid device.

2.4. Interleukin and serum protein assessment

Sample selection for interleukin and serum protein assessment was restricted to non-hemolyzed and non-lipemic sera with adequate volume for duplicate ELISA testing. Based on the Knott's test result and antigen detection before and after heat treatment, samples were allocated into three groups: (i) antigen-blocking group (AB group, n = 8), microfilaremic dogs that were initially antigen-negative but converted to antigen-positive following heat treatment; (ii) *D. immitis*-positive controls (DC group, n = 20), a representative subset of *D. immitis*-infected dogs testing positive for both microfilariae and antigens prior to heat treatment, and (iii) healthy uninfected control dogs (C group, n = 7). The serum concentration of IFN-γ, IL-12 and IL-4 was assessed using ELISA assays specific for canine species (i.e., Canine IFN-γ ELISA Kit, MyBioSource, Inc. San Diego, California, USA, analytical sensitivity 5.9 pg/mL; intra- and inter-assay coefficients of variation were <10 and <12%, respectively; Canine IL-12/P70 ELISA kit, FineTest, Wuhan, Hubei, China, Sensitivity 9.375 pg/mL; intra- and the inter-assay coefficients of variation were 5.19 and 5.05%, respectively; Canine IL-4 ELISA Kit, FineTest, Wuhan, Hubei, China, analytical sensitivity 3.75 pg/mL; intra- and inter-assay coefficients of variation were < 4.64 and 4.62%, respectively) by means of a microtiter plate reader (ELISA microplate Reader BK-EL10B, BIOBASE Bioindustry, (Shandong) Co., Ltd, Jinan City, China).

The serum total protein concentration was determined using the Biuret method via a UV spectrophotometer (SEAC, Florence, Italy), and protein fractions were determined using an automated system (SELVET 24, Seleo Engineering, Naples, Italy) according to the manufacturer's instructions. Electrophoretic separation allowed the main protein components to be highlighted in accordance with the manufacturer's specifications: starting from the anodic (positive) pole, the most pronounced albumin peak was observed, followed by the α1-, α2-, β1-, β2- and γ-globulin fractions.

2.5. Statistical analysis

Data were tested for normality using the Shapiro-Wilk normality test. Normally distributed variables are expressed as mean ± standard

deviation (SD), whereas non-normally distributed variables are presented as median and interquartile range (Q1–Q3). Data related to IFN-γ, IL-12, total proteins and albumin from each group were normally distributed ($P > 0.05$), whereas the values of IL-4, α1-, α2-, β1-, β2- and γ-globulin fractions and albumin-to-globulin (A/G) ratio did not pass the normality test ($P < 0.05$); therefore, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to verify difference in the concentration of IFN-γ, IL-12 total proteins and albumin among groups. Contrariwise, non-parametric statistical analysis (i.e. Kruskal-Wallis test) followed by Dunn's multiple comparisons test with Bonferroni correction was applied on the values of IL-4, α1-, α2-, β1-, β2- and γ-globulin fractions as well as on the A/G ratio to assess differences among the groups. P values < 0.05 were considered statistically significant. The association between AB group vs. DC group and VBP co-positivity was evaluated using Fisher's exact test (two-tailed) and P-values < 0.05 were considered statistically significant. The data were analysed with the software Prism v. 9.00 (GraphPad Software Ltd., San Diego, CA, USA, 2020).

3. Results

The study cohort comprised 101 dogs testing positive for *D. immitis* microfilariae via the modified Knott's test, with loads ranging from 2 to 1400 mfs/mL. Most dogs (74.3%) were infected exclusively with *D. immitis*, whereas co-positivity with other filarioids (superfamily Filarioidea) or other VBPs was detected in 25.7% of the tested dogs (Table 1). Initial testing detected circulating *D. immitis* antigens in 90 samples, whereas 11 of the 101 samples (10.9%) tested negative. Following serum heat treatment, 8 of these 11 serum samples converted from antigen-negative to antigen-positive status (Fig. 1, Table 2). This resulted in an overall frequency of confirmed antigen blocking of 7.9% (8/101) in the study dog population. The three samples that remained negative after heat treatment were classified as non-immunological NAD cases and were excluded from further analyses. All seven healthy control dogs (C group) tested negative for both *D. immitis* mfs and antigens, as well as for all other VBPs evaluated. The analyses revealed a statistically significant association of group on IFN-γ (ANOVA: $F = 5.66$, $P = 0.008$), IL-12 (ANOVA: $F = 27.2$, $P < 0.001$), IL-4 (Kruskal–Wallis: $\chi^2 = 15.8$, $P < 0.001$), and the γ-globulin fraction (Table 3). Specifically, both the AB and DC groups showed significantly higher IFN-γ concentrations than the Control group (AB vs. Control: $P = 0.008$; DC vs. Control: $P = 0.025$), whereas no significant difference was observed between the AB and DC groups ($P = 0.519$). Likewise, IL-12 concentrations differed significantly among groups. Dogs in the DC group showed the highest IL-12 values, which were significantly greater than those observed in both the AB ($P = 0.001$) and Control ($P < 0.001$) groups. In contrast, IL-4 concentrations were significantly higher in the AB group than in both the DC ($P < 0.001$) and Control ($P = 0.002$) groups, whereas no significant difference was detected between the DC and Control groups ($P = 1.000$) (Fig. 2). The γ-globulin fraction values were significantly higher in the AB group than in the Control group, whereas total protein concentrations and the remaining globulin fractions did not differ significantly among groups (Fig. 3). Overall, VBP co-positivity was significantly higher in the AB group (4/8, 50.0%) than in the DC group (2/20, 10.0%) (two-tailed Fisher's exact test, $P = 0.0384$). In the DC group, one dog was co-positive for *L. infantum* and one for *Ehrlichia* spp. whereas *Leishmania* infantum was the most frequently detected VBP in the AB group (3/4, 75%) (Table 2).

Table 1
Descriptive analysis of co-positivity with other vector-borne pathogens in a cohort of *Dirofilaria immitis*-positive dogs (N = 101) from Sardinia and Sicily, Italy.

	Sardinia (n = 73)	Sicily (n = 28)	Total (N = 101)
Dirofilaria immitis monoinfection	51 (69.8%)	24 (85.7%)	75 (74.3%)
Dirofilaria repens	14 (19.2%)	0 (0%)	14 (13.8%)
Dirofilaria repens + *A. reconditum*	1 (1.4%)	0 (0%)	1 (1.0%)
Anaplasma spp.	4 (5.5%)	0 (0%)	4 (3.9%)
Ehrlichia spp.	6 (8.2%)	1 (3.6%)	7 (6.9%)
Anaplasma spp. + *Ehrlichia* spp.	1 (1.4%)	0 (0%)	1 (1.0%)
Leishmania infantum	1 (1.4%)	3 (10.7%)	4 (4.0%)

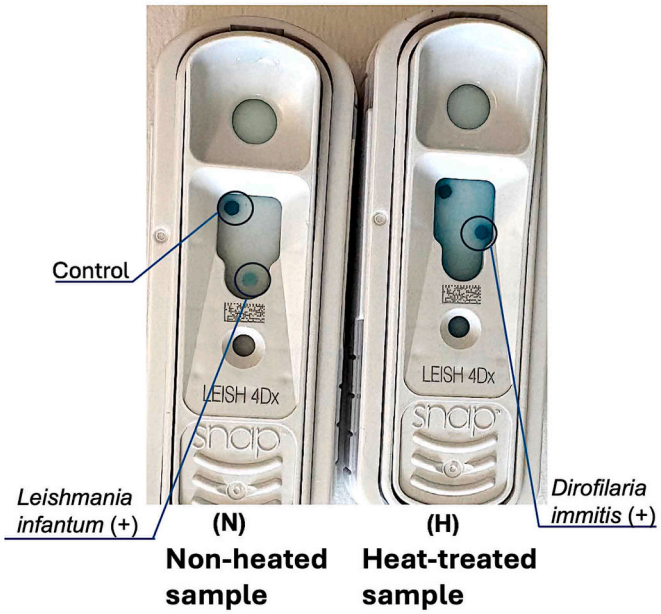

Fig. 1. Antigen test results of the same serum sample tested in parallel using non-heated (N) and heat-treated (H) aliquots. Heat treatment restores *Dirofilaria immitis* antigen positivity (blue spot, right edge), whereas antibody reactivity for *Leishmania infantum* (blue spot, bottom) is lost due to thermal denaturation. The top blue spot in both devices represents the internal control.

Table 2
Microfilaremia loads, co-positivity with other vector-borne pathogens status and interleukins concentrations in a group of 8 dogs with confirmed antigen-blocking (AB group).

ID	Sex	Knott (mfs/mL)	Co-positivity	IFNγ (pg/mL)	IL-4 (pg/mL)	IL-12 (pg/mL)
AB_3	M	Pos (230)	*Anaplasma.* spp.	16.3	28.5	47.5
AB_4	F	Pos (n.d.)	-	2.4	68.0	31.4
AB_8	F	Pos (3)	-	1.9	21.9	21.1
AB_11	M	Pos (2)	*Leishmania infantum*	14.1	80.0	37.1
AB_13	F	Pos (1400)	-	8.7	43.4	14.0
AB_14	F	Pos (227)	*Leishmania infantum*	16.0	64.0	41.1
AB_15	M	Pos (200)	*Leishmania infantum*	28.8	90.0	20.0
AB_16	F	Pos (36)	-	2.8[a]	38.4	22.0

[a] I rounded the numbers to two decimal places so they match all the columns in the table.

4. Discussion

Antigen non-detection represents a key limitation in canine HWD diagnosis, occurring in 10.9% (11/101) of microfilaremic dogs in the present cohort. The successful reversion of 8 out of the 11 cases through heat treatment confirms that heat-mediated dissociation of CIC releases

Table 3
Serum cytokine concentrations in healthy controls (C), antigen-blocked dogs (AB), and *Dirofilaria immitis*-positive control dogs (DC). IFN-γ and IL-12 concentrations are expressed as mean ± standard deviation (SD), whereas IL-4 concentrations are expressed as median (Q1–Q3) due to their non-normal distribution. Comparative analysis was performed among the study groups.

Cytokine (pg/mL)	Control group (n = 7)	AB (n = 8)	DC (n = 20)	Significances
IFN-γ	2.68 ± 1.32	11.37 ± 9.30	8.97 ± 3.62	AB *vs* C ($P = 0.008$), DC *vs* C ($P = 0.025$)
IL-12	10.58 ± 3.74	29.28 ± 11.81	49.85 ± 14.56	AB *vs* C ($P = 0.020$), DC *vs* C ($P < 0.001$), DC *vs* AB ($P = 0.001$)
IL-4	3.80 (3.03–4.28)	53.71 (35.94–71.00)	6.97 (2.64–14.18)	AB *vs* C ($P = 0.002$), AB *vs* DC ($P < 0.001$)

detectable levels of heartworm antigens in cases of antigen blocking, consistent with previous reports (Little et al., 2014). Notably, three microfilaremic samples remained antigen-negative even after heat treatment. This confirms that while heating effectively dissociates CIC, it cannot resolve NAD results driven by non-immunological factors. In these cases, the lack of detection is likely due to few adult parasites or delayed antigenemia, where antigen concentrations remain below the test's analytical sensitivity rather than being immunologically masked (Tonelli, 1989; Rishniw et al., 2012; Velasquez et al., 2014; Drake et al., 2015). Therefore, heat treatment is specifically indicated for cases of exacerbated humoral interference, but it does not replace microfilarial screening (i.e., modified Knott's test). Interleukin analysis indicated that antigen blocking is likely associated with a Th2-type immune response. In canine leishmaniosis, it is widely accepted that the Th2 polarization promotes humoral immunity and antibody production as well as hypergammaglobulinemia (Barbiéri, 2006; Oleaga et al., 2009). Although this association has been more extensively documented in canine leishmaniosis, the predominance of a Th2-type immune response observed in patent *D. immitis* infections suggests that similar immunological mechanisms may also contribute to CIC formation and antigen masking in *D. immitis*-infected dogs (Chamizo et al., 2005; Carrillo & Moreno, 2009). In this regard, the most notable finding of the present study was the higher and significantly different concentration of IL-4 in the AB group compared to both healthy controls and the DC group. As a hallmark Th2 interleukin, IL-4 promotes B-cell activation, differentiation, and antibody production. An exacerbated humoral response may lead to the accumulation of CIC, capable of sequestering *D. immitis* antigens. Such sequestration could impair antigen recognition by the capture antibodies used in rapid enzyme immunoassays, thereby reducing the test's sensitivity and contributing to false-negative results (Tonelli, 1989; Tezuka et al., 2003; Little et al., 2018). Conversely, analysis of the DC group revealed elevated and significantly different IL-12 levels compared to the AB group and healthy controls. Interleukin 12 is a key regulatory interleukin that drives the differentiation of naïve T cells into Th1 effector cells, thereby promoting cell-mediated immunity (Trinchieri, 2003). These findings suggest that *D. immitis* infection, when occurring as a mono-infection or without immunological interference, typically induces a Th1-polarized response. This is likely driven by the *Wolbachia* spp. endosymbionts, which are known to elicit Th1-type immunity (Kramer et al., 2005; Simón et al., 2007; Latrofa et al., 2023). Although the sample sizes of both the AB group (n = 8) and healthy controls (n = 7) were relatively small, primarily owing to the low baseline frequency of the phenomenon and strict serum quality criteria, the robust statistical significance observed for IL-4 and IL-12

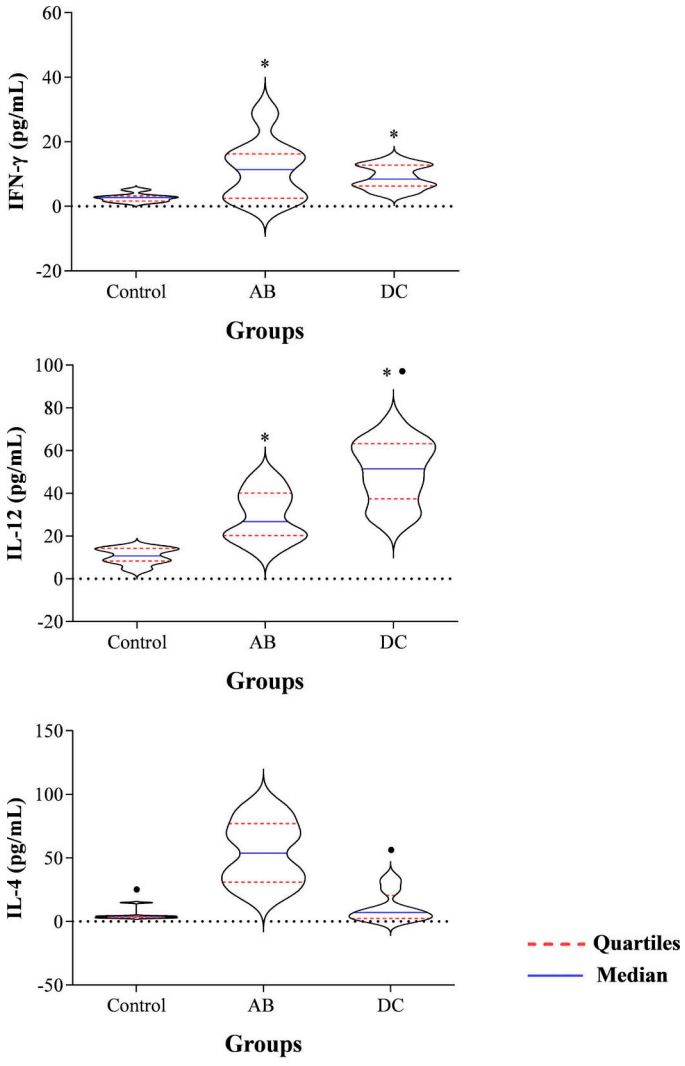

Fig. 2. Violin Plot showing distributions of concentration of interferon gamma (IFN-γ), interleukin 12 (IL-12) and interleukin 4 (IL-4), together with the relative statistical significances among groups (healthy negative dogs, Control group; dogs positive to *Dirofilaria immitis* with antigen blocking, AB group; dogs positive to *Dirofilaria immitis* without antigen blocking, DC group).

supports distinct Th1/Th2 polarization dynamics between groups. Specifically, the elevated IL-12 levels in the DC group indicate a typical Th1 polarization in *D. immitis* infections without antigen blocking. In contrast, the high IL-4 levels in the AB group reflect a predominant Th2 response, potentially exacerbated by seropositivity to other VBPs. Serum electrophoresis confirmed a significantly higher γ-globulin fraction in the AB group compared to the Control group, consistent with marked hypergammaglobulinemia. This finding aligns with the involvement of other pathogenic agents in the AB cohort. Indeed, overall seropositivity to other VBPs was significantly higher in the AB group than in the DC group, with *L. infantum* showing the highest frequency. Given its well-documented role in driving Th2 polarization and hypergammaglobulinemia, *L. infantum* co-positivity may act as an immunomodulatory trigger, providing the immunological substrate for CIC formation and subsequent antigen masking (Rolão et al., 2007; Menezes-Souza et al., 2011; Samant et al., 2021).

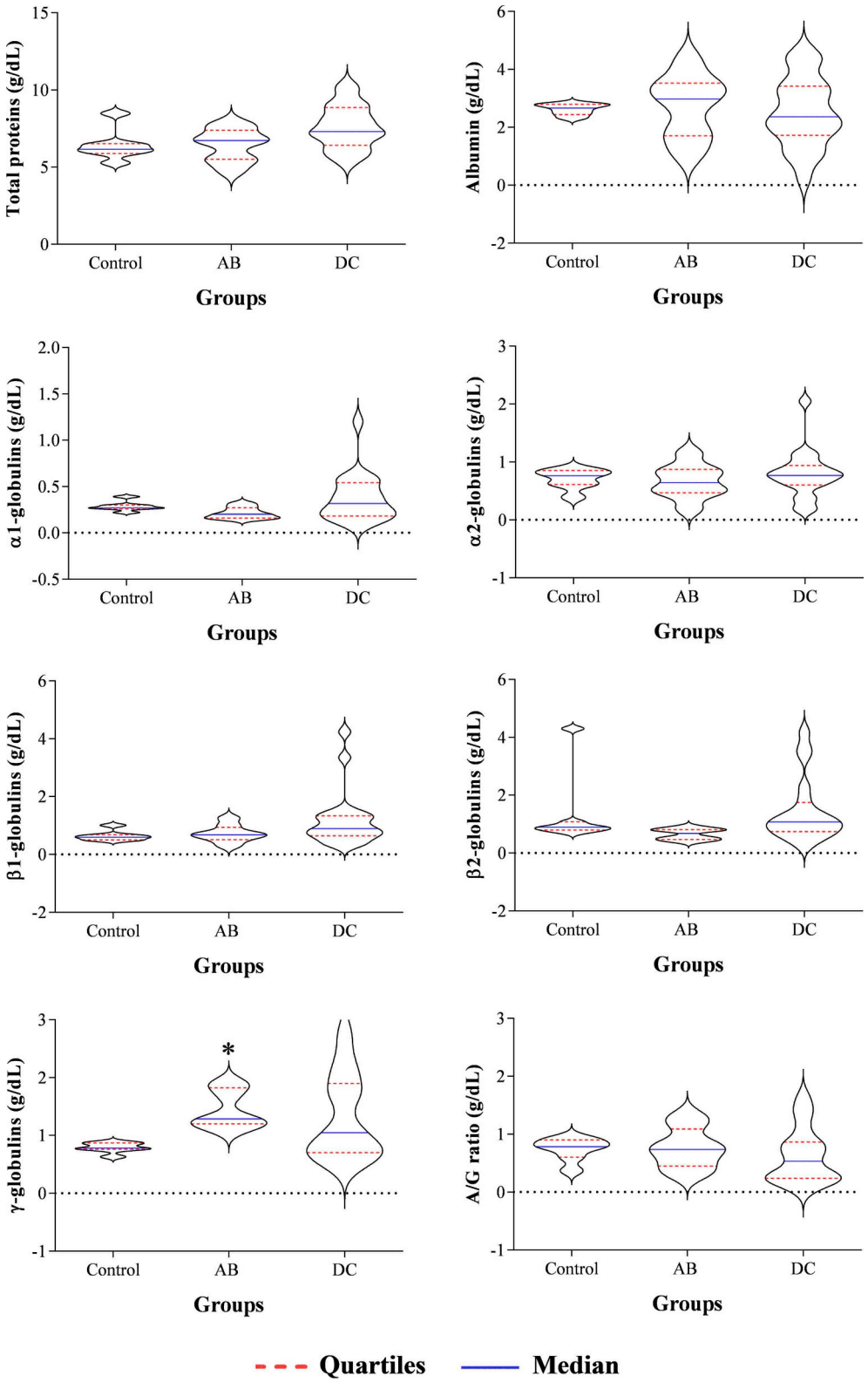

Fig. 3. Violin Plot showing distributions of concentration of total proteins, albumin, α1, α2-, β1-, β2- and γ-globulin fractions, together with the relative statistical significances among groups (healthy negative dogs, Control group; dogs positive to *Dirofilaria immitis* with antigen blocking, AB group; dogs positive to *Dirofilaria immitis* without antigen blocking, DC group).

5. Conclusion

Antigen blocking emerged as a relevant cause of NAD results in canine HWD, occurring in approximately 8% of infected dogs in this study. Heat treatment successfully restored antigen detection in the majority of the antigen negative samples, supporting its usefulness as second-line diagnostic tool in dogs with a high clinical suspicion. However, post-heating results must be interpreted with caution, considering the possibility of cross-reactions with other endemic parasites, and acknowledging that heat treatment will not yield a positive result in cases of extremely low worm burdens or early pre-patent infections. Therefore, antigen detection, regardless of heat treatment, should always be paired with microfilarial screening (e.g., modified Knott's test) to improve diagnostic accuracy. The association between antigen blocking and increased IL-4 concentrations suggests that a Th2-biased immune response may contribute to CIC formation and subsequent antigen masking. This mechanism may be further amplified in some cases by co-positivity with other VBPs and should therefore be considered, particularly in areas where dogs may be exposed to multiple VBPs simultaneously.

CRediT authorship contribution statement

Giulia Sisia: Writing – review & editing, Validation, Formal analysis. **Francesca Arfuso:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Formal analysis, Data curation. **Emanuele Brianti:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization. **Emanuela Sturiale:** Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ettore Napoli:** Writing – review & editing, Resources, Investigation, Formal analysis. **Giovanni De Benedetto:** Writing – review & editing, Resources, Investigation, Formal analysis. **Gabriella Gaglio:** Writing – review & editing, Supervision. **Francesca Nonnis:** Writing – review & editing, Resources, Investigation, Formal analysis. **Antonio Varcasia:** Writing – review & editing, Resources, Investigation, Formal analysis. **Federica Arrigo:** Writing – review & editing, Validation, Formal analysis.

Ethical statement

All experimental procedures were carried out in accordance with the European legislation regarding the protection of animals used for scientific purposes (European Directive 2010/63), as recognized and adopted by the Italian law (D.Lgs. 26/2014).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw data and derived data supporting the findings of this study are available from the corresponding author Emanuela Sturiale on request.

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