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Clinical canine *Borrelia burgdorferi* (*sensu lato*) infections are associated with highly elevated total IgG ELISA titers and convalescent Th2 immune responses

K. Emil Hovius [a,*], Tess van den Bergen [a,b], Osama Almalik [b], Ellen Versmissen [a,1], Victor P. Rutten [c,d], Hein Sprong [e,**], Joppe W. Hovius [f]

[a] *Amphipoda Biology and Veterinary Science, Veldhoven, the Netherlands*
[b] *Department of Applied Biology, HAS Green Academy, 's Hertogenbosch, the Netherlands*
[c] *Section Immunology, Department Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*
[d] *Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Republic of South Africa*
[e] *Laboratory for Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands*
[f] *Center for Infectious Diseases and Molecular Medicine and Amsterdam Institute for Immunology & Infectious Diseases, University of Amsterdam, Amsterdam UMC, Location Academic Medical Center, Amsterdam, the Netherlands*

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ABSTRACT

Lyme disease is caused by *Borrelia burgdorferi* (*sensu lato*), which is transmitted through species belonging to the *Ixodes ricinus* complex. Canine Lyme Disease (CLD) is an established clinical entity in the USA. In Europe, an unambiguous diagnosis is rarely made, although it has been shown that dogs can be naturally infected and develop antibodies against *B. burgdorferi* (*s.l.*). The relation of *Borrelia* total IgG, IgG2, and IgG1 specific antibodies and the incidence of symptoms was studied in a prospective cohort study. In a tick-dense area in the Netherlands, 84 dogs in 4 age cohorts were followed up during 7 consecutive half-years. In addition, 31 Bernese Mountain dogs (BMD), known to have robust anti-*Borrelia* antibody responses, were clinically monitored and serologically examined. Generalized estimating equations (GEE) analysis on repeated half-year measurements of clinical and serological results showed a strong association between the clinical signs fever combined with lameness in time, which in turn was associated with transiently high total IgG titers and elevated IgG1 titers against *B. burgdorferi* (*sensu stricto*). In BMD, we observed seroconversions and persistence of specific high total IgG and IgG1 titers. Although the latter also developed a persistent reaction against the *B. burgdorferi* (*s.l.*) C6 peptide, their tissues tested negative for *B. burgdorferi* (*s.l.*) DNA. This study strongly suggests that dogs - not vaccinated against *Borrelia* spp. infections - that encounter yearly tick infestations are recurrently infected. Some breeds, such as Labrador Retrievers and BMD, in the course of multiple tick-infestation seasons, develop transient symptoms compatible with CLD. Symptoms were strongly associated with temporarily raised total IgG and concomitant or convalescent high IgG1 antibody responses against *B. burgdorferi* (*sensu stricto*). Our findings provide insights into the resistance of dogs against *B. burgdorferi* (*s.l.*) infections and show that transient symptoms of CLD only occur in a subset of infected dogs.

1. Introduction

Lyme disease (LD) in humans was first described in Connecticut, USA (Steere et al., 1977). The causative agent, the spirochete *Borrelia burgdorferi* (*sensu stricto*), transmitted by *Ixodes scapularis* from reservoir animals to humans, was discovered by Willy Burgdorfer in 1982 (Burgdorfer et al., 1982). Thereafter, a high prevalence of canine infections, with a low incidence of clinical symptoms, such as fever combined with lameness (Canine Lyme Disease - CLD) became apparent (Lissman et al., 1984; Kornblatt et al., 1985; Magnarelli et al., 1985).

* Corresponding author.
** Corresponding author.
E-mail addresses: kehovius@iae.nl (K.E. Hovius), hein.sprong@rivm.nl (H. Sprong).
[1] Current address: MSD Animal Health Boxmeer, the Netherlands

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Sero-surveillance of dogs was shown to be a proxy for the risk of infection in humans (Schulze et al., 1987; Lindenmayer et al., 1991) with highest prevalences in north-eastern USA and eastern Europe (Bhide et al., 2004; Krol et al., 2015; Uesaka et al., 2016; Estrada-Peña et al., 2017; Irwin et al., 2017; Zhang et al., 2017; Geurden et al., 2018; Livanova et al., 2018; Evason et al., 2019; Ogden et al., 2019). In the USA, *B. burgdorferi* (*s.s.*) is the most frequently occurring pathogenic genospecies causing LD in humans and dogs (Magnarelli et al., 1996). In Eurasia, infections with *Borrelia afzelii* and *Borrelia garinii* are encountered more often than with *B. burgdorferi* (*s.s.*) (Duray and Steere, 1986; van Dam et al., 1993; Ryffel et al., 1999; Coipan et al., 2016). The different *B. burgdorferi* (*s.l.*) genospecies prefer specific reservoir animals and are transmitted by members of the *Ixodes ricinus* species complex, occurring as vectors in western Europe (Hovius et al., 1998). Co-infections with different *B. burgdorferi* (*s.l.*) species, or with pathogens such as *Anaplasma phagocytophilum*, regularly occur in ticks and dogs, potentially exacerbating disease outcome, and confounding the diagnosis (Mather et al., 1994; Kurtenbach et al., 1998; De Boer et al., 1993; Thomas et al., 2001; Bhide et al., 2004; Hovius et al., 2007). The American College of Veterinary Internal Medicine (ACVIM) states that a small subset of naturally *B. burgdorferi* (*s.s.*)-infected animals may develop CLD and that "it is not proven that European Lyme *Borrelia* cause clinical signs in dogs" (Littman et al., 2018). Both statements illustrate the challenge of reaching an unambiguous diagnosis and reflect the uncertainty and controversy on CLD. Cross-sectional serological studies showing a high prevalence of anti-*B. burgdorferi* (*s.l.*) IgG antibodies are important but inconclusive so far (Goossens et al., 2003; Speck et al., 2007; Gerber et al., 2009a; Barth et al., 2012). Speck et al. (2007) conclude that "a serological diagnoses of CLD on a single consultation is not possible". Dog breeds may differ in sensitivity to infections with *B. burgdorferi* (*s.l.*), displaying a variable course of disease (Schaible et al., 1991; Appel et al., 1993; Barth et al., 2012; Contreras and Lappin, 2016).

Indeed, some studies have shown that Bernese Mountain Dogs (BMD) often show persisting elevated antibody levels against *B. burgdorferi* (*s.l.*) infection (Müller, 1994; Gerber et al., 2007, 2009b; Barth et al., 2012; Pantchev et al., 2015). In a previously executed long-term prospective cohort study (1990–1995; $n = 84$ dogs) investigating the relationship of seasonal infection and clinical outcome, we observed cases with transient fever and lameness (Hovius and Houwers, 2007). These putatively clinically diagnosed dogs showed temporarily high total anti-*Borrelia* IgG titers. Reactivity to *B. burgdorferi* (*s.l.*) in Western blots showed different antibody combinations for preclinical, clinical and convalescent stages (Hovius et al., 2000). Most of these long-term studied dogs showed no disease and remained with the preclinical antibody spectrum. Tissues of some of these dogs contained *Borrelia* spp. DNA, but especially in older dogs, other morbidities confounded the diagnosis (Hovius et al., 1999b). The observations suggest that for most dogs, although seasonally infected, disease-preventing mechanisms exist. Studies on the immunity of vector borne diseases of dogs in general relate the balance between the Th1 and Th2 branches of the immune system to a persistent symptomatic state of infection (Day, 2011). We here investigated the balance between the Th1 and Th2 branches, respectively indicated by IgG2-and by IgG1-isotype antibodies, and clinical symptoms.

For that purpose, in the present study, we statistically evaluated the occurrence of IgG1 and IgG2 *Borrelia*-specific antibodies over time with fever and lameness and titers of total IgG. Four different age cohorts were studied for 3.5 consecutive years, thus 7 alternating half-years of high- and low-risk tick-bite seasons. We describe seasonal fluctuations in the Th1 and Th2 subclass responses, hypothetically impacting susceptibility to *B. burgdorferi* (*s.l.*) infection and the development and course of clinical symptoms. By statistical analysis with generalized estimating equations (GEE) we tested the responsiveness of symptoms compatible with CLD - fever (or malaise) and lameness - for immunological variables and seasonal tick exposure. Moreover, the cohorts of puppies, young-, middle-aged- and elderly dogs of several breeds enabled us to study the influence of age expressed as consecutive seasonal infections and of specific breeds on disease development. The present study ultimately was able to predict the predefined clinical syndrome of CLD with statistical models containing immunological, seasonal and clinical variables.

2. Materials and methods

2.1. Outline of the study

The prospective cohort study with 84 dogs in four age groups was realized from 1990 to 1994. Detailed clinical observations and diagnoses of diseases were collected together with 605 serum samples, on which the total IgG whole cell ELISA, with *B. burgdorferi* (*s.s.*) strain 31 as an antigen, was performed. A majority of dogs with fever and lameness had very high titers in the forementioned assay. In addition, we had available a cohort of BMD ($n = 31$) known to have high titers and a variety of clinical problems. Supplementary file 1: Table S1 describes findings from our previous studies, starting in 1990, and indicates what new data and analyses have been performed during the present study. The main goal of the present study was to investigate the interplay of symptoms malaise, fever and lameness and the relation with immunological, seasonal and clinical variables. These three classes of variables were determined at successive moments in a 3.5-year timeline.

2.2. Enrollment of dogs in prospective age cohorts

From 1990 through to 1994 at the veterinary clinic of the first author (KEH), dogs for each age cohort were enrolled if owners, after written informed consent, complied with at least bi-annual serum sampling (jugular vein) and quarterly clinical examinations for the coming 3.5 years (Hovius et al., 2000; Hovius and Houwers, 2007). Moreover, only dogs that walked in woods and parks, encountering ticks on a regular basis were included. No dogs, except one at the start of the study, were lost to follow-up resulting in 84 enrolled dogs (see Supplementary file 2 for complete information on the enrolled dogs, such as age, clinical data and diagnosis, tick infestation and serological data). Cohort 1 consisted of 22 pups, less than 6 months-old at first sample. All but one were first sampled in winter, the low-risk tick-bite season, before supposedly any tick infestation had occurred. Cohort 2 consisted of 23 young dogs, approximately 12 months of age at first sample, entering the study during their first or second high-risk tick-bite season (April-September) and thus most probably had experienced their first tick encounters. Some had minor clinical signs at inclusion. In retrospect, one case (dog 40) appeared to have had signs of CLD. Cohort 3 consisted of 22 adult dogs, mostly healthy, that entered at their 3rd to 7th tick-season. Cohort 4 consisted of 17 elderly dogs, entering at their 8th to 11th tick-season, most enduring health problems before enrollment. None of these dogs were vaccinated against Lyme disease. The cohorts described above consisted of 30 different dog breeds and reflect the composition of the Dutch domestic dog population at the time of study. Breed groups such as Labrador- and Golden retrievers, Spaniels, Terriers, Dachshunds and Toy- and crossbreeds as well as Mountain dogs were all represented.

2.3. Bernese Mountain Dogs (BMD) and an infected laboratory Beagle

From 1990 through 2012 a total of 31 BMD were presented at the veterinary practice for clinical consultation. Dogs were serologically examined at varying ages and also investigated for prevalence of *Borrelia*-specific antibodies. Eleven of these BMD were clinically and serologically investigated throughout at least three successive years. None of these dogs were vaccinated against Lyme disease. In addition, as a reference to validate all serological assays, we used sera of the Beagle pup (A92-1) experimentally infected by wild collected ticks with an American strain of *B. burgdorferi* (*s.s.*) kindly provided by Dr M.J.G.

Appel, Cornell University, NY, USA (Appel et al., 1993).

2.4. Ticks on vegetation and on dogs, seasonal variables defined

Dogs in this study were walked in forests of the "Kempen" region, extending into Belgium, South-West of Eindhoven, Province Noord-Brabant, the Netherlands. This region, from 1990 onwards, showed increasing infection rates of adult questing ticks and was one of the Dutch high-risk areas for tick bites, and human Lyme disease (Hovius et al., 1998; Gassner et al., 2011; Hofhuis et al., 2015, 2016; Takken et al., 2017; Hartemink et al., 2019, 2021). Ticks on the vegetation and dogs were sampled throughout 1993 during the cohort study (Fig. 1). The highest number of ticks found on the vegetation occurred in May, while on dogs, the highest number occurred in June. No ticks were found on the vegetation in December, January, February and March of the 1990s (Hovius, 1999). Around 95% of the yearly numbers of ticks on dogs were collected between April and August, and occasionally found on the dogs in February, March, September and October. Consequentially, we defined the seasonal effect of the risk of tick-bite: quarters Q2 and Q3 as the high-risk tick-bite season (HR) and quarters Q4 and Q1 (of the following year) as the low-risk tick-bite season (LR). The number of attached ticks on dogs varied from nil to hundreds per dog. Indication of the infestation rates per quarter year per dog in the HR- and LR seasons were obtained by written and verbal questionnaires. Dog owners were also asked to collect attached ticks and deliver them for identification and detection of *Borrelia* spp. prevalence. During serum sampling, the dog's coat and skin were inspected and ticks were collected by the veterinarian (KEH). Four infestation grades were classified each HR season as results of the questionnaires and collections: 0, ticks not observed; +, ticks occasionally (1–2 seasonally) observed; ++, ticks regularly (3–6 seasonally) observed; +++, ticks frequently (more than 6 seasonally) observed. For binary statistics we defined "tick infestation intensities" 0 (as low, the combination of 0/+) and 1 (as high, ++/+++) in Supplementary file 3. Infestation rates and seasons with higher tick abundance were also noted in Supplementary file 2. Seroconversion of total IgG would be expected after tick-bite and was therefore also considered a seasonal variable (see below).

2.5. Canine Lyme disease diagnosis and clinical variables defined

Experimental Beagle dogs inoculated with *B. burgdorferi* by tick-bite, as well as cases of field-studied dogs of various breeds showing malaise and fever (in some cases only malaise) with lameness in synchrony or postponed, which may again recur months later (Lissman et al., 1984; Kornblatt et al., 1985; Greene et al., 1988; Magnarelli et al., 1990; Appel et al., 1993; Straubinger, 1999; Littman et al., 2006). Therefore, we defined CLD as fever or malaise with lameness, with exclusion of the differential diagnosis. A thorough diagnostic workup including the exclusion of the differential diagnosis is mandatory before reaching a presumptive clinical diagnosis of CLD. Dogs were clinically inspected quarterly and, if necessary, attended as usual in the veterinary practice by the first author (KEH). Therefore, clinical examinations and routine diagnostic tests, such as hematology and blood chemistry and, if necessary, X-ray and ultrasound diagnostics were available. Furthermore, external laboratories were consulted for completion of the diagnostic workup, including serology, pathology and a *B. burgdorferi* (*s.l.*) PCR. Clinical data were recorded in the timeline per quarter over the five study years (Supplementary file 2).

For 19 dogs a presumptive diagnosis of CLD could be made before serological results were known. For statistical analysis the diagnosis was presented as two 'response variables': FLb defined as fever with synchronous or postponed lameness, within one quarter year; MFLb defined as malaise or fever followed by lameness within a year. An additional response variable was added, suggestive for the differential diagnosis MFLd-b (malaise, fever and lameness for which the diagnostic workup resulted in an alternative diagnosis). Clinical variables potentially predictive for the above response variables were: (i) comorbidity with possible immunocompromised states based on endocrinological or

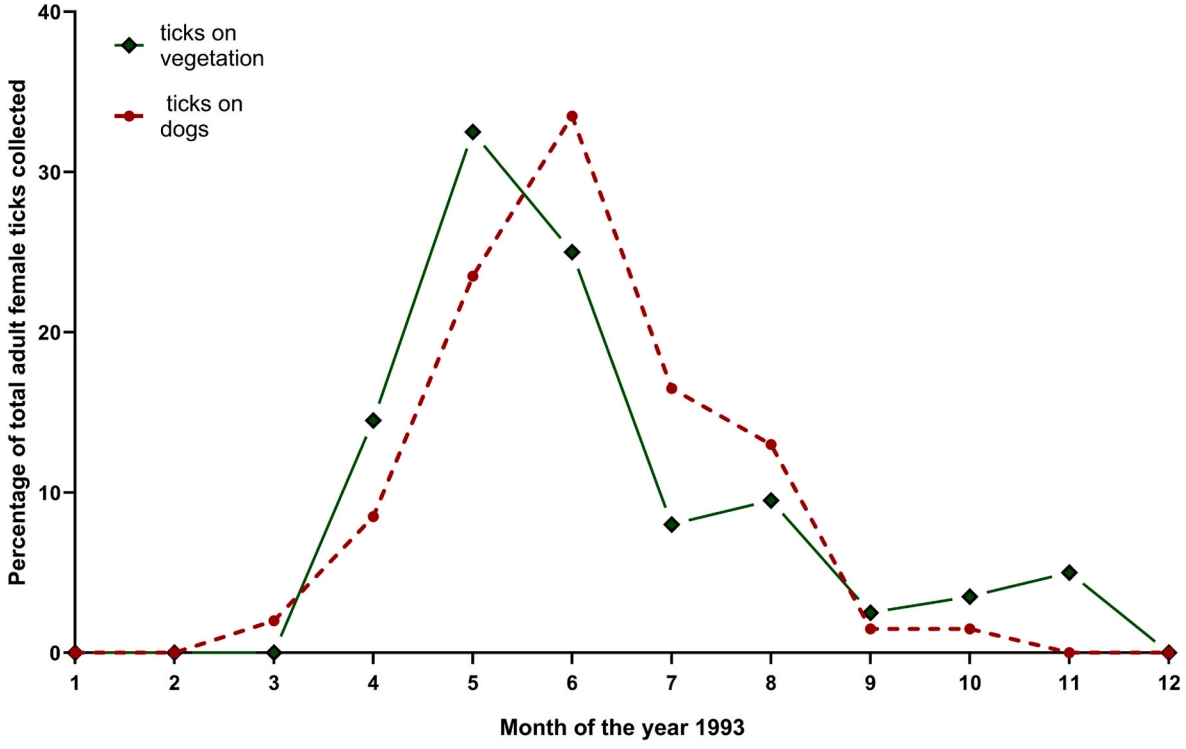

Fig. 1. Monthly distribution of ticks on the vegetation and on dogs in the year 1993, as a percentage of the total yearly catch of adult ticks in one year. The distribution of nymphal ticks on the vegetation throughout the year was bimodal, with a second peak in September (not shown). Most ticks found on the skin of dogs were adult female ticks.

malignant disease; and (ii) antibiotics prescribed for whatever cause, mostly for dogs with repeated urinary or skin problems or combined with fever or malaise. Some of the dogs with severe fever, with or without lameness were given antibiotics. Antibiotics were prescribed for 5 or 10 days with the pharmaceutically recommended dosage (see Supplementary file 2).

2.5.1. Serological assays and definition of serological variables

Serum samples were divided into lots and stored at −30 °C in 2 ml Eppendorf cuffs. The total IgG whole cell ELISA was developed and performed at the Veterinary Microbiological Diagnostic Center (VMDC), Faculty of Veterinary Medicine, Utrecht University. Sera ($n = 605$) were tested for total IgG at the end of the prior study period (1990–1995) in randomly sorted batches at the VMDC. A subset of sera ($n = 328$), mostly collected before, at and after seroconversion of the total-IgG ELISA, was retested with the IgG1 and IgG2 ELISA at Amphipoda Biology and Veterinary science (ABV), the Research Laboratory of the Companion Animal Practice. The antigen for IgG1 and IgG2 whole cell ELISA (see below) was prepared at the Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam (AMC). The antibodies against the C6 peptide were determined with the "in-house ELISA" (4DX Snap test, IDEXX) performed at ABV. Two direct immunological variables for the binary statistical analysis were chosen: total-IgG titer ≥ 8 and IgG1 titer ≥ 2. The IgG2 titer had no predictive value on its own and is represented in a combined variable: the IgG1 titer divided by the IgG2 titer (IgG1/IgG2 ≥ 0.4). The fourth immunological variable is the total-IgG titer ≥ 8 together with the IgG1 titer ≥ 2 in the same half year (IgG ≥ 8 & IgG1 ≥ 2). Thus, four immunological variables were used. The binaries 0 and 1 were used in the statistical analysis, with 1 as a titer above the indicated thresholds. Seroconversion defined as an increase of total-IgG by at least 2 dilution steps (i.e. from titer 4 to 6 or 5 to 7, and not exclusively for titer 6 to 8 - the maximum titer) mostly occurs or is initiated in the high-risk tick-bite season (HR) and was therefore included as a seasonal variable rather than a serological variable. Additional information on the serological and molecular assays as performed in this and the earlier study (Hovius et al., 1998, 1999a, 2000, 1999b) are described below (see also Supplementary file 1: Table S1).

2.5.2. Antigen preparation

Of a thawed *Borrelia burgdorferi* B31 culture (American type culture collection 35210), 0.3 ml was transferred to 30 ml of BSK and incubated at 34 °C for a minimum of three weeks. One ml (around 1×10^7 vital spirochetes) of this pre-culture was transferred to 200 ml of BSK medium and incubated for 3 weeks at 33 °C. When this culture contained *c.*50 spirochetes/field (dark-field microscopy 400×), the 200 ml was pelleted by cooled centrifugation at 30,000 *g* (17,000 *rpm*) and used for antigen preparation. The pellet was washed three times with phosphate buffered saline (PBS) with 5 mM $MgCl_2$, redissolved in PBS and sonicated and centrifugated at 10,000 *g* at 4 °C. The protein concentration of the supernatant was determined according to Lowry et al. (1951) and diluted in coating buffer (0.13 g bicarbonate and 0.31 g sodium bicarbonate in 100 ml distilled water; pH 9.6) to a concentration of 0.4 μg/ml. The *B. burgdorferi* B31 antigen also reacted with European *B. burgdorferi*-, *B. afzelii*- and *B. garinii*-specific antibodies, as shown by immunoblots with specific recombinant antigens of these three species, that reacted against serum of the experimental Beagle A92-1 infected with an American strain (Appel et al., 1993; Hovius et al., 1999a, 2000).

2.5.3. Total IgG whole cell ELISA

The total IgG whole cell ELISA was performed, as described (Hovius et al., 1999a). ELISA plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated overnight with the prepared antigen (in 0.05 M Na_2CO_3, pH 9.5; 100 μl per well concentration of 0.2 μg antigen/ml). Serum dilutions were incubated for 1 h in ELISA buffer (phosphate--buffered saline, 1% w/v Protifar (Nutricia, Zoetermeer, The Netherlands) and 0.05% v/v Tween-80). After washing three times, 1/3000 diluted horse radish peroxidase conjugated goat-anti-dog-IgG was added and after another 1 h incubation after washing, the chromogenic agent (ABTS, Sigma Chemical Co., St Louis, USA) was added. Readouts were scored as titers determined as the last visibly colored well in the 2-fold dilution series in 8 wells 1/20; 1/40 till 1/2560 and annotated as the $^2\log_{20}$ titer: 1, 2, till 8. A dilution above 1/2560, or titer '9' was scored if the two last dilutions 7 and 8 did not diminish in color intensity and only used for the descriptive statistics. The total-IgG whole cell ELISA had been validated by running a recombinant flagellin ELISA in parallel and with canine sera of non-endemic areas (Hovius et al., 1999a, 2000).

2.5.4. IgG1 and IgG2 whole cell ELISA

As with the total IgG whole cell ELISA, the *Borrelia burgdorferi* (*s.s.*), B31 antigen was coated on high binding 96 wells Greiner plates. The above prepared antigen (concentration 0.4 μg/ml) was diluted 500 times for the IgG1 ELISA and 2000 times for the IgG2 ELISA and coated overnight at 4 °C. The same protocol as for the total IgG whole cell ELISA was employed with the exception of the conjugates against dog IgG1 and IgG2 (Duijkeren, 2001). Conjugates were goat-anti-dog IgG1 antibody and sheep-anti-dog IgG2 (both from Bethyl Laboratories Inc., Montgomery, TX, USA). Readouts were scored as titers identical to the total IgG whole cell ELISA. Internal validation of these assays was performed with a selection of the sera that had also been used for validating the total IgG whole cell ELISA as referred to in the previous paragraph. The experimental Beagle dog A92-1 (vectorially infected at 6 weeks of age which resulted in lameness and persistent infection after 2 months) did not react in the IgG1 ELISA at 2, 3 or 4 months post-infection. It did react with increasing IgG2 titers over time.

2.5.5. Antibodies against the C6 peptide

Antibodies of *Borrelia burgdorferi* (*s.l.*) against the conserved IR6 epitope of the VlsE protein, synthesized as the C6 peptide, were tested with the in-house 4Dx-SNAP test (IDEXX Laboratories, Westbrook, ME, USA) that also detects antibodies against *Anaplasma phagocytophilum*, *Ehrlichia canis*, and heartworm antigens. As an internal control, we tested sera from the experimentally infected Beagle dog A92-1, with lameness and a persistent infection, which reacted with increasing color intensity against the C6 peptide in the 4Dx-SNAP test at 2, 3, and 4 months post-infection.

2.5.6. Bactericidal activity assays

A total of 98 sera from 31 healthy and clinically borreliosis-suspected BMDs were tested in the antibody-dependent complement-mediated immobilizing assay. This assay was performed as previously described (Hovius et al., 2000). The *B. afzelii* isolate PkO was grown to a density of 1×10^7 spirochetes in BSK medium. The suspension was added to heat-inactivated sera to be tested in combination with normal canine serum (NCS), as the complement source, in wells of an ELISA plate and incubated at 33 °C. The assay was performed at AMC.

2.5.7. Detection of Borrelia DNA by PCR

As part of the BMD study, DNA detection in a total of 100 tissue samples from 16 dogs was performed. The procedure of amplification and identification of *Borrelia* DNA was similar as for tissues of the other breeds from the prospective cohort study as previously performed and described (Hovius et al., 1999b).

2.6. Data management and statistical analyses

In the first two cohorts from 1991 onward and for cohorts 3 and 4 starting in 1990, at least one sample for serology was planned each half year during the 3.5 study years per dog. The sequential number of tick-seasons was used as a seasonal variable expressing the repetitive frequency of tick bites, presumably with varying intensities of yearly

infections. This allowed the investigation of clinical signs and serology in relation to successive seasonal variables: number of tick-seasons, LR and HR, and the intensity of tick infestation per dog as well as to seroconversion. For statistical analysis on these repeated measurements, the clinical data and serological results were placed in the half-year timeline, with the first half year in the low-risk tick-season (LR is Q4 and Q1 of the following year, see Supplementary file 2). We previously showed that in HR total-IgG titers against *Borrelia* were higher than these titers in LR (Hovius et al., 1999a). Dogs acquire their primary and the yearly repeated infections at variable time points of the first and later HR seasons and may even acquire infections at the beginning of the LR season (October) (see Fig. 1 with monthly tick data in the region during 1993). Furthermore, these infections will have variable bacterial loads and will probably lead to diverse antibody levels and may show the ensuing decline of antibodies with various individual time trajectories. Therefore, the standard deviation of titers, be it calculated per quarter or half-year were expected to be high, acceptable for field data. The association between directly measured seasonal, clinical and immunological variables was determined with contingency tables (Fisher's exact test, GraphPad, Prism 9) with $P < 0.05$ being considered significant. Mixed effects analysis (GraphPad Prism 10) on directly measured data was performed to analyze the effect of consecutive high- and low-risk tick-bite seasons (HR and LR) on the height of measured titers.

By employing generalized estimating equations (GEE) as statistical analysis, models were developed with immunological, seasonal, and clinical variables, predicting the response variables FLb and MFLb of the diagnosed dogs (with the dog as subject and the consecutive half years 1–7 as within-subject factor). Since the response was binary (defined CLD-FLb or MFLb-predicted or not) we applied the binary logistic model within GEE (SPSS IBM version 30). Fulfillment of all half-year values is imperative for GEE; therefore, imputation of serological values was necessary for some half-years as occasionally owners would skip a half-year or the serum-quantity was insufficient for the IgG1-IgG2 assays then an imputed result (in italics) was annotated in Supplementary files 2 and 3. Clinical signs were known for all quarter-years of the study period and had no missing values. The calculation for missing values is presented in Supplementary file 1: Tables S2 and S3.

3. Results

3.1. Clinical symptoms

Of the 84 dogs followed in this study, 19 were clinically diagnosed with presumptive canine Lyme Disease (CLD), defined by either concurrent fever and lameness (FLb), or malaise and/or fever followed by lameness (MFLb) (Table 1, Supplementary file 2 and Supplementary file 1: Table S4).

In total, 37 episodes of fever and 59 episodes of lameness were recorded. These symptoms occurred together within the same half-year significantly more often than expected by chance ($n = 588$ half years; Fisher's exact test, $P < 0.0001$; OR = 13.5). After excluding other potential causes, this co-occurrence was strongly associated with *Borrelia* infection (Fisher's exact test, $P < 0.0001$; OR = 52.4). Most of the 12 dogs diagnosed with FLb experienced fever above 39.2 °C and short episodes of one-sided stifle lameness within the same quarter. The remaining 7 dogs diagnosed with MFLb showed either delayed onset lameness (more than a quarter year after fever) or presented with severe malaise followed by lameness (affecting either the stifle, elbow, or back).

3.2. Serology: Total IgG, IgG2 and IgG1 B. burgdorferi (s.s.) whole cell ELISA titers

At study entry, most younger dogs (cohorts 1 and 2) showed low total-IgG titers but 90% experienced tick infestations and seroconverted during their first tick-season (Fig. 2). Among older dogs (cohorts 3 and 4), approximately half entered the study with low total-IgG titers, while the others already had elevated titers, reflecting prior exposure. During follow-up, many of these older dogs also showed new or repeated seroconversions. Total-IgG and IgG2 titers were normally distributed (Fig. 3A) and showed a moderate correlation (Pearson's $r = 0.3968$, $P < 0.0001$), consistent with a Th1-type response. In contrast, IgG1 titers

Table 1
Dogs from the prospective cohort study with a presumptive clinical diagnosis of canine Lyme disease (CLD), defined as fever or/and malaise followed by lameness.

Dog number and breed	Cohort no.	Tick season (HR/LR) at clinic	Fever (°C)	Lameness	Total IgG and IgG1	
					Clinical	Maximum
Fever and lameness						
1 Alaskan Husky	1	4 LR	39.5	Stifle	6/<1	7/<1
3 Labrador Retriever	1	3 LR	38.9	Stifle	5/<1	8/1
13 Labrador Retriever	1	5 LR	39.2	Stifle	7/3	7/3
29 Golden Retriever	2	4 HR	40.0	Stifle	8/2	8/2
40 Tatra Mountain Dog	2	2 LR	39.6	Elbow; stifle	7/2	8/2
47 Scottish Terrier	3	5 HR	40.9	Stifle	7/<1	8/4
48 Bernese Mountain Dog	3	4 LR	39.9	Stifle	8/7	8/8
58 Great Münsterlander	3	9 LR	40.6	Hind leg	7/<1	7/<1
63 Labrador Retriever	3	8 HR	39.6	Foot; stifle	8/1	8/1
72 Cross Labrador	4	9 LR	39.6	Stifle	8/4	8/4
75 Bouvier de Flanders	4	10 HR	39.5	Stifle	8/1	8/4
78 Irish Terrier	4	13 HR	39.3	Stifle	8/2	8/2
Fever and lameness in separate quarters						
77 Bernese Mountain Dog	4	12 LR	39.4	Stifle	8/3	8/3
83 Dachshund	4	13 LR	39.5	Stifle	5/2	8/3
Malaise and lameness in the same or different quarters						
14 Siberian Husky	1	3 LR	37.8	Elbow	8/<1	8/<1
34 Bernese Mountain Dog	2	4 HR	38.5	Elbow	8/5	8/8
51 Labrador Retriever	3	6 HR	38.6	Elbow	8/<1	8/4
74 Cross Labrador	4	11 HR	39.0	Hind leg	8/3	8/4
79 Irish Terrier	4	12 HR	39.0	Stifle	8/1	8/1

Notes: Consecutive tick seasons, in which the clinical signs on which the presumptive clinical diagnoses were made. HR is the high-risk tick-bite season (Q2 and Q3); LR is the low-risk tick-bite season (Q4 and following Q1). Fever is defined as temperature ≥ 39.2 °C (dog 3 with a 0.3 °C lower temperature being an exception). Column "Lameness" shows the affected joint or leg resulting in lameness. Column "Total IgG and IgG1" shows the results of total-IgG- and IgG1 whole cell ELISA titers observed concurrently with the clinical signs on which the clinical presumptive diagnoses were made; the maximum observed total-IgG- and IgG1 titers are also shown. For more information on the confirmation of the presumptive diagnosis, see Supplementary file 1: Table S4.

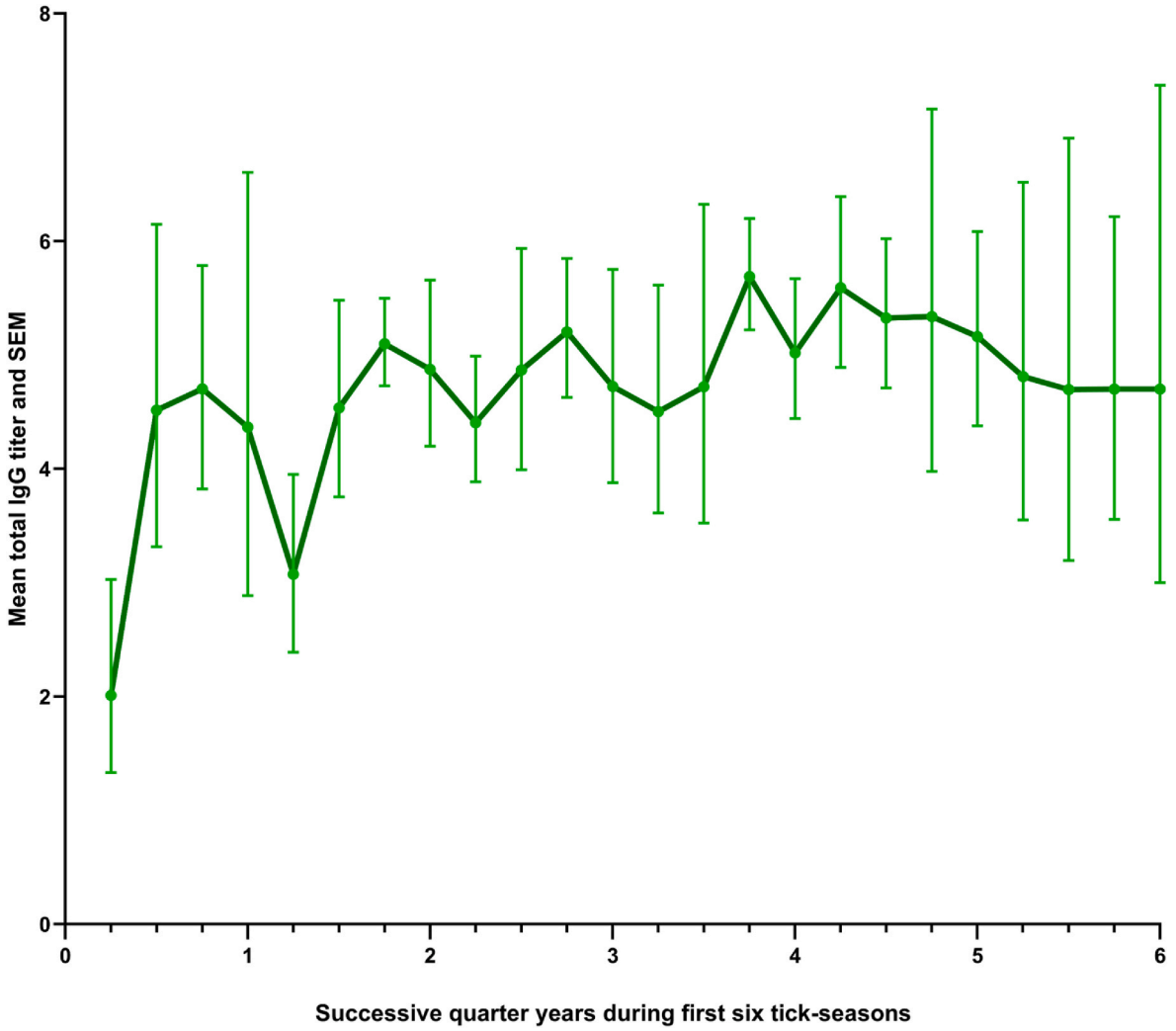

Fig. 2. Mean total IgG whole cell ELISA titers with standard error of the mean (SEM) per quarter year in the first 6 tick-seasons with 355 samples of 56 dogs (cohort 1, 2 and part of cohort 3). Each point is a quarter-year in sequence starting with Q1 - winter - of first tick-season (mean titer 2.0).

followed a lognormal distribution and showed no correlation with total-IgG or IgG2 titers, indicating that IgG1 reflects a separate, likely Th2-driven immune process (Fig. 3B). IgG1 titers ≥ 2 occurred most often in dogs with total-IgG titers ≥ 7 and IgG2 titers ≥ 6, but in some cases, elevated IgG1 was seen in dogs with lower total-IgG- and IgG2 titers. Across all cohorts, average titers tended to increase with age and cumulative tick exposure (Table 2). Younger dogs (cohorts 1 and 2) had comparable mean titers, while slightly higher mean titers were observed in adult dogs (cohort 3). In contrast, cohort 4 (the oldest dogs) included more individuals with exceptionally high total-IgG (≥ 8), IgG2 (≥ 6), and IgG1 (≥ 4) titers. Seasonality also influenced serological patterns. Total-IgG and IgG2 titers tended to peak during high-risk tick-seasons (HR), when tick activity and exposure were highest. In contrast, IgG1 titers tended to peak during low-risk tick-seasons (LR), after the peak transmission period had passed (Table 3).

To statistically assess the influence of seasonality and cumulative exposure, we applied mixed-effects models to the directly measured (non-imputed) ELISA data across all half-years ($n = 328$ samples). These analyses confirmed that total-IgG and IgG2 titers increased significantly with successive tick-seasons ($P = 0.0002$ and $P = 0.0400$, respectively; Table 4). Interestingly, the seasonal effect (higher titers in HR *versus* LR) was modest for these markers. In contrast, IgG1 titers showed a distinctly different pattern, increasing significantly with cumulative tick exposure ($P = 0.0010$) but with a clear seasonal shift: IgG1 titers were significantly higher in low-risk seasons (LR) than in high-risk seasons (HR) ($P = 0.0381$; Table 4). This pattern was even more pronounced when analysis was restricted to the first 6 tick-seasons, reflecting the younger dogs ($P = 0.0059$; Table 5). Because BMD are known to have particularly strong and persistent *Borrelia*-antibody responses, we repeated the analysis excluding BMD to ensure their responses were not driving the observed patterns. Even after removing BMD data, IgG1 titers still showed significantly higher values in LR compared to HR seasons ($P = 0.0489$ for all tick-seasons, and $P = 0.0078$ for seasons 1 to 6; Table 6 and Fig. 4). This confirms that the seasonal shift in IgG1 is a general feature of the canine immune response to *Borrelia* infection, not specific to BMD. Results of Tables 4–6 and Fig. 4 were derived from the directly not imputed serological data from 328 samples for measuring total-IgG titer -, IgG2 titer - and IgG1 titer ELISAs.

3.3. Clinical symptoms and serology

To evaluate the relationship between clinical symptoms and *Borrelia*-specific antibody responses, we applied generalized estimating equations (GEE) models using serological, seasonal, and clinical variables over seven consecutive half-years ($n = 588$ time points). These models tested which factors best predicted the occurrence of fever and lameness (FLb) or malaise/fever followed by lameness (MFLb). Among younger dogs (cohorts 1 and 2), FLb was significantly associated with IgG1/IgG2

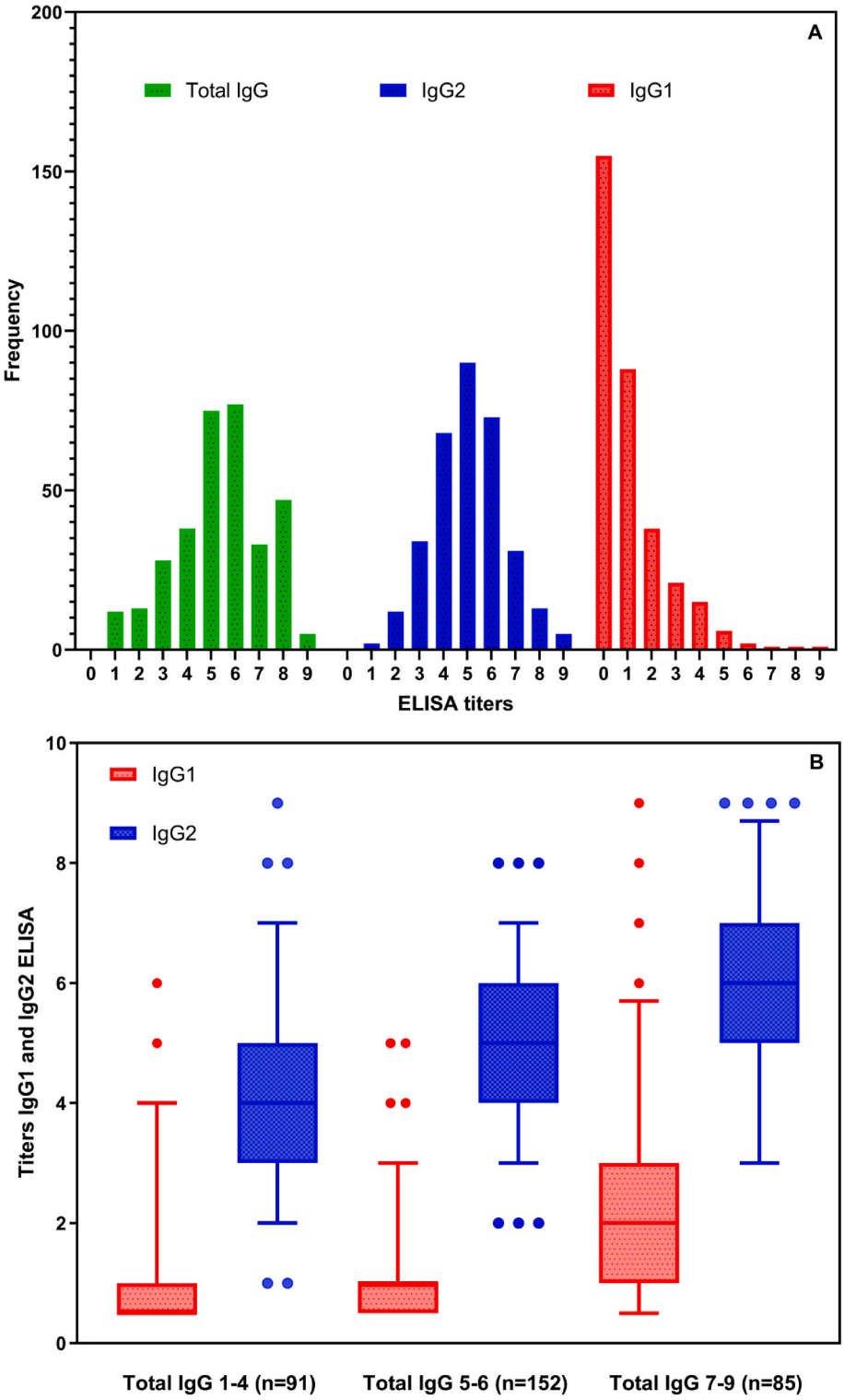

Fig. 3. **A** Frequency distribution of total IgG and IgG2 whole cell ELISA titer results with a normal distribution and IgG1 ELISA results with a lognormal distribution (Kolmogorov-Smirnov test). The 0 result is in fact a titer < 1; total *n* of all titer data is 328. **B** IgG2 and IgG1 results compared to total IgG categories 1 to 4 (1–4), 5 and 6 (5–6), and 7 and 9 (7–9). *Note*: "9" as a titer signifies that the last dilution step in the ELISA plate, titer 8 (dilution 1/2560) is as dark or darker in coloration.

≥ 0.4, while MFLb was predicted by the combination of Total-IgG ≥ 8 & IgG1/IgG2 ≥ 0.4 together with high tick infestation intensity and antibiotic use (Table 7). For all cohorts combined (cohorts 1–4), FLb was best predicted by the combination of IgG ≥ 8 & IgG1 ≥ 2, seroconversion, and antibiotic use. MFLb in the full dataset was similarly associated with IgG ≥ 8 & IgG1 ≥ 2, tick infestation intensity, and the clinical variables comorbidity and antibiotic use. Notably, when limiting the analysis to cohorts 1–3 (excluding the oldest dogs), FLb was still predicted by relatively high IgG1 ≥ 2 and seroconversion, whereas MFLb again as in the young dogs, associated with IgG ≥ 8 together with IgG1/IgG2 ≥ 0.4, tick infestation intensity and antibiotic use.

The models also included a response variable for malaise, fever, and lameness with an alternative diagnosis (MFLd-b) to differentiate presumed CLD from other conditions. Interestingly, MFLd-b was negatively

Table 2
Mean of measured total IgG, IgG2, and IgG1 whole cell ELISA titers in the four cohorts.

C	*n*	Tick-season	Total IgG ELISA titer		Total IgG frequency (%)			IgG2 ELISA titer	IgG2 frequency (%)			IgG1 ELISA titer	IgG1 frequency (%)		
			N	Mean	Titer ≤ 4	Titer 5–6	Titer ≥ 7	Mean	Titer ≤ 3	Titer 4–5	Titer ≥ 6	Mean	Titer ≤ 1	Titer 2–3	Titer ≥ 4
1	22	0–4	87	5.0	33.3	50.6	16.1	5.0	10.3	52.9	36.8	0.7	86.2	12.6	1.1
2	23	1–5	105	5.0	34.3	47.6	18.1	5.1	14.3	49.0	36.7	0.9	84.8	12.4	4.8
3	22	3–10	74	5.4	27.0	48.6	24.3	4.6	25.7	51.4	23.0	1.2	81.1	9.5	9.5
4	17	8–14	62	6.5	9.7	35.5	54.8	5.7	3.2	46.8	50.0	2.5	33.9	45.2	20.1

Notes: Mean total IgG, IgG2 and IgG1 whole cell ELISA titers were measured in four age cohorts (C), and the percentage of low, middle, and high titers is shown.
Abbreviations: C, cohort; *n*, number of dogs; *N*, number of samples.

Table 3
Mean of measured total IgG, IgG2, and IgG1 whole cell ELISA titers and frequencies of extremely high titers for low-risk (LR) and high-risk (HR) tick-seasons.

C	*n*	Tick-season	T-IgG ELISA titer		T-IgG frequency (%)		IgG2 ELISA titer		IgG2 frequency (%)		IgG1 ELISA titer		IgG1 frequency (%)	
			LR	HR	LR	HR	LR	HR	LR	HR	LR	HR	LR	HR
			Mean	Mean	Titer ≥ 8	Titer ≥ 8	Mean	Mean	Titer ≥ 6	Titer ≥ 6	Mean	Mean	Titer ≥ 4	Titer ≥ 4
1	22	0–4	4.8	5.3	8.5	2.5	5.1	4.9	44.7	27.5	0.9	0,5	2.1	0
2	23	1–5	4.7	5.3	10.7	8.2	4.9	5.2	32.1	36.7	1.1	0.7	7.1	2.0
3	22	3–10	5.5	5.3	7.9	13.9	4.5	4.6	26.7	22.7	1.3	1.0	15.8	5.6
4	17	8–14	6.3	6.7	41.4	42.4	5.6	5.7	51.7	48.5	2.4	2.3	24.1	18.2

Notes: Mean total IgG (T-IgG), IgG2 and IgG1 whole cell ELISA titers were measured per cohort (C), split for low-risk tick-season (LR: Q4 and Q1) and high-risk tick-season (HR: Q2 and Q3) and the percentages of exceptional high titers at LR and HR are also shown. For calculation of the mean of the IgG1 whole cell ELISA, the titer result read from the assay as < 1 was set at 0.2.
Abbreviations: C, cohort; *n*, number of dogs; T-IgG, total IgG.

Table 4
Mixed-effects model for total-IgG (T-IgG), IgG2 and IgG1 titers in successive tick seasons 1–12 with high-risk (HR) and low-risk (LR) half-years for cohorts 1–4. Analysis included seasonal data for all dogs, including Bernese Mountain dogs ($n = 84$; samples $n = 328$).

Dependent variable	Predicting variable	Predicted mean titers			*F*-value	*P*-value
		HR	LR	Difference		
T-IgG	Tick-season				$F_{(11,\ 103)} = 3.667$	0.0002***
	Risk of tick-bite	5.834	5.620	0.214	$F_{(1,\ 156)} = 0.791$	0.3753
	Tick-season × Risk				$F_{(11,\ 103)} = 1.373$	0.1966
IgG2	Tick-season				$F_{(11,\ 103)} = 1.960$	0.0400*
	Risk of tick-bite	5.017	5.076	−0.058	$F_{(1,\ 156)} = 0.072$	0.7893
	Tick-season × Risk				$F_{(11,\ 103)} = 0.551$	0.8638
IgG1	Tick-season				$F_{(11,\ 103)} = 3.153$	0.0010**
	Risk of tick-bite	1.133	1.545	−0.412	$F_{(1,\ 156)} = 4.374$	0.0381*
	Tick-season × Risk				$F_{(11,\ 103)} = 1.187$	0.3950

Note: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 5
Mixed-effects model for IgG1 titers in the successive tick seasons 1–12 and 1–6, with Bernese Mountain dogs included.

Tick-season	Predicting variable	Predicted mean titers			*F*-value	*P*-value
		HR	LR	Difference		
1–12	Tick-season				$F_{(10,\ 107)} = 5.0$	<0.0001****
	Risk of tick-bite	1.172	1.585	−0.413	$F_{(1,\ 153)} = 5.7$	0.0186*
1–6	Tick-season				$F_{(4,\ 71)} = 2.4$	0.0598
	Risk of tick-bite	0.777	1.396	−0.619	$F_{(1,\ 92)} = 8.0$	0.0059**

Notes: Tick-seasons 1–12: cohorts 1–4 ($n = 84$ dogs); tick-seasons 1–6: cohorts 1–3 ($n = 54$ dogs). *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

associated with IgG ≥ 8 and IgG1 ≥ 2 meaning that these high antibody titers were significantly less common in dogs where an alternative diagnosis was established. Instead, MFLd-b was best predicted by antibiotic use and comorbidities and not by tick infestation intensity, further supporting the specificity of the IgG1-dominated immune responses in presumed CLD cases. Collectively, these findings indicate that elevated total IgG, IgG1 titers and IgG1/IgG2 ratios, alongside seroconversion and seasonal tick exposure, are strongly associated with transient fever and lameness episodes suggestive of CLD.

3.4. Results for different breeds, and of the C6 antigen test

The incidence of presumptive CLD varied significantly between breeds (Table 8). The highest incidence was observed in Labrador Retrievers (6 out of 9; 67%) and BMD (4 out of 7; 57%). Both breeds belong to genetic cluster 4, which includes breeds genetically most distant from the wolf (Parker et al., 2004). Other affected breeds included Irish Terriers, Dachshunds, and Huskies, all of which experienced very high tick exposures. Dogs in genetic cluster 4 had a significantly higher risk of developing CLD compared to those in clusters 1–3 (Fisher's exact test, $P = 0.0004$, OR = 7.3). Interestingly, among retrievers, Labrador

Table 6
Mixed-effects model for IgG1 titers in the successive tick seasons 1–12 (cohorts 1–4), 1–6 (cohorts 1–3), with Bernese Mountain dogs excluded.

Tick-seasons	Predicting variable	Predicted mean titers			*F*-value	*P*-value
		HR	LR	Difference		
1–12	Tick-season				$F_{(1,\ 111)} = 6.6$	<0.0001****
	Risk of tick-bite	1.049	1.337	−0.288	$F_{(1,\ 148)} = 3.9$	0.0489*
1–6	Tick-season				$F_{(5,\ 78)} = 0.6$	0.7186
	Risk of tick-bite	0.568	1.007	−0.439	$F_{(1,\ 92)} = 7.4$	0.0078**

Notes: Tick-seasons 1–12: cohorts 1–4 (n dogs = 80); tick seasons 1–6: cohorts 1–3 (n dogs = 54). *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

Retrievers were far more frequently affected than Golden Retrievers, despite similar environmental exposures (Fisher's exact test, $P = 0.0029$, OR = 30). Symptomatic Labrador Retrievers showed high total IgG (≥ 8) and IgG1 (≥ 2) titers, especially after the third or fourth tick-season, while non-symptomatic Golden Retrievers typically showed lower total IgG but elevated IgG1 titers.

In the separate BMD cohort (n = 31), fever and lameness occurred more often together than expected by chance (Fisher's exact test, P = 0.0302, OR = 3.5), see Supplementary file 4. In these cases, the symptoms were significantly associated with high IgG2 (≥ 7) and IgG1 (≥ 3) titers (Fisher's exact test, $P = 0.0044$, OR = 15.4). Additionally, 18 of 31 BMD tested positive for C6 antibodies, which were strongly linked to the combination of elevated IgG2 and IgG1 titers (IgG2 ≥ 7 & IgG1 ≥ 3) and the ratio of those titers (IgG1/IgG2 ≥ 0.4) (Fisher's exact test, both $P <$ 0.0001, OR = 8.2) and to high bactericidal activity against *B. afzelii* (Fisher's exact test, P = 0.0003, OR = 26.4). Despite these strong antibody responses, *Borrelia* DNA was not detected in any of the 100 tissue samples tested from 16 BMD, including 5 dogs with concurrent fever and lameness (FLb). This contrasts with other symptomatic breeds in the prospective cohort, where *Borrelia* DNA was detected in tissue samples (as previously described; also see Supplementary file 1: Table S4 and Supplementary file 4). Interestingly, histopathology in symptomatic BMD revealed inflammatory changes, such as reactive hepatitis and membranoproliferative glomerulonephritis, compatible with CLD. Moreover, in two lame BMD, villous synovitis with periarteritis and perineuritis was observed, which is considered pathognomonic for CLD (Summers et al., 2005). Additionally, the strong C6 antibody response in BMD - despite the absence of detectable *Borrelia* DNA in tissues - may suggest that BMD may clear infection efficiently, while persistent immune activation could contribute to inflammatory organ damage.

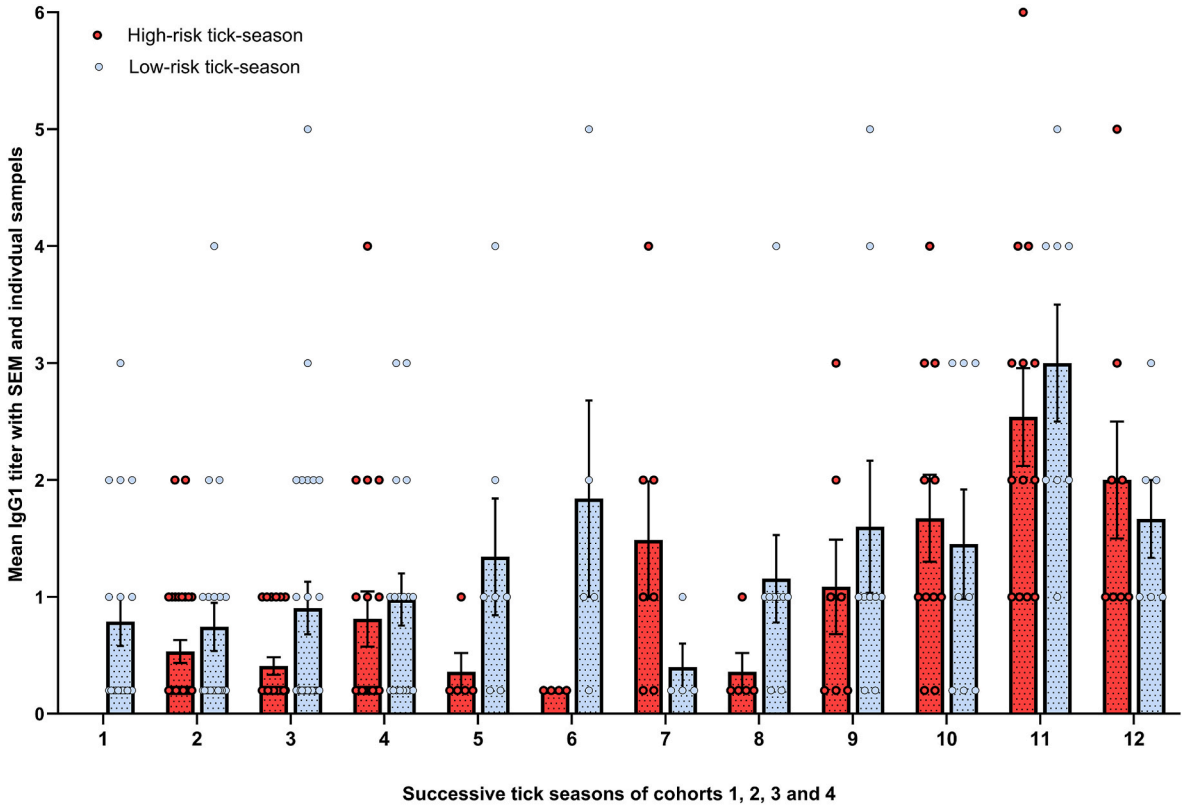

Fig. 4. Mean IgG1 whole cell ELISA titers (bars) with standard error of the mean (SEM). For the cohort dogs during 12 low-risk (LR) and high-risk (HR) tick-seasons, not included the 4 BMD. Presented as tick-seasons 1–12 (cohorts 1–4, n = 80 dogs) and tick-seasons 1–6 (cohorts 1–3, n = 54 dogs). The last tick-season number 12 is a combination of samples from the older dogs that had experienced more than 12 tick-seasons in 1994 for which tick-seasons were grouped together as the 12th season. The tick-seasons are split for high-risk (Q2 and Q3) and low-risk (Q4, and Q1 of the following year) for tick infestations. All data are measured, imputed data are not presented in this graph. The individual data are displayed with the appropriate bar of the mean IgG1 ELISA titers. The range of exceptionally high values at low-risk tick-seasons (light blue colored dots) appears wider than at high-risk seasons (red colored dots) resulting in higher means for LR with larger standard deviations, and SEM at the first 6 tick-seasons. The first LR tick-season is shown in the graph but not incorporated in the statistics (mixed-effect model, Table 6).

Table 7
Models generated with generalized estimating equations (GEE) with predicting variables of 7 successive half-year measurements per dog (total $n = 588$), predicting the response variables. Data from Supplementary file 3.

GEE model/Cohorts (*n*)			Cohorts:	1, 2	1–3	1–4	1, 2	1–3	1–4	1, 2	1–3	1–4
1, 2	1–3	1–4	Response variable	FLb			MFLb			MFLd-b		
			Response variable (*n*)	5	9	14	20	31	51	40	61	84
			Total half-years (*n*)	315	469	588	315	469	588	315	469	588
Immunological												
16	26	54	T-IgG ≥ 8	–	–	–	<0.001	<0.001	–	–	–	–
31	48	147	IgG1 ≥ 2	–	0.014	–	–	–	–	–	–	–
19	33	117	IgG1/IgG2 ≥ 0.4	0.003	–	–	0.004	0.033	–	–	–	–
6	13	36	IgG ≥ 8 & IgG1 ≥ 2	–	–	0.027	–	–	<0.001	–	0.144[a]	0.014 [a]
Seasonal												
56	85	98	Tick infestation high	–	–	–	0.007	0.003	0.030	–	–	–
87	130	173	Seroconversion	–	0.012	0.015	–	–	–	–	–	–
Clinical												
25	46	70	Comorbidity	–	–	–	–	–	0.005	–	0.036	0.046
33	49	77	Antibiotic	–	–	<0.001	<0.001	<0.001	0.010	<0.001	<0.001	<0.001

Notes: Response variables FLb and MFLb (fever and lameness or malaise with or without fever and lameness as symptoms of CLD) and MFLd-b (symptoms not representing CLD) are predicted by one or more immunological, seasonal, and clinical predicting variables. Variables figure together in the model only when statistically significant represented ($P < 0.05$). A variable with a non-significant prediction for the response variable in a combination of cohorts is indicated by "–".
[a] Negative predicting variable for the response variable.

Table 8
Total dogs per breed group studied and frequencies with presumptive CLD.

Cluster no.	Cluster name	Breed group	*N*	Symptomatic breeds	*n* (%)
1	Asian/Ancient	Pole dogs	2	Husky	2 (100)
2 & 3	Asian/Ancient	Toy dogs & cross toy	16	none	0 (0)
2 & 3	Herding	Sight and herding dogs	5	Bouvier de Flandres	1 (20)
3	Hunting	Spaniels and large hounds	12	Friesian Hound	1 (8)
3	Hunting	Golden Retrievers & cross	16	Golden Retriever	1 (6)
3 & 4	Hunting	Terrier	10	Irish and Scottish Terrier	3 (30)
3 & 4	Hunting/Working	Scent Hounds	4	Dachshund	1 (25)
4	Hunting/Working	Labrador Retrievers & cross	9	Labrador & cross	6 (67)
4	Working	Working dogs	3	None	0 (0)
4	Working	Mastiffs and Mountain dogs	7	Bernese- and Tatra Mountain Dog	4 (57)
Total			84		19 (23)

Notes: Cluster numbers 1 to 4 for "genetic clusters" are given as described by Parker et al. (2004); cluster names and breed groups follow Parker et al. (2017).
Abbreviations: *N*, number of dogs studied; *n*, number of symptomatic dogs.

4. Discussion

4.1. Synopsis

This prospective cohort study, spanning 3.5 years and including 84 dogs across different age groups, provides strong evidence that naturally occurring *B. burgdorferi* (*s.l.*) infections in dogs can lead to transient episodes of fever and lameness (FLb) or malaise and lameness (MFLb), particularly after multiple tick-seasons. These clinical episodes were consistently associated with high total-IgG titers, a strong IgG1 response, and recent seroconversion. The seasonal patterns observed - with IgG1 titers peaking in the low-risk tick-season (LR) following periods of high tick exposure - suggest that the development of a Th2-polarized immune response plays a role in diminishing infection and resolving symptoms. These findings indicate that the immunological response to *Borrelia* infection in dogs is dynamic, breed-dependent, and shaped by cumulative tick exposure, emphasizing the complex interplay between infection, immunity, and clinical outcomes in naturally exposed dogs.

4.2. Immune responses and symptom resolution

Our data indicate that the balance between Th1-type and Th2-type immune responses plays a key role in determining clinical outcomes following *Borrelia* infection in dogs. Dogs with transient fever and lameness (FLb) typically showed a strong initial total-IgG and IgG2 response, indicative of a Th1-dominated response during early infection. However, in the subsequent low-risk tick-season (LR), these dogs developed elevated IgG1 titers, consistent with a Th2-shifted response. This pattern closely parallels findings in mice and humans, where a dominant Th1 response is linked to high bacterial loads, tissue inflammation, and symptoms, while a shift toward Th2 is associated with pathogen clearance and convalescence (Olsson et al., 1987; Hechemy et al., 1988; Seppala et al., 1994; Keane-Myers and Nickell, 1995; Aberer et al., 1997; Kang et al., 1997; Anguita et al., 1998; Brown and Reiner, 1999). The model is further supported by the case of the experimentally infected Beagle pup, which failed to mount a Th2-associated IgG1 response and was shown to have persistent infection with detectable *Borrelia* DNA and intermittent symptoms (Appel et al., 1993). It also aligns with our observation that symptomatic dogs with detectable *Borrelia* DNA in tissues typically lacked a robust IgG1 response, further supporting the protective role of Th2 immunity in naturally infected dogs (Hovius et al., 1999b).

4.3. Breed-related differences

Importantly, the incidence of presumed CLD varied significantly between breeds, with Labrador Retrievers and BMD showing the highest susceptibility, although their immune responses followed different trajectories. It has been suggested that differences in the predispositions of BMD, Beagles and Retrievers to CLD and other diseases, are caused by the immunological properties of these breeds, as compared to other breeds (Dambach et al., 1997; Gerber et al., 2007, 2009a, b; Barth et al.,

2012; Baum et al., 2014; Contreras E and Lappin, 2016). In laboratory experiments, 6-week-old Beagle puppies react to tick-induced *Borrelia* infection with fever, pathology and a persistent infection (Appel et al., 1993; Chang et al., 1996; Straubinger, 1999; Summers et al., 2005). However, adult Beagles do not develop persistence of infection and are affected with mild pathological lesions only when repeatedly infected by numerous tick bites (Evans et al., 1995a, 1995b). This latter mode of infection may actually be comparable to the discontinuous and repetitive seasonal infections as observed in our cohort dogs. BMD followed a distinct immunological trajectory, characterized by exuberant and persistent IgG1 and IgG2 responses, combined with strong C6 seroconversions. Despite these strong C6 responses, *Borrelia* DNA could not be detected in their tissues, suggesting that infection is efficiently cleared. However, the persistent immune activation seen in BMD, including immune complex formation, could explain the immune-mediated pathology observed in some of these dogs, such as glomerulonephritis and reactive hepatitis, villous synovitis with periarteritis and perineuritis (Hovius et al., 1999b; Gerber et al., 2009b; Pantchev et al., 2015).

5. Conclusions

Together, our findings highlight that the clinical and immunological outcomes of *Borrelia* infection in dogs are shaped by the dynamic balance between Th1 and Th2 responses, the timing and magnitude of IgG1 induction, and breed-specific immunogenetic factors. Our prospective study provides strong suggestions for 19 transient cases of CLD in 84 dogs (22.6%), based on monitoring the development of predefined symptoms and immunological, seasonal and clinical predictive variables over 588 cumulative half-years, indicating an incidence of 3.2%. Considering only the 12 cases with fever and stifle lameness in the same quarter-year (FLb), the incidence would be 2.0%. As the immunological predictive variables were high total-IgG and IgG1 *B. burgdorferi* (*s.s.*) whole cell ELISA titers, it may be tempting to use extreme high total IgG titers as an indication for the diagnosis, in line with the first reports of CLD in the USA (Lissman et al., 1984; Kornblatt et al., 1985). However, our data suggest that at least two serological samples should be drawn, preferably one in the high-risk and the second in the low-risk tick-bite season for serological confirmation of the observed transient symptoms with total-IgG, IgG2 and IgG1 titers. Our findings also indicate that while the majority of dogs can successfully prevent symptomatic *Borrelia* infections across multiple seasons, a small subset may exhibit transient symptoms after repeated seasonal tick-bite-induced exposures, in which eventually the Th2 immune responses could counteract the infection.

Ethical approval

In the prospective cohort study conducted from 1990 to 1994 at the veterinary clinic of the first author (KEH), dogs from each age cohort were enrolled randomly. In line with the standards of animal welfare and ethical research practices, the enrollment in the cohort study was contingent upon the owners providing written informed consent, agreeing to at least bi-annual serum sampling (jugular vein) and quarterly clinical examinations, with approval of the Veterinary Medical Diagnostic Center, Utrecht. Enrollment, sampling and examinations of dogs not part of the cohort study (Bernese Mountain Dogs) were performed as part of routine clinical practice and thus not requiring approval from a licensing committee, although with informed consent of the owners. We confirm that all methods employed in this study were carried out in accordance with the relevant guidelines and regulations at the time. Although the dogs were enrolled in the study before publication of the ARRIVE guidelines, where applicable we have used methods and procedures reported in those guidelines, ensuring that our data are reported in a thorough and transparent manner and safeguarding the reproducibility and ethical integrity of the work.

CRediT authorship contribution statement

K. Emil Hovius: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Project administration. **Tess van den Bergen:** Investigation, Formal analysis, Visualization. **Osama Almalik:** Methodology, Software, Formal analysis. **Ellen Versmissen:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Victor P. Rutten:** Writing – review & editing, Supervision, **Hein Sprong:** Validation, Investigation, Resources, Writing – review & editing, Supervision. **Joppe W. Hovius:** Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision.

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Declaration of competing interests

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.

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Appendix. A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2025.100258.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary files.

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