Host-pathogen interactions in Lyme disease and their application in diagnostics

Nathalie D. van Burgel

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Stellingen behorende bij het proefschrift

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1. *B. burgdorferi* sl heeft van het eiwit complement regulatory acquired surface protein (CRASP)-1 meerdere paralogen gelegen op het lp54 plasmide. Deze paralogen zijn in staat om factor H van verschillende dieren te binden om zo vorming van het C5b-C9 complex op het celmembraan tegen te gaan. *Dit proefschrift*
2. Het ontwijken van het complementsysteem door middel van expressie van CRASP1-eiwitten is een van de vele factoren die *B. burgdorferi* sl kan gebruiken om een infectie in de gastheer te bewerkstelligen. Voor het bewerkstellingen van een infectie is het echter niet noodzakelijk om deze genen te bezitten. *Dit proefschrift*
3. Het bepalen van anti-C6 peptide immuunglobuline is een voldoende goede screeningsmethode voor detectie van neuroborreliose in de liquor van patiënten met symptomen van neuroborreliose. *Dit proefschrift*
4. Het meten van de hoogte van het chemokine CXCL13 in patiënten met neurologische afwijkingen heeft een sterke aanvullende waarde in de diagnostiek naar Lyme neuroborreliose. Voor het stellen van de diagnose dient CXCL13 echter niet solitair gebruikt te worden, maar een onafhankelijke techniek dient daar aan toegevoegd te worden. *Dit proefschrift*
5. Media geven de voorkeur aan enkele getuigenissen van het lijden van enkele personen met mogelijk persisterende Lyme boven het interpreteren van wetenschappelijke data. *N Engl J Med. 2007 Oct 4; 375(14):1422-1430*
6. Als de snelheid waarmee taxonomie binnen de microbiologie wordt aangepast een directe afspiegeling zou zijn van de toename van erkende relevante pathogenen dan moeten we ons serieus zorgen maken over hoe we de juiste behandeling in de literatuur gaan terugvinden.
7. Infectieziekten hebben een veel grotere schijnbare R0 dan de werkelijk meetbare R0 wanneer ze de kans krijgen zich te verspreiden via glasvezel.
8. Voorheen, toen religie sterk was en wetenschap zwak, dachten mensen dat magie als geneeskunst kon dienen; nu wetenschap sterk is en religie zwak, denken mensen dat geneeskunde magie is. *Thomas Szasz, The second sin, 1974.*
9. Een vergissing in de behandeling van ziektes is dat er een dokter is voor het lichaam en een dokter voor de geest. Bij gebrek aan oog voor een van beide zal het geheel niet beter kunnen worden. *Plato, Charmides, 380 BC.*
10. Ook al zou het leven niets anders zijn dan een droom en de tastbare wereld niets meer dan een geest, dan zal ik deze droom of geest reëel noemen als ik door middel van reden ze nooit zou kunnen falsificeren. *Gottfried Leibniz 1646-1716 (Quoted in The World of Mathematics, JR Newman 1956)*
11. Oog om oog maakt de wereld blind. *Mahatma Gandhi, 1943*
12. Iets lijkt altijd onmogelijk, totdat het gedaan is. *Nelson Mandela.*

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N.D. van Burgel

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Proefschrift

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M Stolker,
volgens het besluit van het College voor Promoties
te verdedigen op woensdag 29 mei 2013
klokke 15.00 uur

door

Nathalie Daniëlle van Burgel

geboren te Gorinchem
in 1979

Our death is not an end if we can live on in our children and the younger generation. For they are us, our bodies are only wilted leaves on the tree of life.

-Albert Einstein, 1926-
Letter to the widow of Heike Kamerlingh-Onnes

Voor mijn moeder

romotiecommissie:

Promotor:	Prof Dr. A.C.M. Kroes
Copromotor:	Dr. A.P. van Dam (OLVG, Amsterdam)
Overige leden:	Prof Dr. P.J. van den Broek Prof Dr. R.A.C. Roos Prof Dr. T.W.J. Huizinga Prof Dr. P. Kraiczy (Johann Wolfgang Goethe-Universität, Frankfurt am Main)

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Chapter 1

General introduction and outline

1.1 History of Lyme disease

The causative agent of Lyme disease, *Borrelia burgdorferi*, was first cultured from ticks and described in 1982 by Willy Burgdorfer et al.[1]. By then many clinicians had already hypothesized a relation between Lyme disease and the tick and many of the clinical presentations had already been described.

Perhaps the earliest work describing Lyme disease is from the reverend John Walker describing a vector-borne illness much like Lyme disease in 1764 that crippled the community of Jura, a Scottish island [2]. More evident reports are from the late 19th century when the physician Alfred Buchwald from Poland described a case of a patient suffering for 16 years from a degenerative skin disorder [3]. This disease is now called acrodermatitis chronica atrophicans (ACA). In 1896 Nikulin, from Moscow, reported the first case of ACA in a child [4]. In 1910 the Swedish dermatologist Arvid Afzelius described the first case of a ring like lesion which he called an erythema migrans (EM). He was the first person to associate this lesion with a recent bite from the European sheep tick (*Ixodes ricinus*) [5]. In 1911 the Swiss pathologist Jean Louis Burckhardt described the first case of what is now known as a borrelia lymphocytoma [6].
Neurological syndromes associated with the EM were described as early as the year 1922 by the French physicians Charles Garin and Charles Bujadoux describing a farmer with a radiculitis after a tick bite and an EM [7]. The association with a tick bite and EM was not recognized until the association was proposed in 1930 by the Swedish dermatologist Sven Hellerström [8]. In 1941 the German neurologist Alfred Bannwarth described several cases of chronic lymphocytic meningitis and painful polyradiculoneuritis [9]. The tick-borne meningopolyneuritis became known as Garin-Bujadoux-Bannwarth syndrome.

The first reports of effective treatment of these clinical syndromes by the use of penicillin date from 1949 [10, 11]. The effective treatment of the syndromes with penicillin prompted scientists to try to culture bacteria in order to fulfill Koch's postulates in which they continuously failed. For many years European doctors described the different syndromes in more detail but the actual causative agent was not isolated [12, 13].

The first report of an EM in North America dates from 1970 [14]. Soon after that, in 1975 in the towns Lyme and Old Lyme, a remarkable cluster of juvenile rheumatoid arthritis associated with an EM was identified. The disease was epidemiologically associated with tick bites and named Lyme disease [15]. The clinical manifestation of Lyme carditis was first described in 1980 [16]. The first

placebo controlled trials for treatment with antibiotics were performed shortly after the discovery of the clusters [17-19]. It was in 1982 that the Lyme disease agent, *B. burgdorferi* sensu lato (sl) was isolated from *Ixodes scapularis* ticks [20]. In that same year *B. burgdorferi* was isolated from the blood of several Lyme disease patients [21, 22]. In 1983 the first report of cultured spirochetes from Swiss *I. ricinus* ticks was published [23]. The difference in clinical presentation between the North American and European patients was soon clear. Since then much research has been done on the pathogenesis, epidemiology, diagnostics and treatment of Lyme disease.

1.2 B. burgdorferi transmission and pathogenesis

Microbiology

B. burgdorferi is a spirally shaped gram negative, microaerophilic bacterium that belongs to the family of the Spirochaetaceae. The genus *Borrelia* consists of several species among which vector transmitted relapsing fever *Borrelia* and the tick transmitted *B. burgdorferi* sl. The *B. burgdorferi* sl complex consists of at least 17 genospecies, among which B. *burgdorferi* sensu stricto (ss), *B. afzelii*, *B. bavariensis*, *B. garinii* and *B. spielmanii* are known to cause disease in humans. Possibly *B. valaisiana*, *B. lusitaniae* and *B. bissettii* can also be pathogenic [24]. Other genospecies in this group are *B. andersonii, B. japonica, B. turdi, B. tanukii,* and *B. lonestari* [25].

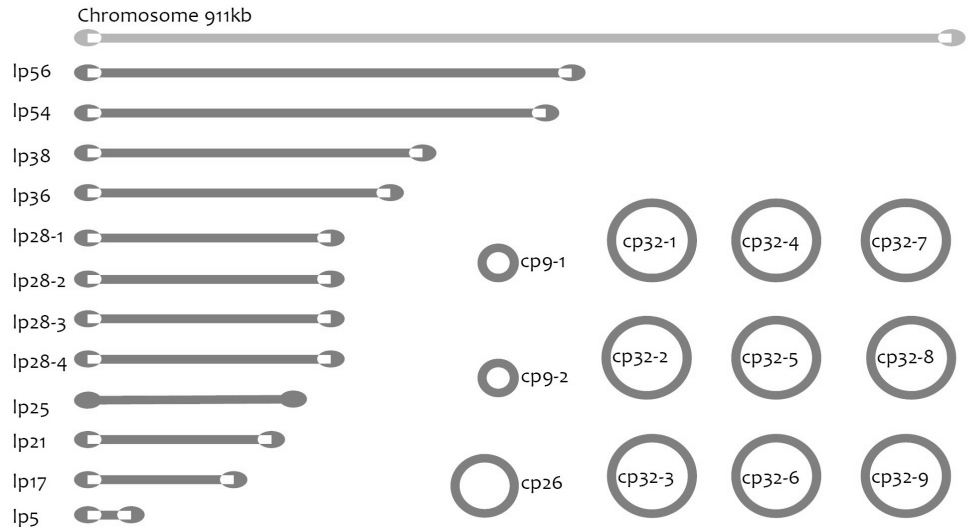

Figure 1: The genome of reference strain *B. burgdorferi* ss B31

The genome of *B. burgdorferi* sl has unique features. Primarily it has the largest number of plasmids known for any bacterium. An example of reference strain *B. burgdorferi* ss B31 is shown in figure 1. *B. burgdorferi* sl has a chromosome of about a million base pairs and is estimated to contain 853 genes that encode a basic set of proteins. The genome further consists of a large number of linear and circular plasmids with a combined size of more than 533,000 base pairs, containing approximately 926 genes [26-30]. The plasmids have approximately one copy number per chromosome [31]. The plasmids are unusual in that they contain many paralogous sequences and a large number of pseudogenes [30]. Some of the plasmids contain essential outermembrane proteins necessary for infectivity and immune evasion in the vertebrate and arthropod host [32]. In addition, a number of the cp32 plasmids have features suggesting that they are prophages, which can facilitate exchange of genes [33].

The vector lifecycle

B. burgdorferi sl is transmitted to the mammalian host by infected ticks. Ticks belong to the phylum of the arthropoda and the class of arachnida. Ticks are classified as hard-bodied or soft-bodied ticks. Several hard body ticks can carry the different Borrelia species and they belong to the *Ixodes ricinus-persulcatus* complex, including *I. scapularis* (deer tick) in the Northeastern and Midwestern United States, *I. pacificus* in the Western United States, *I. ricinus* (sheep tick) in Europe, and *I. persulcatus* in Asia [34]. In North America the only pathogenic species found is *B. burgdorferi* ss, while the biodiversity of pathogenic species in Eurasia is much larger [35].

Hard-bodied ticks have three distinct stages, for each stage only one blood meal is taken to molt into the next stage (figure 2). From the eggs larvae emerge, they usually obtain their blood meal from small rodents or birds. Typically a blood meal takes 4-7 days until the ticks drop off. After obtaining a blood meal they molt to the nymphal stage. Nymphs usually feed on medium to large sized vertebrate hosts and molt to the next and final stage - the adult. After feeding once more on a medium to large sized vertebrate host the adult female hard ticks lay one batch of thousands of eggs and then die. In central Europe nymphal ticks usually feed during spring and early summer, larval ticks later in spring and early autumn (figure 3). Activity in the summer is slightly lower and completely absent in winter, but this is very much depending on the local climate [35]. Many hard bodied ticks can live for several months without feeding and the entire lifecycle can range from 2 to 6 years [36].

Transovarial transmission of *B. burgdorferi* sl does not occur, so the larva stage is assumed to be *B. burgdorferi* sl free [37]. When the primary vertebrate host is infected with *B. burgdorferi* sl the larvae can become infected.

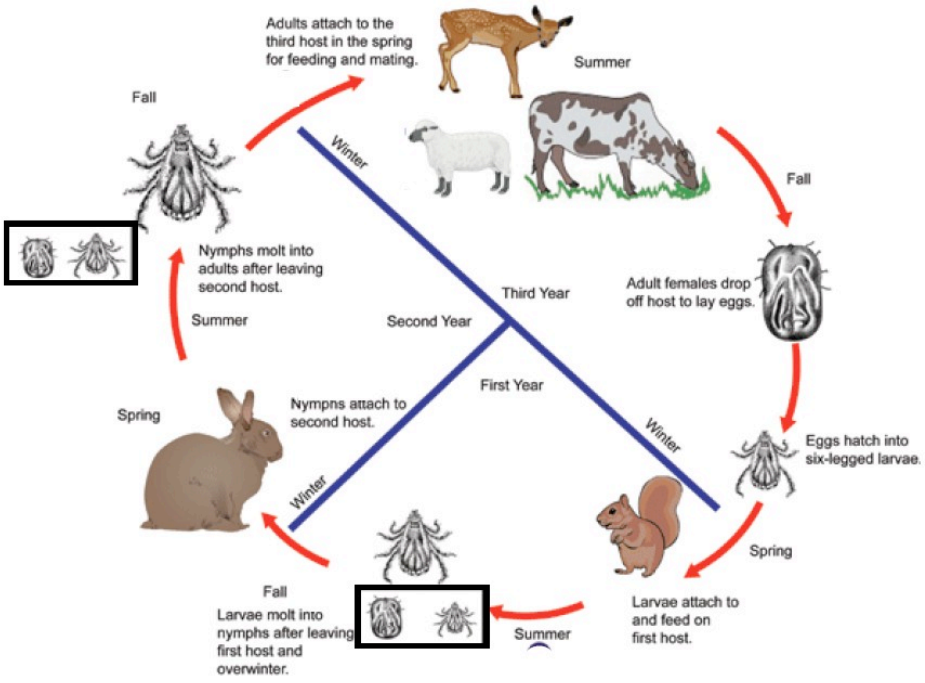

Figure 2: Tick lifecycle, adapted from www.cdc.gov

Pathogenesis

When the tick is feeding it acquires *B. burgdorferi* from the blood of the infected vertebrate host. Spirochetes are transferred from the vertebrate host to the tick and start to express high levels of outer surface protein A and B (OspA/B) which enable the spirochete to attach in the tick gut [38-40]. In between blood meals the spirochetes reside in the tick midgut. Over a period of months the tick molts into the next stadium. During the next feeding, the temperature of the tick rises and the pH in the midgut drops. This is a signal for the bacteria to migrate to the salivary glands. This is achieved by downregulating OspA/B and upregulate many proteins among which OspC [41-43]. Tick saliva already contains substances that can suppress the host immune response [44]. In the salivary gland OspC can bind to tick salivary protein (SALP) 15 which protects the spirochete against the host immune system [45-48]. During feeding the tick inoculates the spirochete in the blood of the host. After inoculation by the tick

the spirochetes give a local infection after which they disseminate. During infection spirochetes can be found throughout the infected host. However during prolonged infection the spirochetes can reside in preferred locations, for example joints or the central nervous system. It is generally presumed that *B. burgdorferi* are extracellular microorganisms, mainly located in the extracellular matrix.

On the membrane of the spirochete many proteins are expressed that are of importance for pathogenesis in the vertebrate host. Many proteins, of which some essential for effective transmission, are involved in binding host extracellular proteins like decorin (DbpA an DbpB), fibronectin (RevA and BBK32) and laminin (ErpX and BmpA-D) [49-53].

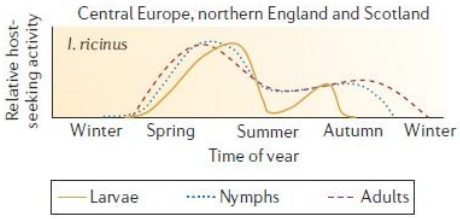

Figure 3: Seasonal activity of tick feeding in Europe. Adapted from Kurtenbach et al [35]

Immune evasion

Other proteins that are expressed at the surface of the spirochete are responsible for immune evasion later in infection. The best studied protein is the lipoprotein VlsE [54, 55]. The *vls* locus consists of 15 silent vls cassettes and the VlsE lipoprotein. VlsE consists of six variable and six invariable regions (IR) [56]. By applying unidirectional recombination events VlsE can display antigenic variation. This recombinational variation can potentially derive millions of antigenic variants and complicates detection by the immune system which facilitates persistent infection of the mammalian host [57].

Borrelia can also bind to tick salivary proteins by OspC which can inactivate the mammalian host immune system like tick salivary protein (SALP) 15 [46]. Immediately after transmission many of the immunogenic outer surface proteins are downregulated, among which OspA/B (see figure 4) [58].

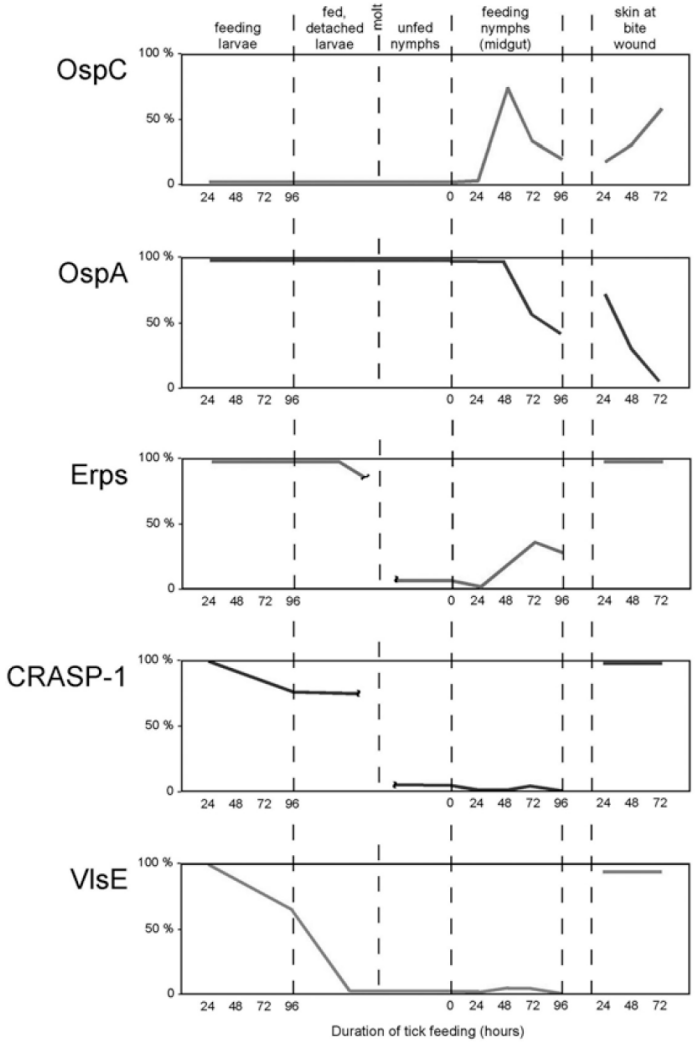

Figure 4: *B. burgdorferi* sl gene regulation at different time points in tick and host infection. Adapted from Stevenson et al. [58].

1.3 B. burgdorferi complement evasion

Complement is one of the first lines of defense against entering bacteria in the mammalian host. *B. burgdorferi* is primarily an extracellular pathogen [59]. When a bacterium enters the mammalian host, three pathways can be activated; the alternative pathway (AP), the lectin pathway (LP) or the classical pathway (CP). The CP is activated by antibodies binding to the membrane of the pathogen. The LP is activated by binding of mannose binding lectin (MBL) to carbohydrates of the pathogen. The convertase for both these pathways

(C4bC2b) is composed of C4b and C2b. The alternative pathway is activated constantly and the convertase of the alternative pathway (C3bBb) is formed by C3b and factor Bb. Both C4bC2b and C3bBb cleave C3 to C3a and C3b. If activation progresses C3b is deposited on the nearest membrane. On the membrane of mammalian host cells deposition is prevented or C3b is inactivated by soluble regulators. If activation progresses C3b binds to the C3 convertases which generates the C5 convertase (C4bC2bC3b or C3bBbC3b respectively). By this convertase C5 is cleaved in C5a and C5b, the latter can activate the membrane attack complex (MAC) which consists of C6, C7, C8 and multiple C9 components. The assembly of the MAC leads to pore formation and cell lysis [60].

Borrelia can evade activation of the MAC. However, the extent to which spirochetes can resist complement differs markedly between the several *B. burgdorferi* sl species; *B. afzelii*, *B. burgdorferi* and *B. bavariensis* are relatively resistant to human serum, while *B. garinii* and *B. valaisiana* strains are usually sensitive. However in sera from other mammalian and avian species this resistance profile can be completely different [61-65].

Early this century, it was discovered that borrelia can inactivate human complement by binding two host derived fluid phase regulators of complement; factor H (CFH) and factor H-like protein 1 (FHL-1), also known as reconectin [66-68]. CFH and FHL-1, the main immune regulators of the alternative pathway of complement activation, are structurally related proteins composed of several protein domains termed short consensus repeats (SCRs) [60]. CFH is a 150-kDa glycoprotein composed of 20 SCR domains. In contrast, FHL-1 is a 42-kDa glycoprotein corresponding to a product of an alternatively spliced transcript of the *CFH* gene and consists of seven SCRs and is present only in human serum in ten to 50 times lower concentrations than CFH [69]. The seven N-terminally located SCRs of both complement regulators are identical with the exception of four additional amino acids at the C-terminus of FHL-1 [70]. CFH and FHL-1 in the human host are responsible for preventing binding of factor B to C3b, supporting the dissociation of the C3bBb complex and acting as a cofactor for factor I-mediated cleavage of C3b, the central component of all three complement activation pathways [70-73] (see figure 5)

Serum resistant borreliae acquire CFH, FHL-1 and/or complement factor H-related proteins (CFHR) by direct interaction with outer surface proteins designated CRASPs (Complement Regulator-Acquiring Surface Proteins) [74]. Previously, five different CRASPs have been described for *B. burgdorferi ss* and *B. afzelii*. No CRASP has been described for the also complement resistant *B.*

bavariensis. The CFH and FHL-1 binding CspA protein, also designated CRASP-1, is encoded by *cspA*, a gene located on the lp54 plasmid. Although the lp 54 plasmid of *B. burgdorferi* and *B. afzelii* carries multiple genes encoding a number of paralogous proteins, only the CspA is capable of binding human CFH and FHL-1 [75-77]. CspA can also bind other proteins like plasminogen and different other extracellular matrix proteins [78]. CspA is upregulated by spirochetes during the tick-mammalian transmission stage and downregulated during persistent infection [79, 80]. Antibodies to CspA could be detected in sera from infected mice and from Lyme disease patients suggesting a potential prolonged expression of CspA in the mammalian host [81-83]. Previously only CspA from complement resistant *B. burgdorferi* ss and *B. afzelii* has been described, but not a CspA from the human complement resistant species *B. bavariensis*.

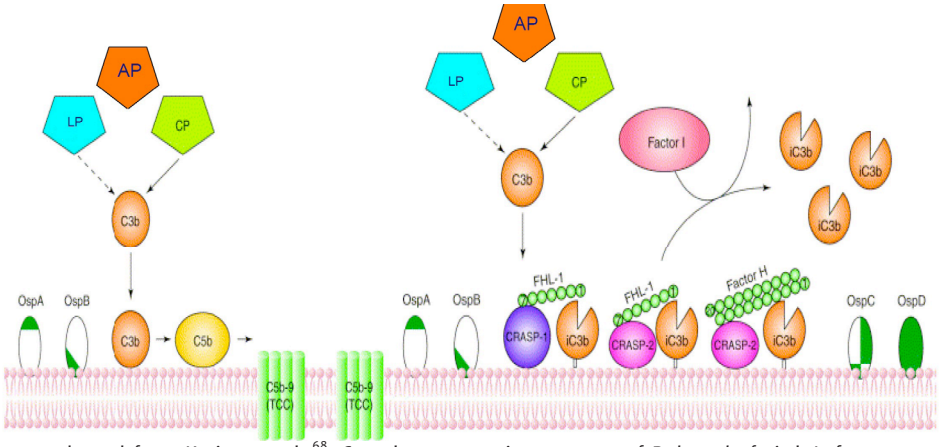

Figure 5: adapted from Kraiczy et al. [68]. Complement evasion strategy of *B. burgdorferi* sl. Left: a normal situation of activation of complement with the eventual deposition of C3b on the membrane and assembly of the membrane attack complex (C5b-C9). Right: By expressing FHL-1 and factor H binding proteins on the membrane C3b is inactivated (iC3b) and the membrane attack complex is not formed. (LP: lectin pathway, AP: alternative pathway, CP: classical pathway)

Another protein that can bind human CFH and FHL-1, CspZ, also designated CRASP-2, is a distinct protein encoded by the *cspZ* gene located on plasmid lp28-3 and is expressed at higher levels during the mammalian infection than in bacteria residing in ticks or during laboratory cultivation [79]. Anti-CspZ antibodies can be detected as early as two weeks post infection in mice infected by ticks [84]. CspZ has been shown to bind other yet unknown proteins and therefore can have multiple functions [63, 80, 84, 85]. However, the protein is not essential to cause effective transmission to, and infection of, the mammalian host [86]. The CFH-binding CRASP proteins BbCRASP-3, -4, and -5 belong to the OspE-related proteins (Erp) paralogous family and their respective genes are located on different cp32 circular plasmids [87]. Erp proteins are expressed in

tissues in the host during disseminated mammalian infection. Erp proteins have also been shown to be able to bind to CFHR proteins and plasminogen [53, 88-97].
In a mouse model where CFH deficient mice were infected with *B. burgdorferi* ss there were no major effects of the infectivity of *B. burgdorferi* ss injected in factor H deficient mice concluding that mice lacking factor H were as efficiently infected by *B. burgdorferi* as WT mice [98]. Since in these mice, complement-mediated killing of spirochetes would not be inhibited by CFH binding, these findings suggested that complement did not play an important role in host defense against Borrelia infection. A problem with that model, however, is the fact that factor H deficient mice practically do not have C3, compensating for their factor H deficiency and can not kill invading spirochetes by complement activation [99].
Additionally, when entering the host *B. burgdorferi* can bind proteins from the tick saliva that can inactivate or modulate complement activation [100-102]. Also expression of a CD59-like protein is described to modulate complement activation [103].

It is not clear whether the inactivation of complement by *B. burgdorferi* is essential for the infection of the mammalian host. It is also not known whether resistance to complement aids *B. burgdorferi* sl to cause a persistent infection and whether it causes a specific pattern of dissemination in the mammalian host.

1.4 Clinical presentation of Lyme borreliosis

The clinical presentation of Lyme Borreliosis (LB) can vary greatly between patients. The clinical presentation of patients that are infected in North America differs significantly from patients infected in Europe, most likely due to the different species that are prevalent and associated with specific clinical manifestations [104-106]. There are several ways of classification of LB. One commonly used classification is the classification in three stages, stage I is an early localized infection like an erythema migrans (EM). Stage II is an early disseminated disease like multiple EM lesions. Stage III is the late persistent disease [107]. Other classifications primarily make a difference between early localized and later manifestations [108, 109].

For a diagnosis of LB to be considered the patient must have been exposed to the risk of a tick bite. This means having been to woody, grassy or bushy area in an endemic country [108, 110]. The history of actual tick bites is important for the

clinical diagnosis. However, in clinical practice only a minority of patients remembers getting a tick bite [111-114]. There is usually a bimodal distribution of patients with LB mainly affecting children and elderly people, most likely due to risk behavior for attracting ticks (playing, gardening, hiking) [112, 114-117].

Incidence of LB in Europe seems to be gradually increasing within the last decades; this might be due to increased awareness, as well as to an actual rise in infections [118-120].

Erythema migrans

Erythema migrans (EM) is a typical sign of LB, usually presenting several days to weeks after a tick bite. The classical EM lesion is a round to oval expanding erythema that fades in the center, leaving only the annular border erythematous; the classical bulls eye (see figure 6). For some patients however, a homogeneous erythema may persist. The size of the ring, usually 5-10 cm, or the distance the lesion has migrated is positively related to the duration of the disease [121]. Sometimes the EM may also remain stationary for a long time. Successful cultivation of spirochetes from skin biopsies have proved that atypical variants such as vesicular, scaling, or purpuric-hemorrhagic lesions can occur [122]. Slight itching, burning or dysthesia may occur at the site of the EM [123].

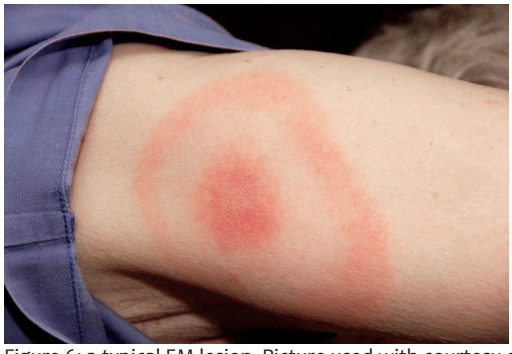

Figure 6: a typical EM lesion. Picture used with courtesy of the CDC.

In 30-68% of the cases the EM presents with a flu-like illness [123-125]. The lesion will usually resolve within a month, but may persist for a year. Relapsing lesions may occur [126, 127]. Reports of a previous EM in LB are reported in 28-90% of all patients. Multiple EM lesions are more often found in North-American patients and are rare in European individuals [113, 114, 128-132].

Borrelia lymphocytoma

Borrelial lymphocytoma (BL) is a rare cutaneous manifestation of LB that is diagnosed in 3-5% of patients with early Lyme borreliosis in Europe. It is a benign B-cell lymphoproliferative process which appears after about 3 weeks (2 days to 6 months) after a tick bite. It presents as a painless red to bluish papule or nodule with a diameter of up to a few centimeters typically localized usually on the ear lobe of children, on the nipple–areola mammae in adults or other localization such as the scrotum. This lesion can appear in any stage of LB. It is a more common manifestation among children than in adults. Borrelial lymphocytoma can be the only manifestation of the disease or it can be accompanied by other symptoms most frequently by EM, but concomitant ACA can be observed. An untreated BL can persist for several months to more than one year [133-136].

Acrodermatitis chronica atrophicans

ACA is a characteristic late manifestation of LB in Europe. Although ACA rarely has been reported in North America, it may be seen in approximately 10% of European cases of LB [121, 137, 138]. ACA most often presents in elderly women and is anecdotally reported in children [121, 139, 140]. ACA is most commonly caused by the genospecies *B. afzelii*, but can sometimes be caused by other genospecies [141-143, 143]. ACA is usually distributed on the lateral aspects of the distal extremities, but can also be present on the trunk. The disorder is characterized by a chronic, progressing course, beginning with an early inflammatory stage with bluish red discoloration and doughy swelling of the skin without sharp demarcation. Over weeks to months, the disease gradually develops into a late atrophic stage, in which the skin becomes thin, wrinkled, dry, and transparent because of the loss of epidermal and dermal structures. Underlying vessels become visible and multiple telangiectases occur [121, 140]. Cutaneous neuropathy with abnormal electroneurographic test results can be present in up to 60% of cases and have been elaborately described [144-147]. In 10% to 20% of patients with ACA, localized increase of dermal collagen leads to fibrotic nodules, which can lead to limitations of joint movement [148]. Upon treatment the infection readily comes to an arrest, but in late stage or prolonged ACA the elaborate atrophy of the skin and peripheral neuropathy will not completely recover [145].

Very often, ACA has already been present for months to years at the time of diagnosis, with a median of 9 months [149], or two years in another study [112]. This is most likely due to its insidious course and the frequent misinterpretation, especially as chronic venous insufficiency or skin aging, but also as a vascular disorder, deep vein thrombosis, superficial thrombophlebitis, arterial occlusive vascular disease, acrocyanosis or livedo reticularis [121].

Lyme arthritis

Lyme arthritis (LA) can present 12-50 weeks after a tick bite and is considered a late manifestation of LB. Spirochetes disseminate and invade synovial joints, where they induce an inflammatory response in synovial tissue by inducing infiltration of mononuclear cells, neutrophils, immune complexes, complement, and cytokines. The inflammatory response induces synovial hypertrophy and vascular proliferation with eventually cartilage damage [150]. LA is a common sign of LB, especially in North America, where it is one of the mayor clinical presentations [15].

In Europe 8- 25% of patients with Lyme disease complain of having arthralgias, often accompanied by other clinical manifestations of LB [112, 116, 117, 151, 152]. In a European study monoarthritis was observed in 71%, oligoarthritis in 20% and polyarthritis in 10% of the cases [112]. Manifestations of Lyme arthritis in Europe, as in North America, comprise recurrent attacks or long-lasting objective joint swelling/synovitis sometimes without arthralgia [153]. The joint most commonly affected is the knee (70-95%), but often also elbow, wrist, ankles, shoulders and hip [15, 112, 116, 153, 154]. Oligoarthritis of the large joints often presents unilateral or symmetric in LA [153]. The recurrent arthritis can persist for months to years. In most cases (79%) the arthritis will eventually resolve without treatment, but in some cases a chronic synovitis will develop. When a patient develops chronic synovitis elaborate destruction of the joint with erosive lesions and permanent joint disability will occur in 50% of the cases [155].

Children with LA are regularly misdiagnosed as suffering from pauciarticular juvenile rheumatoid arthritis or an acute bacterial septic arthritis [156].
Differential diagnosis in adults includes rheumatoid arthritis, palindromic arthritis, septic arthritis or a reactive arthritis.

Carditis

Cardiac manifestations in LB rarely appear to be a solitary symptom [157]. Most frequently it can be observed in early LB, or in association with neurological symptoms or arthritis. Conduction abnormalities with varying degrees of atrioventricular conduction defects are typical manifestations. In particular, Lyme carditis should be suspected in younger individuals showing conduction abnormalities without other apparent risk factors, and who have a history of recent exposure to ticks. Other rhythm disturbances, endomyocarditis and pericarditis have been infrequently reported [158]. Palpitations, bradycardia, or bundle branch block alone are not sufficient for diagnosis and alternative explanations for the cardiac condition presented must be excluded. Temporary

pacemakers are frequently used, but permanent pacemaker implantation is rarely needed [16, 157, 158].

Neuroborreliosis

Lyme neuroborreliosis (LNB) is a manifestation of an infection with *B. burgdorferi* sl in up to 20% of cases in Europe and up to 5% in North-America [112, 122, 159-162]. LNB commonly presents as cranial neuritis, meningitis or radiculoneuritis. The classic triad of radiculitis, peripheral paresis and inflammatory changes of the cerebrospinal fluid (CSF) are also known as Garin-Bujadoux-Bannwarth syndrome.

Headache is a common general symptom, as is fever, though both are more pronounced in children. Nuchal rigidity is not a common symptom and when it is present it is only mild and mainly present in children. Often there is more than one manifestation of LNB present [111, 114, 163, 164]. Cerebrovascular stroke and transient ischemic attacks are a rare complication of LNB [111, 114].

In the presentation of cranial neuritis the nerve most commonly affected is CNVII, also known as the facial nerve. In children, facial nerve paralysis (FNP) often is the first symptom of early LNB. Unilateral FNP in children is thought to be caused by *B. burgdorferi* sl in 35-75% in Lyme endemic countries, with the peak incidence in spring or late summer [165, 166]. Bilateral FNP in children is caused almost exclusively by *B. burgdorferi* sl [167, 168].
In adults the most common cause of FNP is idiopathic and only about 1,5 -2,2% of all FNP in Europe are caused by *B. burgdorferi* sl [113, 169]. The differential diagnosis of FNP consists of HSV or VZV, trauma/tumor, diabetes mellitus, pregnancy, autoimmune disorders, EBV and HIV [169].
Other cranial nerves can be involved in LNB and in particular involvement of more than one cranial nerve is a reason to suspect Lyme disease. Other cranial nerves commonly (co-)affected are the CNVIII (vestibule-auditory) the CNIII and the CNVI (oculomotor and abducens). CNII (optic nerve) and CNV (trigeminal) are uncommon nerves to be affected [114, 170-175].

Radiculitis is a common clinical manifestation of LNB in adults, but is rare in children. It can present one week to months after a recorded tick-bite, but is generally considered a late manifestation [114]. It starts with a painful radiculitis, eventually leading to focal motor signs. In two-thirds of the patients the pain started at the primary site of the EM. Radicular pain is easily distinguished from back pain as it is severe, burning and deep accompanied with areas of dyseasthesia. It has typical nocturnal exacerbations. In about a third of the

untreated patients the symptoms resolve within several months. Neurological examination often reveals involvement of multiple nerve roots [111, 114, 176].
The clinical presentation is sometimes mistaken for herpes zoster neuralgia, cervical or lumbar nerve root compression, visceral disease, brachial plexus neuropathy, Guillain-Barré, polymyalgia rheumatica or kidney concrements.

Progressive encephalomyelitis can manifest in approximately 4-6% of patients with LNB. The disease is often present for a longer duration (6 months to several years) before progressing to this stage. Clinical presentations associated with LNB encephalitis are; an ataxic gait, spastic bladder paresis with progression to spastic para- or tetraparesis, general confusion, opsoclonus-myoclonus, ocular flutter and apraxia [111, 114, 177, 178].

In the differential diagnosis of LNB many diseases can be included, especially auto-immune disorders; Multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), acute disseminated encephalomyelitis (ADEM). In literature many case reports describe misdiagnosis of Lyme disease for the abovementioned diseases, but also visa versa. This is why Lyme disease has been suggested to be the new "great imitator" after neurosyphilis that can also have a spectrum of presentations mimicking other neurologic diseases [179].

Ocular manifestation

Although ver rare, infections of the eye presenting as uveitis, conjunctivitis, papillitis, episcleritis, retinal vasculitis or keratitis/keratopathy have been described. Ocular involvement usually takes place in the late phases of disease [180-182].

Other manifestations

Lyme disease has been associated with acute hepatitis and elevated liver enzymes in active infection have frequently been reported [111, 183]. Myositis as an additional presentation of Lyme disease has been described on several occasions [184-187]. *B. burgdorferi* infection has been associated in one case with acute disseminated encephalomyelitis (ADEM) [188].

Host dependent factors

Several animal experiments have shown an increased rate of infectivity and an increased burden with a diminished rate of treatment success in rodents with an immunodeficiency [189-192]. However, in Lyme disease patients treated with immunosuppressive agents, no significant effect on clinical course and

response to treatment was observed [193, 194]. Treatment of patients with rituximab during the acute infection can lead to seronegative Lyme disease [195]. Treatment of Lyme disease patients in pregnancy is not associated with adverse outcome in the mother. One study however showed that an untreated maternal *B. burgdorferi* sl infection may be associated with an adverse outcome of the pregnancy (OR 7) compared to a treated pregnant population. It is therefore advisable to treat the *B. burgdorferi* sl infection during pregnancy [196, 197].

1.5 Laboratory diagnosis of Lyme borreliosis

Diagnosing Lyme disease is sometimes troublesome. Definite diagnosis is made by directly detecting living spirochetes, but this is often not possible. The diagnostic methods and their pitfalls in Lyme disease diagnostics are described in this paragraph.

Microscopy

Spirochetes can be detected directly by silver stain like the Warthin-Starry or a modified Steiner's stain [198, 199]. Indirect immunofluorescence is also possible (see figure 7). Spirochetes can be detected in EM and ACA lesions, in synovial biopsies, (heart)-muscle biopsies and CSF. Literature on these findings is mainly anecdotal [187, 198-210]. Electron microscopy has been attempted in several cases and detection of spirochetes has been reported. However, in some of these cases presence of active Lyme disease is doubtful [211-214]. For all forms of microscopy the limitation is the low amount of spirochetes in the tissue making it an unsuitable method for diagnosing Lyme disease.

Culture

Culturing *B. burgdorferi* sl has been a tedious task. The first success was by W. Burgdorfer in 1981 [1]. For years there were attempts to get a good standardized culture medium and modified Barbour–Stoenner-Kelly medium (BSK-H) is now commercially available [215-217]. However, culturing spirochetes from patient material is labor-intensive, usually slow and not very sensitive.

Culture has been attempted on a large subset of patient materials. Success of blood cultures for *B. burgdorferi* is very much dependent on the volume of blood used for culture. In the early days often only small volumes of blood, serum or plasma were used, yielding a very low sensitivity [22, 218-223]. Using higher volumes of blood, serum or plasma yielded a higher sensitivity (>40%) in several studies [224-227]. Large volumes of plasma have yielded the best results, followed

by serum and then whole blood [225, 228]. Culture from blood has a higher positivity rate in patients with cutaneous early manifestations of the disease, like EM. For diagnosing extracutaneous later manifestations blood culture sensitivities vary (5-50 %), but are generally lower than for EM patients [229-231]. Most studies that use blood culture are performed in North America. Studies performed in European patients gave significantly less positive findings than in North American patients. A hypothesis is that this is due to the high prevalence of localized *B. afzelii* in the skin lesion, but not in the blood of European patients [232, 233].

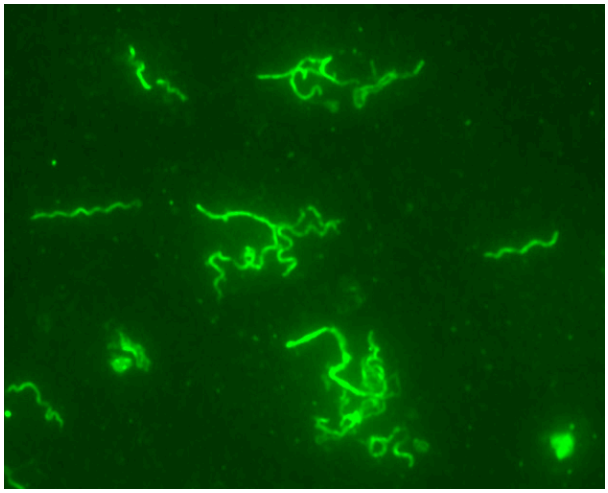

Figure 7: *B. burgdorferi* indirect immunofluorescence stain.

Better results are obtained with cultures from biopsies of EM lesion from European and North American patients (40-88%) [234, 235]. There is no significant difference in yield between a biopsy from the center, the edge or beyond the edge of the EM [236-238].
In biopsies from acrodermatitis chronica atrophicans lesions the sensitivity of culture is 22-60% [105, 141, 239]. Even ten years after the primary infection spirochetes can be cultured from an ACA [121]. In a biopsy from a BL the sensitivity of culture ranges from 24 to 33% [133, 135]. Cultures from CSF yield a sensitivity of 8-10% [235, 240]. From LA synovial fluid or synovial membrane biopsies spirochete recovery is rare [241, 242]. After start of effective antibiotic treatment cultures rarely become positive [235] .

Antigen detection

Antigen capture tests for Lyme disease have been developed for urine and CSF. Applicability in a clinical setting has been insufficiently validated, but reports up to now describe a high rate of aspecificity [243-246].

-lymphocyte stimulation assays

Some research has been performed on applying T-lymphocyte stimulation assays on the T-cells of Lyme disease patients. Higher reactivity to *B. burgdorferi* antigens has been shown, but results have been variable. The application of T-lymphocyte stimulating assays does not seem to increase the sensitivity or specificity of the already generally applied assays, like serology [247-253]. Currently, there is no place for T-lymphocyte stimulation assays in Lyme disease diagnostics, mainly because of concerns regarding the specificity and standardization of the assay [254].

Molecular detection

Direct molecular detection of *B. burgdorferi* from patient material has mainly consisted of polymerase chain reaction (PCR)-based methods. Because of the low amount of spirochetes in the tissue correct sample collection, transport and storage is essential for yielding reliable and consistent results. Several PCR targets have been employed; the 16S rRNA gene, p66, flaB, 23S RNA, recA from the chromosome and OspA from the plasmid DNA. Plasmid DNA is believed to be more stable than chromosomal DNA; therefore a plasmid located target has had the preference [255, 256]. However, for PCR in general the performance of individual in-house PCR's can differ greatly. Currently there is no standard and there are no efforts to develop one. It is therefore difficult to compare the results of performance of PCR from the literature.
DNA isolation protocols are generally also not standardized, but using large volumes of patient material for DNA isolation generally gives a better yield than small volumes [235, 257].

Several different PCR techniques have been evaluated, among which: conventional PCR, nested PCR, competitive PCR and real-time-PCR. Overall nested PCR obtained the highest sensitivity, but this technique is very prone to contamination and therefore not generally used in diagnostic practice [257].

On skin biopsies from EM and ACA good results have been obtained. In EM lesions the sensitivity ranged from 36-88%, but is generally about 70%. From ACA lesions the sensitivity ranged from 54-100%, generally yielding 80%

sensitivity [235]. The sensitivity of PCR on CSF from patients suffering from LNB generally yields 38% sensitivity (range 12-100%) [258-260]. Sensitivity of PCR on synovial fluid and synovial biopsies is much higher than culture for this material, usually detecting DNA in 78% of patients (42-100%). In some studies PCR on biopsies of synovial tissue yields better results than synovial fluid [255, 256, 261-266]. However, also after adequate treatment with clinical response the synovial tissue can stay positive for borrelia DNA for several months in animal experiments as well as in patients [267, 268].
Blood, serum or plasma give low sensitivity in PCR assays (45%). The specificity of PCR on blood has been insufficiently studied [257].
Attempts have been made to amplify DNA from urine from Lyme disease patients. The reports on the yield have been highly variable; furthermore there have been reports of nonspecific amplification [269-271]. Urine is not an adequate material for PCR for diagnosing Lyme borreliosis.

Due to the fact that PCR can stay positive after treatment for several months in combination with the fact that culture is insensitive, attempts have been made to detect viable spirochetes by detecting 16S mRNA in patient material [272]. In pre- and post treatment EM lesions it has been shown to give results comparable to that of the DNA PCR. However in EM lesions persistent PCR positivity after treatment is not found.
In patients that have been treated for LA the persistent PCR positivity of synovial fluid has been a diagnostic problem and specifically in this material the pre-treatment mRNA is not highly present. The hypothesis is that DNA of non-viable Borrelia is shedded in the synovial fluid from the actual (previous) site of infection [268].

Serology

Serology for Lyme disease is the most often performed test to diagnose Lyme disease. The development of serologic tests for Lyme disease has evolved rapidly in the last 3 decades. Many serological techniques have been applied, among which immunofluorescence assays (IFA), enzyme immunoassays (EIA) and Western blots.
IFA is based on (cultured) *B. burgdorferi* fixed onto glass slides. Specific antibodies from patient sera are bound to the *Borrelia* membrane and subsequently detected with fluorescein labeled anti-human IgG. The problems with this technique are the inter-assay variability and the subjectivity of the interpreter [235, 273].

In general practice EIAs are more commonly used, advantages are the objective numerical value it yields and the capability of automation. Upon the discovery of *B. burgdorferi* in the late 1970's EIAs were first developed as ELISA's with whole-cell sonicates of *B. burgdorferi* sl. This approach has several technical setbacks. For *B. burgdorferi* sl it is the question which species or strains should be used in the ELISA [274]. Several important immunodominant antigens in vivo are not expressed under standard in vitro culture conditions and that some of the immunodominant plasmid-encoded antigens may be lost when the strains are passed multiple times [275, 276]. Furthermore, using whole bacteria caused a high rate of aspecific results because of similar antigens shared between Borrelia and other bacteria. The EIA gives a cumulative result and it is not possible to distinguish against which antigens the detected antibodies are directed. IgG EIAs are more reproducible than IgM EIAs, because the latter often give false positive results, for instance in patients with autoimmune disorders, or acute infection with other pathogens [277, 278].
In general, the EIA yields a sensitivity of approximately 30-50% in early localized manifestations of Lyme disease. Sensitivity rises to about 70-80% in early disseminated stages of the disease, like LNB. In late manifestations, or prolonged duration of disease the sensitivity can rise to nearly 100%. In early cases a convalescent serum will increase detection of antibodies [129, 234, 279-281]. Specificity varies markedly between assays.

In recent years much work has been done on the development of assays of combined purified proteins with more *Borrelia* specific antigens like borrelia flagellin (p41), BmpA (p39), OspA or p83/100. Furthermore the development of EIAs has focused more on immunodominant conserved antigens that are expressed by *B. burgdorferi* sl specifically in early infection in the mammalian host, like OspC, DbpA (p18) and VlsE (IR6/C6-peptide) [282-285]. In most assays commercially available now either combinations of these antigens with whole cell sonicate or combinations of one or more recombinant antigens are used. These EIAs often have a comparable sensitivity for detecting anti-borrelia antibodies, but often a higher specificity [114, 284, 286-291, 291, 292].

Another common test for detecting antibodies against *B. burgdorferi* is a Western blot. A Western blot can give a more specific result than an EIA, because antibodies against more than one antigen can be detected separately. The EIA is more sensitive for early manifestations of Lyme disease [293].
The conventional Western blot is a technique in which a whole-cell sonicate of *B. burgdorferi* sl is separated by SDS-PAGE and blotted on a nitrocellulose membrane. It is important to use a strain that expresses immunodominant epitopes [274, 294, 295]. Antibodies from patient serum are incubated with the

nitrocellulose strip. Antibodies are detected by labeled anti-IgG or IgM antibodies. This allows for a more specific determination of the different antibodies against *B. burgdorferi* within the individual patient sera. The difficulty with interpreting a conventional blot is the presence of a high amount of cross-reactive bands, apart from the difference in the quality of reproducibility of the batches of blots [296-299]. Cross-reactive IgM is often a problem in interpreting the blots [300]. Several algorithms for interpretation of blots have been proposed [234, 301-303]. The criteria for interpreting Western blots as they are used in North America do not yield a good sensitivity in European patients [294, 303, 304]. It is important to realize that the prevalence of different genospecies is region specific which can lead to preferential rules for specific geographical regions [303].
In the recent years much research has been done on line-blots, instead of conventional or native blots. Line-blots are produced with purified recombinant proteins which are placed on a nitrocellulose band in a specific concentration. Advantages of this technique are the specific amount of protein that can be applied, the specific proteins expressed in vivo that can be elected and elimination of the background of cross-reactive proteins.
The use of different homologues proteins, from different genospecies can be important to obtain optimal results [285, 305, 306]. Many of the line-blots commercially available in Europe now use combinations of immunodominant antigens like OspC, flagellin internal conserved fragment, several DbpA homologues, VlsE, p83/100 and BmpA as specific and sensitive Borrelia antigens [234, 306-308]

Two-tier testing is a common serologic approach for diagnosing LB. Common practice is performing an EIA which is, if positive, followed by an immunoblot as a confirmatory assay. In general the immunoblots yields a higher specificity, but a lower sensitivity in early manifestations of LB [291, 296, 302, 309-312]. In those cases a convalescent serum taken several weeks later can aid in confirming the diagnosis.
It has been proposed that the use of only one specific recombinant antigen in an EIA alone can give comparable sensitivity and specificity as the two-tier testing strategy [234, 291, 310, 313, 314]. This strategy is currently not yet generally accepted in many guidelines. A two-tier testing strategy that has been proposed is the use of two EIAs, a whole-cell sonicate EIA and a VlsE/C6-peptide based EIA [315, 316].

It is important to realize that serology is an indirect test and development of antibodies can take several weeks, sometimes resulting in negative serologic results in patients presenting with early Lyme disease [123, 235, 292, 317, 318]. It is

therefore generally advised not to perform serology when a patient presents with a specific early symptom as for instance an EM. If the clinician is in doubt it is advisable to also test a convalescent serum several weeks later, in order to increase sensitivity of antibody detection [291].
Furthermore, it is generally not possible to distinguish between active infection from past Lyme disease with serology. In the general population in endemic countries in Europe the prevalence for antibodies against Borrelia is 4-7% [111, 319-321]. This can be up to 25% in specific populations that have a high exposure rate to ticks [320, 322-324]. Most likely the majority of infections in the European population resolve without symptoms, accounting for the high level of seropositivity without clinical manifestation [325].

Serology after treatment

After treatment anti-Borrelia IgG, but also IgM can persist during several years. For IgG this can be lifelong, for IgM persistance has been described for at least one year [317, 326-330]. On the other hand, after treatment of early Lyme borreliosis it is possible that the patient will show only very weak or no seroconversion at all, further complicating serologic diagnosis.
When the diagnosis has been made and treatment has been started follow-up serology by Western-blot is not helpful, because in most patients a decline in antibody titer is not detectable [329, 331-333]. Furthermore, there is no clear correlation between decline of antibodies after treatment and clinical response [290, 334].

C6, Vlse IR6 Epitope

In recent years the interest in the C6 peptide for diagnosing Lyme borreliosis has increased. C6 is part of a larger protein: VlsE. To maintain chronic infection Borrelia can apply antigenic variation. One of these membrane expressed lipoproteins is Variable Mayor Protein-like sequence, expressed (VlsE). VlsE is encoded on the lp 28-1 where VlsE and its promoter are situated next to a cassette of 15 unexpressed VlsE lipoproteins (See figure 8) [57, 335, 336].

During infection promiscuous recombination events take place from the cassette leading to antigenic variation of the expressed VlsE, potentially leading up to 10^{30} different combinations [56]. VlsE consists of six variable regions (VR) and six invariable regions (IR). The VR are highly immunogenic and antibodies directed to the VR are able to effectively kill spirochetes that express the corresponding antigens. Spirochetes escape killing by expressing different variable domains as rapid as four days post infection [54, 337]. IR are essential for structural conformation of proteins applying antigenic variation and are highly

conserved and highly expressed among *B. burgdorferi* sl complex. Usually IR are not immunogenic, but IR6 of VlsE, is highly immunogenic [338-341]. Incorporated in VlsE on the membrane C6 is not accessible for Abs and Abs to C6 are not bactericidal in vitro [342]. It has been hypothesized that chronic host exposure to immunodominant C6 diverts the immune system from responding to less antigenic but functionally more important Ags or epitopes, thus serving as a protective strategy for persistent infection [341]. In non-human primates IgG responses are detectable as early as three weeks post inoculation, IgM responses are rarely detectable and do not appear earlier than the IgG response[339]. In humans detection of anti-VlsE Abs in an early phase of disease has been described [282].

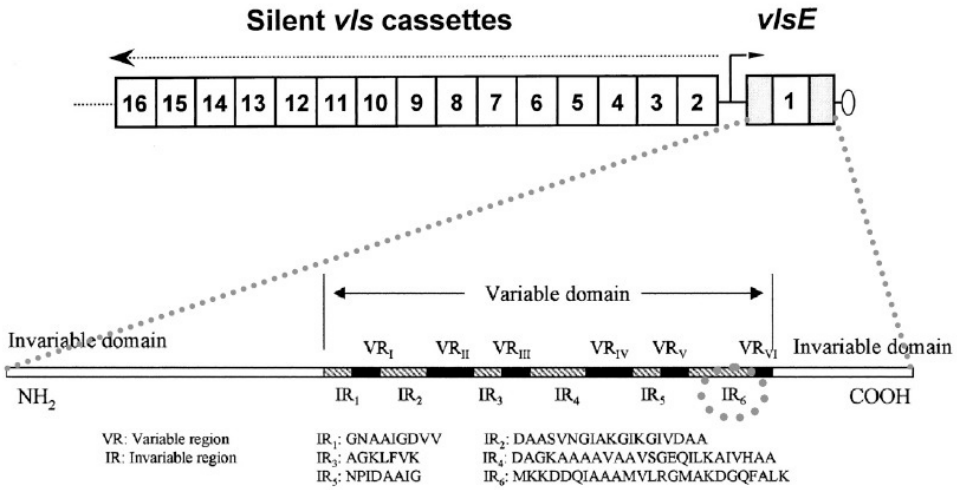

Figure 8: The arrangement of the VlsE gene at the lp-28. Picture modified from Liang et al.and Zhang et al.[56, 338]

Of all of the six IR, IR6 is the most immunogenic and highly conserved among *B. burgdorferi* sl, but can still vary up to 5 amino acids between species [335, 340, 342-345]. IR6 consists of only 26 amino acids, making it an easy to manufacture and cheap recombinant protein for diagnostic procedures. The IR6 peptide is unique, and hardly any cross reactivity is reported, on GenBank no identical sequence has been described thus far [339, 341]. Some *B. burgdorferi* strains lack part of the lp28-1 plasmid, but these strains still bear the VlsE cassette and express VlsE [346]. Only rarely patients do not seem to make an early anti-C6 response, while the cassette is still present and expressed in the infective strain [310].

Waning of C6-peptide antibodies

It has been proposed that after adequate eradication, the C6 antibody titers wane. In that case, covalescent antibody titer measurements might be an instrument to monitor therapy efficacy as is the case with the VDRL in syphilitic patients. In a study by Philipp et al. 7 rhesus macaques showed rapid anti-C6 decline after infection and subsequent treatment for LB. Week 34 after infection all serological levels dropped to near detection level. The ELISA for anti-p39 did not show such rapid decline. In 15 patients all serum anti-C6 titers dropped more than 4-fold 20 weeks after treatment [347]. Philipp et al. followed 105 New York patients after treatment for early localized LB. In all 105 patients C6 levels declined, in 96 (91%) a decline below baseline, or a more than fourfold decrease in C6 titers was found. However, in patients that had multiple EM, and therefore were likely longer exposed to *B. burgdorferi*, the decline was less prominent [348]. Anti-VlsE titers can also wane over time, after treatment for EM shown in 67 patients [349]

This was also confirmed in a later study in which serum from 11 EM and 34 disseminated LB patients was tested, 80 % showed the more than fourfold decrease in titers after six months. Nine patients did not show the more than fourfold decrease in titers after 6 months, they all belonged to the disseminated disease group [350].

The decline in titer does not mean C6 antibody titers become undetectable in all adequately treated patients. Peltomaa et al. showed in 45 patients (n=24 early disseminated disease, 21 late disseminated disease) titers decreased more than fourfold in 67% of patients with early disseminated disease and only 14% in patients with late disseminated disease. Years (median 11 years) after treatment without signs of relapse 50% of patients with early disseminated and 83% of patients with late disseminated disease still had positive C6 serology [351].

In PTLDS patients, no correlation was detected between quality-of-life markers, clinical outcome, response to therapy and anti-C6 presence. The sheer presence of C6 Abs does not necessarily indicate active infection [352].

Diagnosing neuroborreliosis in cerebrospinal fluid

Primarily when a clinician has a LNB in the differential diagnosis several CSF markers will make LNB more probable. The CSF generally shows sign of inflammation; pleiocytosis is usually present, dominated by the presence of mononuclear cells [111, 353-355]. Some cases have been described where pleiocytosis was absent and the diagnosis of LNB highly likely due to a combination of a tick bite, a positive antibody index and a clinical picture resembling known LNB manifestations [111, 112, 356-358].Other signs of inflammation in the CSF of a patient

with LNB include; elevated total protein levels, elevated CSF/serum albumin ratio (80-90%), presence of oligoclonal bands (40-50%), elevated lactate (55%) and low glucose (10%) [111, 354, 357].

In the CSF antibodies against Borrelia can be detected, commonly used EIAs are whole cell sonicate EIAs or a purified flagellin EIA. IgM and/or IgG against Borrelia in CSF are invariably positive at time of presentation with a LNB [356, 359]. Research on the presence of intrathecal anti-C6-peptide yielded conflicting results. The sensitivity in these studies ranged from 61-98% [360-362]. This is remarkable because in the studies applying the C6-peptide EIA in serum an early anti-C6-peptide response is usually present.
Detection of intrathecal anti-Borrelia IgG alone is not enough, because passive diffusion from the serum can take place. The way to correct for this phenomenon is to test a paired serum CSF sample and to calculate the antibody index (AI) [356, 363]. By diluting the serum and CSF to the same concentration of total IgG and performing an ELISA the fraction of specific anti-Borrelia IgG can be compared. If the AI index is larger than two, indicating the amount of intrathecal antibodies is relatively twice as high, the AI is positive. It is of minimal relevance whether a whole-cell sonicate or purified flagellin EIA is used and several calculation methods yield identical results [364, 365]. A second technique is a capture EIA which captures total IgG or IgM and then detects the fraction of anti-flagellin IgG or IgM in serum and CSF samples. The AI is then calculated directly form these results. Advantage of this assay is the lack of necessity to determine amount of total IgG and albumin and the fact that no additional dilution is necessary [366].

When the anti-Borrelia AI is elevated the diagnosis of LNB is highly probable (89-97%). The Borrelia AI can sometimes be positive in patients with relapsing remitting multiple sclerosis [367]. The sensitivity of a positive AI for diagnosing LNB has been reported to vary from 38-92%, with a median of approximately 70% [114, 354, 356, 368]. The sensitivity of AI is lower in early manifestations of LNB [366, 368, 369]. In contrast with this finding, in previous studies it was also demonstrated that patients with LNB can have an earlier response to antigens intrathecally than in serum [292, 366, 370, 371]. For the clinical manifestation of late LNB in North American patients, it seems that the AI is less sensitive than in European patients [356, 359].

IgG is the most reliable immunoglobulin for calculating the AI. In some cases the IgM-AI is more sensitive in early manifestations of LNB [354, 366, 372]. In LNB patients with a negative AI at diagnostic lumbar puncture a convalescent AI can

be helpful for detecting delayed intrathecal synthesis of IgG, but the AI will stay negative in 33% of previously AI negative cases [368, 373].
In most cases the signs of inflammation in the CSF and the AI will decline after treatment. However, a positive AI index can stay positive for decades after effective treatment without any sign of clinical failure [374, 375].

Another method for detecting intrathecal production is by Western blot. However the use of immunoblots to determine intrathecal antibody responses is problematic, due to difficulties in interpreting the intensity of bands, variety displayed in individual patients and the lack of good criteria for interpretation [354, 376-378].

Biomarkers

Biomarkers for detecting LB have been of interest in Lyme disease diagnostics. It has been proposed that specific cyto- and chemokines play a role in LB. A chemokine of particular interest is CXCL13.
Intrathecal levels of CXCL13 have been suggested as a potential biomarker for diagnosing LNB. CXCL13 is produced by antigen presenting cells and is a selective chemoattractant for B-cells and B-helper T-cells. Toll like receptor 2 is most likely the receptor involved in induction of CXCL13 production in LB [379]. CXCL13 has been found to be expressed at high levels in pooled cerebrospinal fluid (CSF) from human LNB patients (219ng/g total protein) by a cytokine array, while in pooled CSF from subjects with non-inflammatory neurological disease levels were barely detectable (<1.7ng CXCL13 /g total protein). Patients with multiple sclerosis had a slightly higher level of CXCL13 intrathecally than healthy controls [380]. In a patient cohort of acute LNB patients CXCL 13 levels were highly elevated in all 37 definite LNB cases and no or minimal elevation was seen in the non LNB controls (n=8) [381]. In another study 28 LNB cases had significantly elevated levels of CXCL13 compared to neurological and infectious controls. Some infectious controls had high levels of CXCL13 intrathecally, but overall sensitivity and specificity for LNB using a cut off of 337ng CXCL13 /g total protein were 96% and 97% respectively [382]. Another study used a combination of CXCL13 and anti-C6-peptide antibodies for diagnosing LNB in children. This study used a cut-off of 163ng CXCL13/g albumin (equivalent to 142 pg CXCL13/ml CSF), which gave 98% sensitivity (n=124 LNB) and 95% specificity (controls n=92). Adding the detection of antibodies against C6-peptide in CSF increased the specificity for detecting LNB to 97%. This study also included several groups of possible LNB according to EFSN criteria; in the group of serologically antibody positive, CSF antibody negative but pleiocytosis positive group 100% of patients were children and had elevated levels of CXCL13. This was not the case in all pleiocytosis negative, or serologically negative probable LNB [372].

A prospective study used CXCL13 to diagnose adult patients with untreated LNB (n=13), controls were 178 patients with pleiocytosis due to other diseases. In this specific study the cut-off was chosen at 1229 pg CXCL13/ml CSF. Patients presenting with an intrathecal lymphoma often had very high levels of CXCL13 intrathecally [383]. No consensus on a cut-off has been reached to date. It has been shown that CSF CXCL13 is a biomarker that declines rapidly after treatment for LNB, making it a possible biomarker for evaluating treatment for neuroborreliosis [382, 383].

CXCL13 is a chemokine that is produced during presentation of antigens by antigen presenting cells. The question remains whether levels of CSF CXCL13 are specific for LNB or also present in other CNS infections or in other autoimmune diseases in which elevated levels of serum CXCL13 have frequently been reported [384-388].

CXCL13 is also highly present in Borrelia lymphocytoma lesions [389]. Detecting CXCL13 in serum of LB patients has been attempted but this has been shown to have no diagnostic value for LB [382, 390].

1.6 Treatment and prevention strategies

Choice of antibiotics

The first cases of effectively treated LB with penicillin date back to the 1950's [10, 11]. In vitro, *B. burgdorferi* sl is sensitive to a very large spectrum of antibiotics, including β-lactam antibiotics, tetracyclines and macrolides [391-395]. There is a lack of standardized methodology to determine MIC/MBC's [396]. A standardized microdilution method has been developed [397]. Aminoglycosides, older generation quinolones and aztreonam are inappropriate antibiotics for *B. burgdorferi* sl [391, 396, 398, 399]. Antibiotics that have a gram positive spectrum like vancomycin and linezolid surprisingly show activity against Borrelia spp in vitro, though it has never been proven that they are adequate therapeutic agents in vivo [392, 400]. A glycylcycline (tigecycline) has low MIC/MBC's against *B. burgdorferi* sl in vitro but its effectivity in vivo has been insufficiently studied [401, 402]. The in the Netherlands commonly applied antibiotics amoxicillin, ceftriaxone and azithromycin have the lowest MIC_{90}'s and MBC's (≤ 2 μg/ml) in vitro against several *B. burgdorferi* sl tested [394, 395, 403].
Despite a low MIC of *B. burgdorferi* sl for macrolides clinical trials have shown failure of the older generation macrolides like erythromycin [19, 404]. Azithromycin,

however, has shown to be non-inferior or even superior to treatment with doxycycline in early stage LB [405-408]
In some studies patients treated with cephalosporines have equal or higher response rates than patients treated with penicillines in late manifestations of LB [409-411]. For the treatment of LNB doxycycline per os (po) was non-inferior to intravenous (iv) ceftriaxone in an European cohort [412]. The data about susceptibility patterns between species differs, but there are some studies that conclude that *B. burgdorferi* ss and *B afzelii* are more resistant to some classes of antibiotics. Treatment failures have been described for almost any class of antibiotic [396]. However, culture confirmed failure of treatment for EM with β–lactams and tetracyclines is rare (<1.7%) [413].

Commonly advised antibiotics in the guidelines for treatment of LB in Europe and North America are doxycycline and ceftriaxone. For oral therapy in children doxycycline is usually replaced by amoxicillin [414-417]. In early manifestations oral therapy should be sufficient. For later manifestations intravenous therapy is recommended for specific clinical manifestations.

Duration of treatment

The advised duration of treatment varies mildly between guidelines. For early manifestations of LB duration of treatment of about two weeks (10-21 days) is commonly advised in European and North American guidelines. For later, disseminated manifestations of LB duration of treatment of about 3 weeks (14-30 days) is commonly advised [414-419].

Early manifestations of Lyme borreliosis

For the treatment of a solitary EM 10 days of treatment is generally sufficient, longer treatment has no effect on outcome [420]. In the case of an early disseminated LB most studies on treatment have been performed in North America. Most patients with an early disseminated disease present with multiple EM, but this is not a clinical picture that is prevalent in Europe. In the case of carditis no sufficiently powered study has been done. Generally a treatment of 2 weeks with ceftriaxone iv or 3 weeks of doxycycline po is adequate [421].

Acrodermatitis chronica atrophicans

The manifestation of an acrodermatitis chronica atrophicans is rare and mainly present in Europe. There are not many studies that compare duration of treatment in well conducted studies. Longer treatment up to 30 days seems to be indicated for this chronic manifestation of LB. Intravenously or orally

administered β–lactams and doxycycline po seem to be equally effective regimens [422, 423].

Lyme arthritis

LA is a late manifestation of LB. In the studies conducted on the effectivity of antibiotics the success-percentage varies greatly. Treatment regimens of longer duration have a higher response rate than treatment duration of 2 weeks. Intravenously administered β–lactams and doxycycline po are both effective regimens [411, 424-427]. After treatment up to 25% of the patients remain with complaints of arthritis or arthralgias for several months, despite the fact that there are no signs of persistent infections [153, 268, 428]. In the case of persistent symptoms with no sign of persistent infection expert opinion is to attempt treatment with anti-inflammatory agents or an arthroscopic synovectomy [150, 272, 429, 430].

Lyme neuroborreliosis treatment

In several studies cephalosporines have been compared to penicillin in the treatment of early LNB. Ceftriaxone has been a preferential treatment due to good penetration in the CSF and the favorable pharmacokinetic properties. Penicillin and ceftriaxone seem to be equally effective, though no large randomized study has been performed [409, 410, 431-433]. A treatment period of at least 14 days is advisable, because with treatment regimens of 10 days relapses were observed in a small population [431].

Treatment with β-lactams is usually intravenous, but an option is the treatment of LNB with orally administered doxycycline. All randomized studies conducted in Europe showed non-inferiority of the doxycycline po compared to ceftriaxone iv in early LNB [412, 434]. Many other studies have compared the two treatment regimens unblinded, retrospectively or non-randomized. Mean overall success rate of ten studies with LNB patients treated with a β-lactam antibiotic iv was 95% and for doxycycline po 99%, this difference was not significant. In these studies it can not be excluded that the patients with a milder form of LNB were treated with the oral therapy leading to a selection bias [419, 435].

Research has been performed on addition of corticosteroids for FNP, but the data is not conclusive for Lyme disease and does not significantly favor use of corticosteroids for the recovery of the function of the nerve [436-441]. Anecdotal reports suggest that post antibiotic treatment with corticosteroids of other LNB manifestations might reduce residual complaints [442, 443].

In general, many LNB patients after treatment have residual complaints that need months to years to resolve even after adequate treatment. Longer duration of complaints before treatment is associated with slower recovery [357, 434, 444, 445]. Seventy to eighty percent of patients report complete recovery within 6 months [446, 447]. About 25% of all LNB patients report neurological problems years after the primary infection, of which half of the patients report it is interfering with daily activities [437, 448-452]. However, in an age-matched control population the prevalence of neurological complaints and symptoms reported by post treatment LB patients are not significantly different [450, 453, 454]. In two studies of age-matched adolescents this difference in performance or residual complaints was significant [437, 448].

Prolonged treatment for residual complaints

Treatment failure with currently advised regimens is very rare [124, 420, 455, 456]. It is on the other hand difficult to diagnose persistent active infection after completed treatment, because culture confirmed relapse is rare. In one study it was shown that 22 paired consecutive episodes of EM were due to reinfection rather than relapse [457].

DNA of spirochetes can persist in joints for several months before it is completely cleared after therapy without clinical failure [272]. It has also been described that spirochetes can go into a dormant state when the bacteria are in a hostile environment, for instance during treatment. These forms are named cyst form. It has been shown that the cyst forms can be taken up by ticks and transmitted to SCID mice [458]. When SCID mice are infected with *B. burgdorferi* they have extensive infection and inflammation [459]. However, infection with the cyst forms do not give any histological inflammation and the numbers seem to slowly decline in the host concluding that the cyst forms are avirulent and do not cause disease [460].

The prolonged treatment of patients that have no objective sign of persistent infection is controversial. Studies done on this subject are difficult to interpret due to the difficulties with the definition of post Lyme disease syndrome. Some studies take patients with any kind of residual complaint; other studies only include patients with objective complaints. Several double-blind, randomized, placebo-controlled studies found no effect of prolonged treatment of patients with persistent symptoms after LB [461-465]. Furthermore there have been several observations in which it was clear that antibiotic refractory arthritis does not resolve with prolonged treatment [272].

As there is no significant effect of prolonged treatment in placebo controlled trials it is generally advised not to treat patients with persistent symptoms after

treatment according to guidelines [414-417, 419]. It is advisable to further examine patients with complaints after LB, because in a significant proportion of the cases another illness can be diagnosed [466, 467].

Correct and evidence-based of treatment of an sufficiently proven LB is important, because the prolonged intravenous treatment is not without risks. Severe complications and deaths due to, the sometimes prolonged, treatment of supposed LB have been described in case reports [461, 468-472].

Vaccination against Lyme disease

Vaccine development has been a hot item in the last few years. Several vaccine proteins have been applied among which OspA, OspB, OspC, DbpA, RevA, BBK32, BB0323 and ACGal [473].
The OspA vaccine using aluminum as adjuvant was approved by the FDA and applied [474]. However, after wide application it was shown that titers were not sufficient in 5% of the population and that additional boosters were essential for a prolonged effective titer. Furthermore there were some concerns about the safety of the vaccine because of an hypothesis that molecular mimicry between the vaccine antigen and an auto-antigen triggered autoimmunity [475]. The vaccine was withdrawn from the market due to insufficient sales. Since then new OspA vaccines have been under development [476, 477].
However, an interesting candidate is the OspB vaccine that has shown a complement independent bactericidal effect [478, 479]. Combining more than one antigen, for instance OspA/B/C, DbpA or BBK32, in a vaccine has shown promising results [480, 481].
Vaccine research has also been done on tick immunity, which is elicited by repeated feeding on a host. Tick immunity can interfere with transmission of *Borrelia* spp. though it does not give complete protection [482-485]. This strategy however could also give protection to other arthropod borne infections, like *Francisella* spp. [486]. It is still a long way to developing a suitable vaccine against *Borrelia* spp, but perhaps a strategy combining some of the before mentioned epitopes is going to elicit an effective vaccine [473].

Preventive measures

A highly effective way of preventing an infection with *B. burgdorferi* is preventing the tick bite itself. Wearing light colored, tightly woven clothing, tucking pants into socks and shirt into pants, using insect repellents and frequent checking to remove crawling or attached ticks reduce the risk of tick-borne diseases if conscientiously practiced [487-489].

In North America deer control is one of the most effective measures in diminishing the tick population [490]. In Europe however this strategy is less effective because of the different tick and host species that are prevalent. The parasitic Ichneumon wasp *Ixodiphagus hookeri* has long been investigated for its potential to control tick populations. It lays its eggs into ticks; the hatching wasps kill their host. Another natural form of control for ticks is the use of the guinea fowl, a bird species which consumes mass quantities of ticks. Just 2 birds can clear 2 acres in a single year [490]. Furthermore research is being performed on using molds that can infect ticks [491].

Phenothrin in combination with Methoprene, both acaricides, were a popular topical flea/tick therapy for felines. Acaricides might be applied in an individual setting, but is not effective in reducing the tick population when it is mass applied [492, 493]. Furthermore topical (drops/dust) flea/tick medicines may be toxic to animals and humans [494].

□rophylaxis after a tick bite

Prophylaxis after a tick bite remains controversial, although some guidelines will propose use of prophylaxis after a tick bite. The risk of contracting Lyme disease after a tick bite of a tick that is not promptly removed is very low, also in the Netherlands (<1-2%) [495, 496]. Furthermore, there is no clear evidence on what the prophylaxis should consist of. Some studies apply a 10-day course of antibiotics while others give one dose of 200 mg doxycycline [497-504]. On the basis of pharmacodynamic studies one dose of 500mg azithromycin should be as effective as one dose of 200mg doxycycline [505]. Results of efficacy of the prophylaxis vary greatly between studies. A meta-analysis combining data from North America showed there was a significant reduction in Lyme disease in the population who received prophylaxis consisting of one dose of 200mg doxycyclin. The number needed to treat was 50 to prevent one case of EM in a highly endemic area in North America [506].

1.7 Scope of this thesis.

As discussed in the previous section *B. burgdorferi* has a wide variety of strategies to hide from the host immune system. Complement regulatory binding proteins have been described for almost all complement resistant *B. burgdorferi* sl, except for the complement resistant *B. bavariensis*, one of the species that is known to frequently cause Lyme neuroborreliosis.
In **chapter 2** it is attempted to identify CRASP-1 proteins in *B. bavariensis*, formerly known as *B. garinii* OspA serotype 4. Potential CRASP-1 proteins will be cloned and studied for their ability to interact with host derived fluid phase regulators of complement.

The specific role of complement resistance in early effective infection and dissemination of *B. burgdorferi* sl has not been well investigated. Can complement resistance lead to a better and more effective infection and dissemination? In **chapter 3** an in vivo experiment in which the infectivity and dissemination patterns of complement sensitive and complement resistant *B. burgdorferi* sl in a C3 deficient mouse model is described.

After effective transmission from the tick to the host the next challenge in *B. burgdorferi* infection is rapid and accurate detection of the pathogen. Diagnostics of Lyme disease is often compromised due to specific pathogen properties combined with technical shortcomings of bacterial serology.
Two indirect detection methods which can aid in diagnosing patients suffering from Lyme neuroborreliosis were studied. In **chapter 4** the performance of the C6-peptide ELISA for detecting antibodies in CSF in Lyme neuroborreliosis patients is studied. While in **chapter 5** levels of CXCL13 in several patient populations as a potential biomarker for the diagnosis of Lyme neuroborreliosis is studied.
For both indirect markers of presence of B. burgdorferi the specificity in clinically resembling and neuroinfectious diseases is of key importance. Several other infectious and inflammatory diseases that have a clinical presentation that can resemble Lyme disease are included in the analysis.

Diagnosing Lyme disease can be difficult in some populations, first because Lyme disease is a relatively rare infection, resembling a large spectrum of other autoimmune and inflammatory diseases. Clinicians could often consider testing for Lyme disease. It is also important to do this in specific preselected populations, because the positive predictive value of a test, but specifically indirect tests such as serology, in a random population, is low.

In **chapter 6** all patients that present with complaints of arthritis at the early arthritis clinic are tested for Lyme arthritis. The prevalence of *B. burgdorferi* seropositivity in this population is studied. Another aim is to identify clinical factors which should urge the doctor to test, or explicitely not test, for Lyme disease in a patient presenting with arthritis in Europe.
In **chapter 7** a case of an HIV positive patient presenting with a meningo-encephalitis caused by *B. burgdorferi* is described. The literature on HIV and Lyme neuroborreliosis co-infections is also reviewed.

Chapter 2

Identification and functional characterization of Complement Regulator Acquiring Surface Protein-1 of serum resistant Borrelia garinii OspA serotype 4

Nathalie D. van Burgel[1]
Peter Kraiczy[2]
Tim J. Schuijt[1]
Peter F. Zipfel[3]
Alje P. van Dam[1]

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[1] Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands
[2] Institute of Medical Microbiology and Infection Control, University Hospital of Frankfurt, Frankfurt, Germany
[3] Department of Infection Biology, Leibniz-Institute for Natural Products Research, Jena, Germany

Abstract

B. burgdorferi sensu lato (sl) is the etiological agent of Lyme borreliosis in humans. Spirochetes have adapted themselves to the human immune system in many distinct ways. One important immune escape mechanism for evading complement activation is the binding of complement regulators Factor H (CFH) or Factor H-like protein1 (FHL-1) to Complement Regulator-Acquiring Surface Proteins (CRASPs).

We demonstrate that *B. garinii* OspA serotype 4 (ST4) PBi resist complement-mediated killing by binding of FHL-1. To identify the primary ligands of FHL-1 four CspA orthologs from *B. garinii* ST4 PBi were cloned and tested for binding to human CFH and FHL-1. Orthologs BGA66 and BGA71 were found to be able to bind both complement regulators but with different intensities. In addition, all CspA orthologs were tested for binding to mammalian and avian CFH. Distinct orthologs were able to bind to CFH of different animal origins.

B. garinii ST4 PBi is able to evade complement killing and it can bind FHL-1 to membrane expressed proteins. Recombinant proteins BGA66 can bind FHL-1 and human CFH, while BGA71 can bind only FHL-1. All recombinant CspA orthologs from *B. garinii* ST4 PBi can bind CFH from different animal origins. This partly explains the wide variety of animals that can be infected by *B. garinii*.

Introduction

B. burgdorferi sensu lato (sl), the etiologic agent of Lyme borreliosis, is a genetically diverse species. The different genospecies of *B. burgdorferi* sl appear to be associated with different manifestations of the disease [105, 507]. *B. burgdorferi* sensu stricto (ss) is more common in North America but also found in Eurasia and is associated with arthritis, while *B. garinii* and *B. afzelii* are only present in Eurasia and are more commonly associated with Lyme neuroborreliosis and cutaneous manifestations, respectively. Specifically *B. garinii* OspA serotype 4 (ST4) strains, a genetically homogenous group, are frequently observed as a causative agent of neuroborreliosis in adults in Europe [508-511]. Recently it has also been proposed, though not yet generally accepted, to delineate the *B. garinii* ST4 strains as a separate species, *B. bavariensis*, due to large differences compared to *B. garinii* non-ST4 in multilocus sequence analysis (MLSA) on several housekeeping genes [512].

The different human pathogenic genospecies are associated with certain human serum resistance profiles; the majority of *B. burgdorferi* ss and *B. afzelii* strains are relatively resistant to human serum, while most *B. garinii* strains are highly sensitive to complement-mediated killing in vitro. Among *B. garinii*, the ST4 strains showed a similar resistant profile as *B. burgdorferi* ss and *B. afzelii* [61, 64, 65].

B. burgdorferi sl has developed a variety of immune evasion strategies, among which the binding of two host-derived fluid-phase regulators of complement: Factor H (CFH) and Factor H-like protein 1 (FHL-1). CFH and FHL-1 the main immune regulators of the alternative pathway of complement activation, are structurally related proteins composed of several protein domains termed short consensus repeats (SCRs) [60]. CFH is a 150-kDa glycoprotein composed of 20 SCR domains. In contrast, FHL-1 is a 42-kDa glycoprotein corresponding to a product of an alternatively spliced transcript of the *CFH* gene and consists of seven SCRs. The seven N-terminally located SCRs of both complement regulators are identical with the exception of four additional amino acids at the C-terminus of FHL-1 [70]. CFH and FHL-1 in the human host are responsible for preventing binding of factor B to C3b, supporting the dissociation of the C3bBb complex and acting as a cofactor for factor I-mediated cleavage of C3b, the central component of the three complement activation pathways [70-73].

Serum resistant *Borrelia* acquire CFH and/or FHL-1 by direct interaction with outer surface proteins designated CRASPs (Complement Regulator-Acquiring Surface Proteins) [74]. Previously, five different CRASPs have been described for *B. burgdorferi* ss and *B. afzelii*. The CFH and FHL-1 binding CspA protein is (also

designated CRASP-1) encoded by *cspA*, a gene located on the lp54 plasmid. Although the lp54 plasmid of *B. burgdorferi* and *B. afzelii* carries multiple genes encoding a number of paralogous proteins, also called the gbb54 orthologous family, only the CspA is capable of binding human CFH and FHL-1 [76]. CspA is upregulated by spirochetes during the tick-mammalian transmission stage and down regulated during persistent infection [79, 80] . CspZ is a distinct protein encoded by the *cspZ* gene located on plasmid lp28-3 and is expressed at higher levels during the mammalian infection than in bacteria residing in ticks or during laboratory cultivation [79]. Anti-CspZ antibodies can be detected as early as two weeks post infection in mice infected by ticks [84]. CspZ has been shown to bind other yet unknown proteins and therefore can have multiple functions [63, 80, 84, 85]. The CFH-binding CRASP proteins BbCRASP-3, -4, and -5 belong to the OspE-related proteins (Erp) paralogous family and their respective genes are located on diverse cp32 prophage DNA molecules [87]. Erp proteins are expressed in tissues in the host during disseminated mammalian infection. Erp proteins have also been shown to be able to bind to factor H related proteins-1 (CFHR1) and plasminogen [53, 92, 94-97].

In contrast to *B. burgdorferi* ss and *B. afzelii* most *B. garinii* strains are unable to bind human complement regulators [513]. Two CspA orthologs from *B. garinii* ST6 ZQ1, named BgCRASP-1α and BgCRASP-1β, have been shown to bind weakly to FHL-1 but not to human CFH [77]. Little data is published on complement evasion strategies of human serum resistant strains of the *B. garinii* ST4 strains. The gbb54 orthologous family of *B. garinii* ST4 has not been studied before.

It has been elaborately shown which gbb54 ortholog from *B. burgdorferi* ss and *B. afzelii* can bind human CFH, but little is known about the function of the other orthologs. It has been described previously that CspA derived from *B. burgdorferi* ss interacts with human CFH; however none of the closely related protein of the gbb54 family, interacts with human CFH [514]. Wallich et al.characterized all gbb54 orthologous members of a *B. afzelii* and *B. garinii* strain wherein none of the remaining orthologs could bind human CFH/FHL-1 [76, 77]. We hypothesize that orthologs from the gbb54 family have the ability to bind to CFH from several animal origins.

The aim of the present study was to investigate the mechanism for complement evasion by *B. garinii* ST4 strains and to isolate and functionally characterize the specific gbb54 orthologs binding to human CFH/FHL-1 and also to other mammalian and avian CFH. We could prove binding of 2 ST4 gbb orthologs, BGA66 and BGA71, to human FHL-1, whereas BGA66 also bound CFH. Moreover, both these and other orthologs from the gbb54 family were also able to bind CFH from various animal species.

Materials and methods

Borrelial strains and culture conditions

B. garinii strains PBi and VSBP as well as *B. burgdorferi* ss strain B31 were cultured until mid-log phase (5 x 10^7 cells per ml) at 33°C in modified Barbour-Stoenner-Kelly (BSK-H) medium (Sigma). Aliquots of 1 ml were then diluted 1:1 with glycerol peptone (8% glycerol, 1% w/v Proteose Peptone 3 (Brunschwig chemie, Amsterdam) in distilled water), dispensed into screw-cap tubes (Nunc, Wiesbaden, Germany), frozen at –80°C, and used as stock cultures. Prior to use, a frozen suspension of spirochetes was thawed and inoculated into fresh BSK-H medium.

Serum bactericidal assay

Serum susceptibility of *Borrelia* was determined as described previously [64]. Briefly, serum obtained from a non-immune human donor (NHS) was frozen at -80°C and thawed on ice prior to use. Heat inactivated (HI) serum was incubated for 1 hour at 56 °C in order to inactivate complement. *B. garinii* ST4 PBi, *B. garinii* non-ST4 VSBP, and *B. burgdorferi* ss B31 were cultured until mid-log phase in BSK-H. An aliquot of 50 µl containing 10^7 live *Borrelia*/ml was added to 50 µl of serum and incubated for 1 and 3h at 33°C. After incubation aliquots of 5 µl were drawn from the suspensions and mobility and blebbing of the spirochetes was assessed under dark-field microscopy. One hundred spirochetes were examined, motile cells as well as non-motile cells were counted and the percentage of survival was calculated. The experiment was repeated three times.

Immunofluorescence assay

Immunofluorescence microscopy was performed as described previously [100]. Briefly, freshly cultured *B. garinii* strains PBi, VSBP, and *B. burgdorferi* ss B31 were incubated for 30 minutes in BSK-H medium containing 25% NHS. Subsequently spirochetes were washed twice with PBS/1% BSA, resuspended in the same buffer and air dried on microscope slides overnight. After fixation in 100% methanol, slides were incubated with human immune serum containing anti-*Borrelia* antibodies (1:2000) and a mAb recognizing a neoepitope of the terminal C5b-9 complex (1:1000) (DAKO). Slides were washed with PBS-1% BSA and incubated with an anti-human immunoglobulin G-fluorescein isothiocyanate-labeled antibody (1:100) (bioMérieux) and an anti-mouse immunoglobulin G Cy3-labeled antibody (1:1000) (Jackson). Afterwards slides were washed three times and mounted with Mowiol (Hoechst). Spirochetes

were visualized by confocal microscopy using an Axioscop 2 mot plus fluorescence microscope (Carl Zeiss).

Serum adsorption experiments

Borrelia (2 x 10^9 cells) were grown to mid-log phase, harvested by centrifugation (5,000 x *g*, 30 min, 4°C), and resuspended in 100 µl of veronal-buffered saline (supplemented with 1 mM Mg^{2+}-0.15 mM Ca^{2+}-0.1% gelatine, pH 7.4). To inhibit complement activation, NHS was incubated with 0.34 mM EDTA for 15 min at room temperature. The spirochete suspension was then incubated in 1.5 ml of NHS-EDTA for 1 hour at room temperature with gentle agitation. After three washes with phosphate-buffered saline (PBS) (supplemented with.15 M NaCl, 0.03 M phosphate, 0.02% sodium azide, pH 7.2), 0.05% Tween 20. The proteins bound to the cells were eluted by incubation with 0.1 M glycine-HCl, pH 2.0, for 15 min. Cells were removed by centrifugation at 14,000 x *g* for 20 min at 4°C, and supernatants were then analyzed by Western blotting.

Protease degradation assay

To characterize protease-susceptibility of CFH and FHL-1 binding proteins of *B. garinii* ST4 PBi, cells were treated with two different proteases as described previously [75]. Briefly, spirochetes were grown to mid-log phase, sedimented by centrifugation at 5,000 x *g* for 30 min, washed twice with cold PBS containing 5 mM $MgCl_2$ (PBS-Mg), and resuspended in 100 µl PBS-Mg. To the *Borrelia* cell suspension (at a concentration of 10^8 in a final volume of 0.5 ml), proteinase K or trypsin was added to a final concentration of 12,5 µg/ml to 100 µg/ml. Following incubation for 1 hour at room temperature, proteolytic degradation with proteinase K or trypsin was terminated by the addition of 5 µl of phenylmethylsulfonyl fluoride or by the addition of 5 µl of phenylmethylsulfonyl fluoride and 5 µl of 4-(2-aminoethyl)-benzenesulfonyl fluoride, respectively. *Borrelia* were then gently washed twice with PBS-Mg, resuspended in 20 µl PBS-Mg, and lysed by sonification five times using a Branson B-12 sonifier (Heinemann, Schwäbisch Gmünd, Germany). Equal volumes of *Borrelia* lysates were subjected to Tris/Tricine SDS-PAGE, and proteins were transferred to nitrocellulose membranes as described previously [74]. Susceptibility of proteins to proteolytic degradation was assessed by Western or ligand affinity blotting with the appropriate monoclonal or polyclonal antibodies, followed by incubation with a horseradish peroxidase-conjugated IgG antibody, and then visualized by the addition of 3, 3', 5, 5'-tetramethylbenzidine.

PCR cloning, expression and purification of recombinant CspA orthologous proteins

Sequences of genes encoding for CspA B31 and orthologs from *B. garinii* ST4 PBi were obtained from genbank (NC_006129 and NC_001857). Primers were designed using primer3 (MIT) and listed in table 2.

Oligonucleotides	Sequence (5'-3')	Target
BBA68s	ATGCGGCCGTGTTGTGTTTTAGTTTGGAT	BBA68
BBA68as	GTGGGATCCCATGCGCACCTTTTAGCAA	BBA68
BGA66s	ATGCGGCCGTGTTTTTAGTTTGGGCTCT	BGA66
BGA66as	GTGGGATCCCATGTGCCGTTAATAAAAATTG	BGA66
BGA67s	ATGCGGCCGATCAAGTGCAACGTATTTTT	BGA67
BGA67as	GTGGGATCCCATGTGCCGTTAATAAAAATTG	BGA67
BGA68s	ATGCGGCCGACATTATTGTTTTTAGTTTGGACTCT	BGA68
BGA68as	GTGGGATCCCATGTGCTGATAAAACC	BGA68
BGA71s	ATGCGGCCCATTGTTGTTTTTGGTTTAGACTC	BGA71
BGA71as	GTGGGATCCCATGTGTGCTGTTGATAAAATAG	BGA71
qFlaBs	GCTTCTGATGATGCTGCTG	FlaB
qFlaBas	TCGTCTGTAAGTTGCTCTATTTC	FlaB
qFlaB Taqmanprobe	GAATTRGCAGTAACGG-FAM	FlaB
qBGA66s	AGTTGTGCAGCAGCAATTTT	BGA66
qBGA66as	ATCCAGATCCTTTAAAGAC	BGA66
qBGA71s	TTCATATAGGTTGCTAATGCG	BGA71
qBGA71as	TTGCACACTCAAAACCAAAAA	BGA71

Table 2: Oligonucleotides used in this study

Amplification reactions were performed in a 50 µl final volume, containing 25 µl IQ Supermix (Bio-Rad, Veenendaal, the netherlands), 15 pmol forward primer, 15 pmol reverse primer, and 10 µl of a DNA isolate of cultured B31 or PBi. Following an enzyme activation step for 3 min at 95°C, amplification comprised 50 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C. Genes lacking their leader sequences were ligated in frame into the pGEX-5X3 vector (Amersham Bioscience, Freiburg, Germany). The ligation mixtures were used to transform *Escherichia coli* MC1061. Plasmid DNA was prepared from the presumptive clones with the QIAprep kit (QIAGEN, Hilden, Germany), and the *Borrelia* DNA inserts were sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems International, Foster City, CA, USA) in accordance with the manufacturers' recommendations. Plasmids were used to transform *E. coli* BL21. Expression of the GST fusion proteins was done by induction of the respective BL21 clones induced for 5 hours with 1mM IPTG, followed by affinity purification with glutathione-Sepharose 4B (GE Healthcare, Netherlands). Expression and purity of generated GST fusion proteins were confirmed by

employing SDS-PAGE, and protein concentrations were determined by a Bradford assay (Bio-Rad, Munich, Germany).

Real Time-PCR analysis

For determining expression in vitro cultures of PBi spirochetes grown to mid log phase were isolated. Nucleic acid was extracted with a QiaAmp *Mini Blood DNA* kit (Qiagen, Hilden, Germany). Total nucleic acid was treated with DNAse and 1µg RNA was reverse transcribed using iScript (Bio-Rad) according to the manufacturer's protocol. Primers and probe for the *flaB* gene were designed from an interspecies conserved region of *flaB* using the Beacondesigner and listed in table 2. Amplification reactions were performed in a 50-µl final volume, containing 25 µl IQ Supermix (Bio-Rad, Veenendaal, the netherlands), 15 pmol forward primer, 15 pmol reverse primer, 2.5 mM $MgCl_2$, 0.3 µM FlaB-probe, or 1x Sybrgreen (Molecular Probes), and 10 µl cDNA. Following an enzyme activation step for 3 min at 95°C, amplification comprised 50 cycles of 30 sec at 95°C, 30 s at 55°C and 30 s at 72°C in an iCycler IQ real-time detection system (Bio-Rad). The FlaB assay was optimized using a TA vector into which the complete *flaB* encoding gene from *B. burgdorferi* ss B31 had been cloned and had an analytical sensitivity of 1 copy per PCR in 0.9% saline. Quantitative DNA analysis was performed using the Icycler IQ5 PCR system. The relative starting copy number was determined by cycle threshold detection using Icycler relative quantification software (Roche).

SDS-PAGE, ligand affinity blot analysis, and Western blotting

Purified recombinant fusion proteins (500 ng) were subjected to 10% Tris/Tricine-SDS-PAGE under reducing conditions and transferred to nitrocellulose as previously described [74, 515]. Briefly, after transfer of proteins onto nitrocellulose, nonspecific binding sides were blocked using 5% (w/v) dried milk in TBS (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1% Tween 20 for 1 hour at room temperature. Subsequently, membranes were rinsed four times in TBS and incubated for 1 hour at room temperature with TBS containing recombinant FHL-1, pooled non-immune human serum (NHS), or non-immune animal sera. To detect the fusion proteins a goat anti-GST antibody (dilution 1:2,000) (GE Healthcare, Germany) was used. Polyclonal rabbit anti-SCR1-4 antiserum (□SCR1-4) (dilution 1:1,000) used for the detection of FHL-1 and monoclonal antibody (mAb) VIG8 (undiluted) against the C-terminus of CFH, are described elsewhere [72, 516]. After four washings with 50 mM Tris-HCl (pH 7.5)-150 mM NaCl-0.2% Tween 20 (TBST), membranes were incubated for 1 hour with either a polyclonal rabbit antiserum recognizing the N-terminal region of CFH (□SCR1-4) or mAb VIG8, directed against the C-terminus of CFH. Following four

washes with TBST, strips were incubated with a peroxidase-conjugated anti-rabbit IgG antibody or with a peroxidase-conjugated anti-mouse IgG antibody (DAKO, Glostrup, Denmark) for 1 hour at room temperature. Detection of bound antibodies was performed by using 3, 3', 5, 5'-tetramethylbenzidine as substrate.

ELISA

Recombinant proteins (500 ng/well) were immobilized on wells of a microtiter plate overnight at 4°C. Unspecific binding sites were blocked with 0.1% gelatin in PBS for 6 h at 4°C. CFH (Calbiochem), or recombinant FHL-1 was added to the wells and left overnight at 4°C. Polyclonal goat anti-CFH antibody (Calbiochem) was added for 3 h at room temperature, protein complexes were identified using secondary horseradish peroxidase–coupled antiserum. The reaction was developed with 1,2-phenylenediamine dihydrochloride (Dako-Cytomation), and absorbence was measured at 490 nm.

Binding domains of CFH and FHL-1 to CspA orthologs To identify domains of CFH and FHL-1 responsible for binding of BGA66 and BGA71, 500ng purified recombinant protein was separated by 10% Tris/Tricine SDS-PAGE and transferred to nitrocellulose. Membranes were then incubated with either recombinant FHL-1 (FH1-7), deletion constructs of CFH (FH1-2, FH1-3, FH1-4, FH1-5, FH1-6, FH8-20, FH19-20), or human serum as source for CFH. Bound proteins were visualized using polyclonal goat anti-CFH antibody (Calbiochem), or mAb VIG8.

Statistical analysis

All statistical analyses were done using SPSS 16.0 and Microsoft Excel software. The two-tailed Student t-test was used to analyze ELISA results. Values of $p<0.05$ were considered to be significant.

Results

Serum susceptibility testing of borrelia strains

To assess and to compare serum susceptibility of *B. garinii* PBi and VSBP as well as *B. burgdorferi* ss B31, spirochetes were incubated for 3 h with either 50% NHS or 50% HI NHS. As shown in figure 1, >75% of the cells of *B. garinii* ST4 PBi and *B. burgdorferi* ss B31 survived in serum, indicating that both strains resist complement-mediated killing. In contrast, *B. garinii* non-ST4 strain VSBP was highly sensitive to complement as 99% of the cells were immobilized and showed blebs after 3 hours. Incubation of strains PBi, VSBP, and B31 with HI

NHS resulted in no or very little immobilization. Summarizing *B. garinii* ST4 PBi and *B. burgdorferi* ss B31 are resistant to human serum when incubated with active human complement, while *B. garinii* non-ST4 VSBP is not human serum resistant.

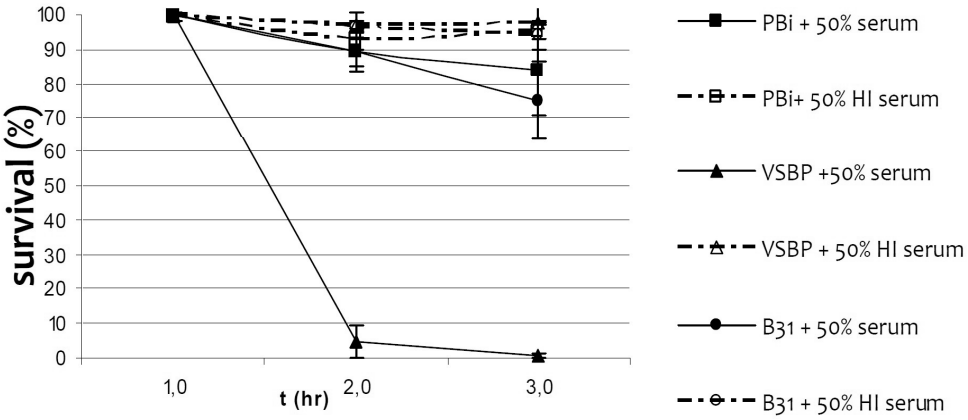

Figure 1: In vitro serum susceptibility of B. garinii ST4 PBi, B. garinii non-ST4 VSBP, and B. burgdorferi ss B31. Resistance to complement was determined by counting motile spirochetes by dark-field microscopy and values obtained were represented as percentages of survival. All strains were tested in triplicate with 50% NHS and HI NHS. VSBP is rapidly killed by complement, while >75%of B. burgdorferi ss B31 and B. garinii ST4 PBi are alive after 3 hours of incubation.

The detection of the membrane attack complex deposited on borrelial cells after complement activation

To test whether membrane attack complex (MAC) was formed on the surface of different strains after complement activation, spirochetes were incubated with 25% serum and deposition of the MAC was detected by immuno-fluorescence microscopy (IF) (figure 2). The majority of the cells of *B. garinii* ST4 PBi and *B. burgdorferi* ss B31 stained negative for the MAC while all *B. garinii* non-ST4 VSBP were fully covered with MAC. This finding indicates that *B. garinii* ST4 PBi and *B. burgdorferi* ss B31 allow formation of the MAC on their bacterial surface only to a limited extent in comparison to *B. garinii* non-ST4 strain VSBP.

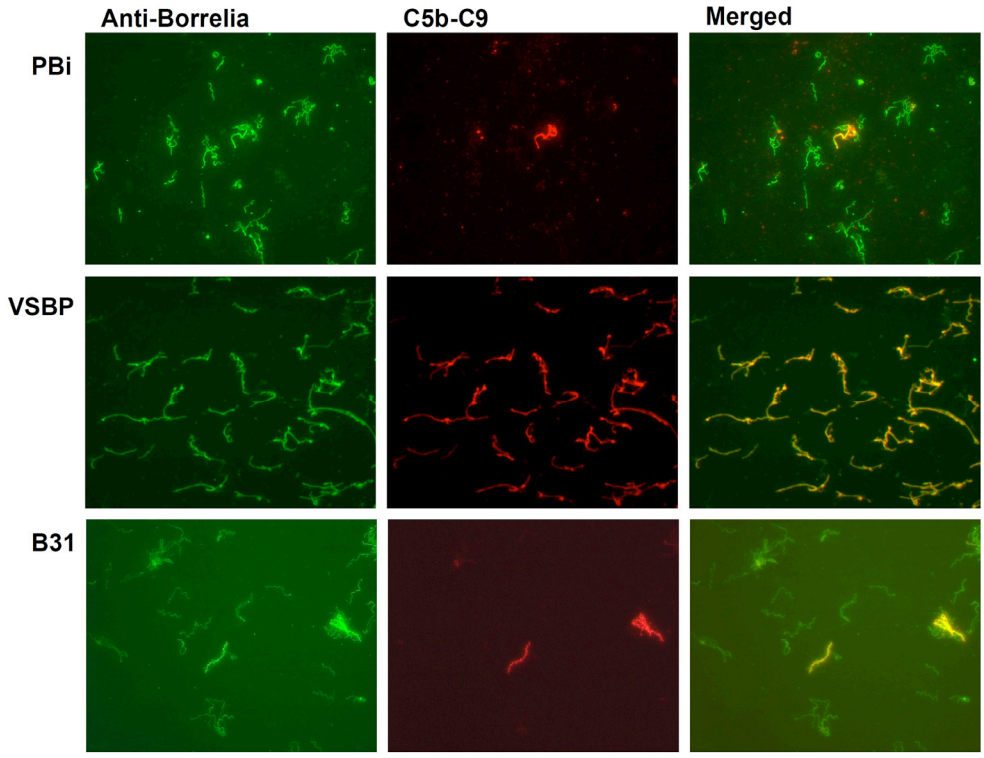

Figure 2: Detection of deposited C5b-9 complex on the surface of Borrelia by Immunofluorescence microscopy. B. garinii PBi and VSBP and B. burgdorferi ss B31 were incubated with 25% NHS and deposition of C5b-C9 was detected by a MAb. Few cells of B. garinii ST4 PBi stained positive for C5b-C9, while almost all spirochetes were covered with C5b-C9 using B. garinii non-ST4 VSBP. The absence of deposition of C5b-C9 onto B. burgdorferi ss B31 is comparable to B. garinii ST4 PBi.

Detection of bound complement regulators to different borrelial strains

In order to elucidate the capability of serum resistant *B. garinii* ST4 PBi to bind complement regulators CFH and FHL-1 to the surfaces in a non-denatured state, intact spirochetes were incubated with NHS which was supplemented with EDTA to prevent complement activation. Complement regulators were allowed to adsorb to the *Borrelia* surface and bound proteins were subsequently eluted with acidified 0.1 M glycine. The wash and the eluate fraction were analyzed for the presence of CFH and FHL-1 by Western blotting. As shown in figure 3, FHL-1, but not CFH could be detected in the eluate fraction indicating that *B. garinii* ST4 PBi specifically interact with FHL-1.

Accessibility and surface exposure of CFH/FHL-1 binding proteins of B. garinii ST4 PBi

In order to identify FHL-1 binding proteins produced by *B. garinii* ST4 PBi and to determine whether these proteins are exposed to the extracellular space, spirochetes were treated with increasing concentrations of proteinase K or trypsin and proteolysis was detected by ligand affinity blotting. Cell lysates obtained after protease treatment were separated by SDS-PAGE, transferred to nitrocellulose and the respective proteins were detected. As shown in figure 4, four distinct binding proteins could be detected in untreated serum-resistant *B. garinii* ST4 PBi. Treatment with proteinase K at the lowest concentration resulted in the complete elimination of CFH/FHL-1 binding. Upon treatment with trypsin, degradation was only achieved at a concentration of 100µg/µl. As expected, the intracellular protein flagellin was resistant to trypsin and proteinase K treatment, even at the highest concentration. These data demonstrate that *B. garinii* ST4 PBi produced up to four surface-exposed CFH/FHL-1 binding proteins, in the range of 19-26 kDa. This is in concordance to the findings of McDowell et al, where *B. garinii* ST4 PBi expressed a 20.5 and 26 kDa protein that were found to interact with CFH [517]. The CspA orthologs tested in this study are in the range of 25-27kDa, the smaller proteins detected appear to belong to the Erp protein family.

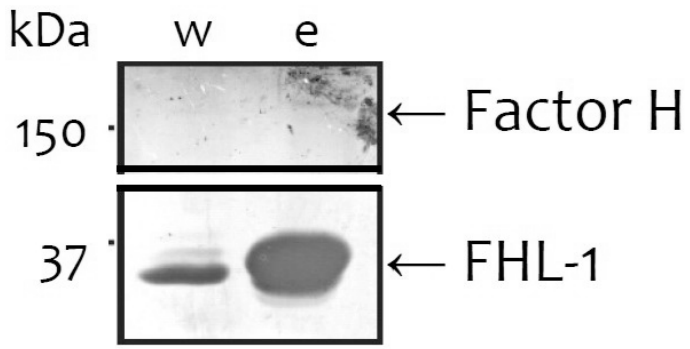

Figure 3: Detection of bound complement regulators by B. garinii ST4 PBi
After incubation of spirochetes with NHS-EDTA, bound proteins were eluted. The wash (w) and the eluate (e) fraction were separated by SDS-PAGE. The last wash and eluate fraction were subjected to SDS-PAGE and separated proteins were blotted on nitrocellulose. CFH and FHL-1 were visualized using a polyclonal goat anti-factor CFH antiserum (Calbiochem). It is shown that B. garinii ST4 PBi is able to bind FHL-1 on its membrane.

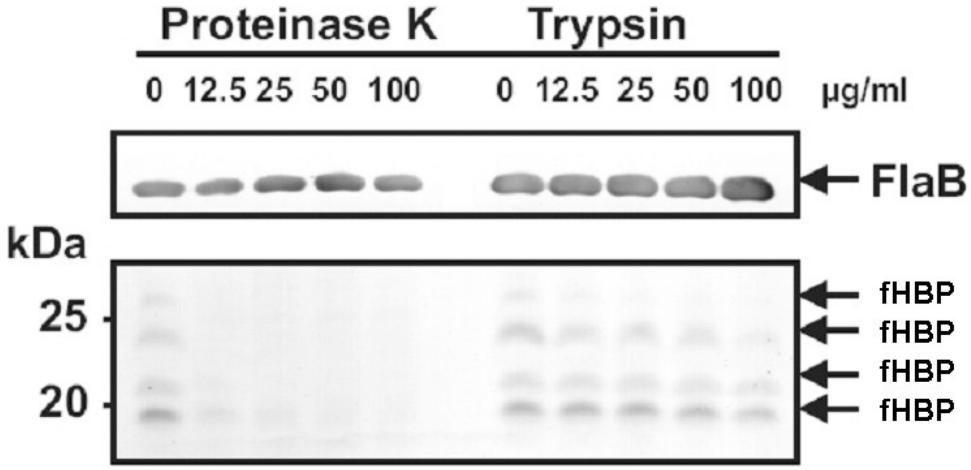

Figure 4: Accessibility of CFH/FHL-1 binding proteins of B. garinii ST4 PBi by different proteases. Spirochetes of B. garinii ST4 PBi were incubated with either proteinase K or trypsin at concentrations of 12.5 to 100µg/ml or in buffer without any protease (0). After 1h of incubation, cells were lysed by sonification as described in Materials and Methods. Each protein lysate was then subjected to 10% Tris/Tricine SDS-PAGE, blotted to membranes, and analyzed by Western or ligand affinity blotting. CFH/FHL-1 binding proteins were identified using NHS and a polyclonal anti-CFH antibody. Equal sample loading was assessed by detection of flagellin (FlaB) using MAb L41 1C11 1C11 at a dilution of 1:1000. Mobility of molecular mass standards are indicated to the left. Four proteins able to bind CFH/FHL-1 and they are readily digested by proteinases and therefore located on the membrane.

Cloning and identification of the CFH/FHL-1 binding proteins of B. garinii *ST4 PBi*

Assuming that the genes encoding CFH/FHL-1 binding proteins of *B. garinii* ST4 PBi share similarity to CspA encoding *cspA* gene of *B. burgdorferi* ss B31, *B. afzelii* MMS and *B. garinii* ZQ1, a database search was conducted. Four genes revealed a high degree of similarity with either CspA of *B. burgdorferi* ss B31, *B. afzelii* MMS or *B. garinii* ZQ1 as described previously [75, 77]. BGA66, BGA67, BGA68 and BGA71 showed similarity to previously described CspA of about 50%. Comparative sequence analysis revealed that orthologs BGA66 and BGA71 were found to have the highest degree of similarity within the putative CFH/FHL-1 binding regions of CspA (region 1-3) [518-520]. BGA66, BGA67, BGA68 and BGA 71 as well as CspA of *B. burgdorferi* ss strain B31 were cloned and expressed as GST fusion proteins.

Determination of binding of CspA orthologs to CFH and FHL-1

Binding of CFH and FHL-1 to non-denatured purified recombinant proteins was evaluated by ligand affinity blot. Proteins were separated under denaturing conditions and subsequently blotted on a nitrocellulose membrane. As shown in figure 5, BbCspA used as positive control bound strongly to CFH and FHL-1 as

described previously [75]. Orthologs BGA66 and BGA71 were capable of binding to both complement regulators, however, with reduced intensities compared to CspA.

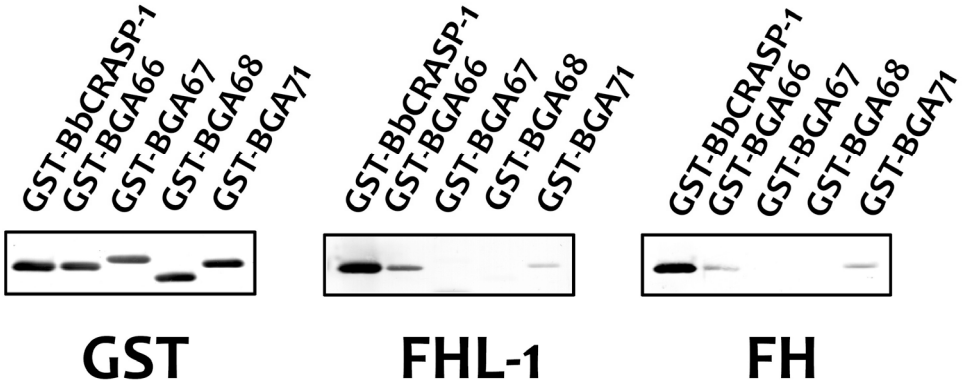

Figure 5: Binding capacities of CFH and FHL-1 to CspA orthologs of B. garinii ST4. Purified GST fusion proteins, BbCspA, BGA66, BGA67, BGA69, and BGA71 (500 ng/lane) were subjected to 10% Tris/Tricine SDS-PAGE and blotted to nitrocellulose membranes. Membranes were then incubated with recombinant FHL-1 or with NHS. GST-fusion proteins were detected by using anti-goat GST antibody and binding to CFH and FHL-1 were visualized using mAb VIG8 specific for the C-terminal region of CFH and ?SCR1-4 antiserum specific for the N-terminal region of FHL-1. Binding of CFH and FHL-1 is visible for BGA66 and BGA71.

To further confirm binding of CspA orthologs an ELISA was conducted. CspA orthologs BGA66, BGA67, BGA68, and BGA71 were immobilized on a microtiter plate and binding of CFH and FHL-1 was evaluated (figure 6). BbCRASP-1 used as a positive control strongly bound to CFH and FHL-1. Of the four CspA orthologs analyzed, BGA66 was capable of binding to both complement regulators, this binding was significantly higher than the baseline ($p<0.05$). Ortholog BGA71 specifically bound to FHL-1 ($p<0.05$) but less efficiently than CspA and BGA66. In contrast, orthologs BGA67 and BGA68 were not able to bind to CFH or FHL-1 at all.

These data confirmed that orthologs BGA66 as well as BGA71 derived from *B. garinii* ST4 PBi were capable of binding FHL-1. Binding of CFH in both assays is evident for BGA66, but not for BGA71.

Mapping of the binding domains of CFH and FHL-1 to CspA orthologs

In order to map the binding regions of CFH and FHL-1 interacting with BGA66 and BGA71, various deletion constructs of CFH and FHL-1 were used for ligand affinity assays (figure 7). BGA66 bound to full-length CFH and FHL-1, but to none of the deletion constructs lacking SCRs 5-7. BGA71 bound FHL-1 as well as deletion constructs SCR1-5 and SCR1-6. Thus, SCR5-7 of both CFH and FHL-1 are required for binding to BGA66 and BGA71.

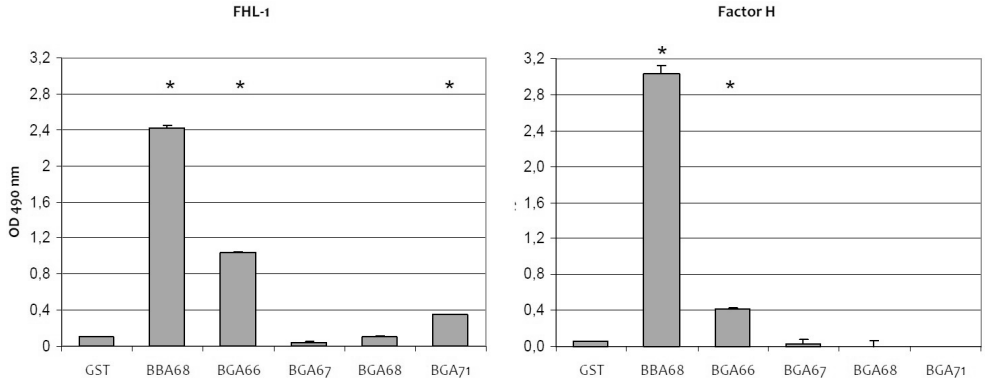

Figure 6: Binding of CspA orthologs to FHL-1 and CFH. Recombinant proteins (500ng each) were coated onto an ELISA plate and incubated with purified FHL-1 (A) and CFH (B). Binding was assayed by ELISA using polyclonal SCR1-4 that recognized CFH and FHL-1. All experiments were performed at least in triplicate. * ($p<0.05$ compared to baseline (GST) OD)

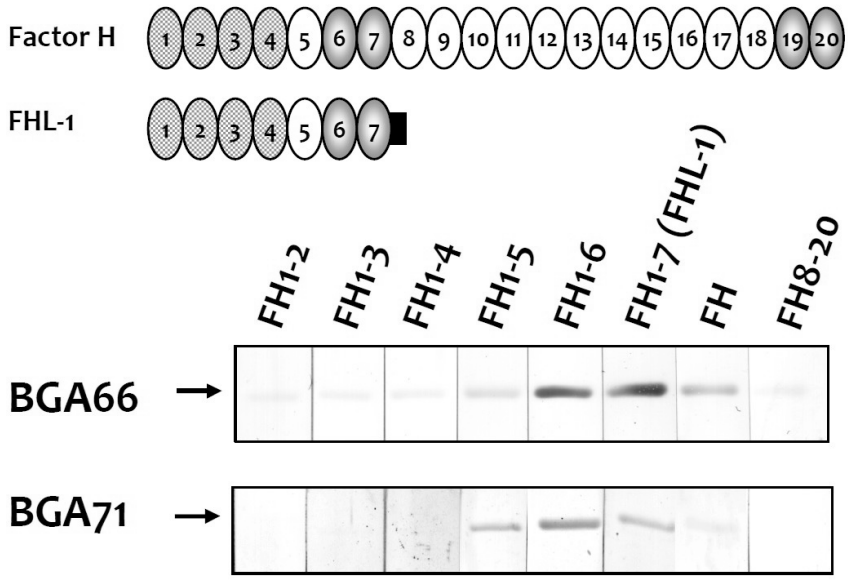

Figure 7: Mapping of the binding domains of CFH and FHL-1 for BGA66 and BGA71. Schematic representation of the CFH and FHL-1 protein and ligand affinity blot analysis of fusion proteins. The complement regulatory domains SCR 1-4 are in checked. Purified recombinant protein was separated by 10% Tris-Tricine-SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with either recombinant FHL-1 (FH1-7) or several deletion constructs of CFH (FH1-2, FH1-3, FH1-4, FH1-5, FH1-6, FH8-20) or with human serum (FH). Bound proteins were visualized using polyclonal goat anti-CFH (Calbiochem), or MAb VIG8 directed against the C-terminus of CFH. SCR 5-7 are essential SCR for binding of BGA66 and BGA71 to interact with CFH/FHL-1.

Expression of BGA66 and BGA71 by real-time RT-PCR

cDNA prepared from in vitro cultured B. garinii ST4 PBi were tested in a quantitative real time PCR. Cultures repeated in sexplet demonstrated a mean

expression of BGA66 of 34 copies/1000 copies flaB (SD 22) and BGA71 21 copies/1000 copies flaB (SD 18). All spirochetes cultivated in vitro expressed BGA66 and BGA71 simultaneously.

Analysis of CFH binding of different animal sera to CspA orthologs

A variety of sera obtained from different animals were used to analyze binding of CFH to CspA, BGA66, BGA67, BGA68, and BGA71 by ligand affinity blotting. As shown in Fig 8, CspA orthologs displayed distinct capacity of binding to CFH from a wide variety of sera from different mammals and poultry. All orthologs exhibit binding of CFH from bovine, equine and canine serum with different intensities. BGA68 and BGA71 showed a weak binding capacity to murine CFH. In addition, BGA68 but not CspA nor other orthologs bound to avian CFH. Porcine and feline serum proteins did not bind any of the CspA orthologs of *B. garinii* ST4 PBi while feline CFH appears to bind only to BbCspA.

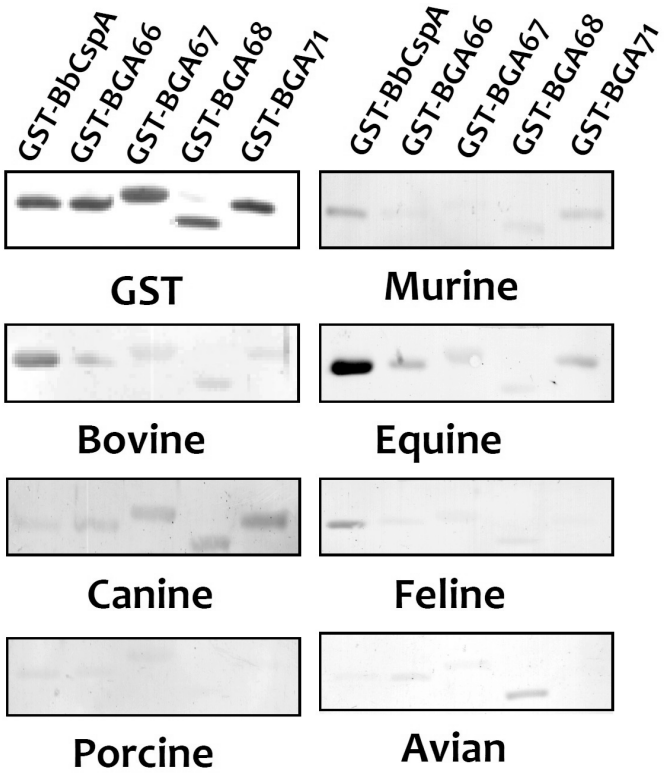

Figure 8: Interaction of different CspA orthologs to interact with animal CFH. Purified recombinant CspA and B. garinii ST4 CspA orthologs were subjected to 10% Tris/Tricine SDS-PAGE and blotted to nitrocellulose membranes. Recombinant proteins were visualized by an anti-GST antibody. Additional membranes were incubated with sera obtained from diverse animals. Interacting proteins were then visualized using a polyclonal anti-CFH antibody.

Discussion and conclusions

We are the first to demonstrate that *B. garinii* ST4 PBi is serum resistant and is able to acquire FHL-1 but not CFH from human serum. In addition, we identified two distinct CspA orthologs, BGA66 and BGA71 as potential ligands of complement regulators CFH and FHL-1. These proteins were produced under in vitro conditions as demonstrated by real time PCR. Finally, we demonstrated distinct binding capacities of CFH of different mammalian and avian origin to different CspA orthologs of serum resistant *B. garinii* ST4 PBi.

In Europe four human pathogenic genospecies are endemic. *B. burgdorferi* ss, *B. afzelii*, and *B. spielmanii* display a human serum resistant phenotype while *B. garinii* strains are often serum sensitive [61, 64, 65, 521, 522]. Within the OspA typing scheme, *B. garinii* ST4 strains represent a distinct branch as shown by random amplified polymorphic DNA (RAPD) analysis. On the basis of MLSA analysis it has recently been proposed, though not yet generally accepted, to delineate this subgroup in a separate species; *B. bavariensis* [512, 523]. *B. garinii* ST4 is remarkably often associated with dissemination to the CNS [508, 510, 511, 524]. In a previous study it was confirmed that *B. garinii* non-ST4 strains, including strains isolated from CSF, are sensitive to complement while *B. garinii* ST4 strains were resistant to human complement [64]. In this report we confirm with an in vitro killing assay and IF that *B. garinii* ST4 is resistant to human complement killing and that it does not allow formation of MAC on the spirochetal membrane.

It has been extensively shown that CspA fulfils a key role in complement resistance of *B. burgdorferi* ss [525, 526]. In the present study, a comparative binding analysis was conducted to isolate and characterize CspA orthologs from the serum resistant, *B. garinii* ST4 strain PBi. We hypothesized that binding of CFH and/or FHL-1 via CspA orthologs contributes to serum resistance of *B. garinii* ST4 PBi. We identified orthologs BGA66 and BGA71 but not BGA67 and BGA68 as being potential ligands for FHL-1 and CFH. In vitro cultured spirochetes bound FHL-1 but not CFH on their surface. The affinity for FHL-1 appeared to be stronger than for CFH, it can be concluded that FHL-1 competes with CFH for the same binding site and thus CFH could not be detected in the cell binding assay. When employing ELISA on recombinant proteins, BGA66 bound both complement regulators while BGA71 only bound FHL-1. By ligand affinity blotting BGA71 bound FHL-1 as well as CFH. A logical explanation for this phenomenon might be the denaturing conditions of the Western blot, suggesting that native BGA71 specifically interacts with FHL-1 only.

Furthermore, it was previously shown that CspA forms homodimers and three regions of CspA have been implicated in formation of a functional binding site of CspA to CFH/FHL-1 [518-520, 526]. Previously it has been hypothesized that the C-terminal YKXXDXXXP motif is important in binding of CFH and FHL-1, as well as the lysine residue at position 246 of CspA [77]. Recently it was also shown that a leucine residue at position 146 within the proposed CFH binding region 1 as well as Tyr240, Asp242 and Leu246 within the proposed binding region 3 of CspA were important in binding of CFH and FHL-1 [518]. The C-terminus of all known human CFH/FHL-1 binding CspA and the *B. garinii* ST4 gbb54 orthologs is shown in table 2. Comparative sequence analysis revealed that the C-terminus of BGA66 and BGA71 are highly homologous to the C-terminus of all known human CFH/FHL-1 binding CspA. Ortholog BGA66 contains the C-terminal motif as well as the Leu246, while BGA71 contains the C-terminal motif but has a phenylalanine instead of a leucine residue at position 246. Positions 146 and 240 are unchanged in BGA66 and BGA71 both orthologs show substitutions at position 242; the Asp242 in BGA66 and BGA71 is replaced by a glutamic acid and a threonine residue, respectively. A substitution of Asp242 by a neutral alanine residue within CspA did not have a significant effect on binding, while the replacement of aspartic acid by tyrosine at this position influenced binding of FHL-1 and is associated with a loss of binding of CFH [518]. Lack of binding of native BGA71 to CFH is likely to be due to the non-synonymous mutation of aspartic acid by threonine, while BGA66 can still bind both CFH and FHL-1 due to the synonymous mutation of aspartic acid to glutamic acid. It is likely that absence of CFH binding by BGA71 might be a result of an effect of the mutation on protein folding and conformation. Our finding that under denaturing conditions BGA71 can bind CFH, but not under native folded conditions supports this hypothesis.

Protein		**pos 240**										**pos 250**	
BbCspA	Y	□	□	*D*	F	□	T	*L*	K	P	A	F	Y
BaCspA	N	□	□	*D*	L	□	S	*F*	N	P	I	N	-
BgCspAα	N	□	□	*E*	F	□	P	*L*	N	L	D	Y	-
BgCspAβ	N	□	□	*T*	L	□	S	*F*	K	S	I	N	-
BGA66	N	□	□	*E*	H	□	S	*L*	K	P	I	Y	-
BGA67	N	□	□	*E*	F	N	S	*L*	K	P	I	Y	-
BGA68	N	□	□	*N*	L	H	S	*F*	K	T	V	Y	Y

Table 2: C-terminus of all CspA and *B. garinii* ST4 CspA orthologs. C-terminal end of CspA orthologs described in this study and previously determined. Positions 242 and 246 depicted in italic. The sequence for CspA derived from *B. burgdorferi* ss B31, BaCspA from *B. afzelii* MMS, ZQA68 (BgCspAα) and ZQA71 (BgCspA β) from *B. garinii* ZQ1, BGA66, BGA67, BGA68 and BGA71 from *B. garinii* ST4 PBi.

A number of Gram-negative as well as Gram-positive bacteria have already been shown to be able to bind CFH in order to protect themselves from complement-mediated lysis [527-529]. CFH possess three binding sites for complement C3b, however the only essential binding site is SCR1-4 [67, 70, 530]. Here we show that BGA66 as well as BGA71 bind SCR5-7 of CFH and FHL-1, thus leaving the N-terminus free for maintaining their regulatory activity in factor I-mediated inactivation of C3b [75]. Our finding indicates that *B. garinii* ST4 strains can bind functionally active CFH and FHL-1 on the membrane by BGA66 and BGA71 in order to evade complement activation.

B. burgdorferi sl has developed an intriguing system to respond to changes of the microenvironments by coordinated expression of proteins. In vitro experiments usually do not completely mirror the expression patterns of CspA during the tick to mammal infectious cycle and might also vary in cultured population [58]. CspA shows a distinct expression profile as it is mainly expressed during transmission of spirochetes from the tick-to-mammal and mammal-to-tick infection cycle [80]. Previously antibodies to CspA could be detected in sera from infected mice and from Lyme disease patients suggesting prolonged expression of CspA in the mammalian host [81-83]. In the present study we demonstrated that in vitro *B. garinii* ST4 PBi is capable of expressing BGA66 and BGA71. Experiments regarding expression of BGA66 and BGA71 during tick-to-mammal transmission and mammalian infection are ongoing and will give more insight in their function in vivo.

Although all five CRASPs of *B. burgdorferi* sl are primarily identified as ligands of human complement regulators, several studies clearly showed that CspA can also bind CFH from other mammalian hosts [63]. CFH binding of several animal CFH sources has also been reported in a recent article where new CFH binding proteins were identified [531]. It is still not quite clear how the wide variety of complement resistance is obtained in strains that do not interact with human CFH. The *B. burgdorferi* ss and *B. afzelii* orthologs of CspA were previously not studied for binding to CFH of non-human origin. In this study all CspA orthologs of *B. garinii* ST4 PBi were tested with whole sera from different animals. BGA67 and BGA68 lack binding to human CFH but were able to interact with CFH from other hosts, of which some are not competent reservoir hosts for *Borrelia*. It is likely that several members of the gbb54 paralogous family are designated to bind CFH from other species in the infectious cycle and are therefore not redundant but essential for infection of a wide range of hosts. The interaction of mammalian CFH with CspA orthologs of *B. burgdorferi* sl might unveil a part of the serum resistance patterns obtained from in vitro experiments.

In this study we demonstrated *B. garinii* ST4 PBi is able to evade complement killing and it can bind FHL-1 to membrane expressed proteins. Recombinant proteins BGA66 can bind FHL-1 and human CFH, while BGA71 can bind only FHL-1. All recombinant CspA orthologs from PBi can bind CFH from different animal origins. This can partly explain the wide variety of animals that *B. garinii* can infect.

Acknowledgements
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Chapter 3

Infectivity of Borrelia burgdorferi sensu lato is unaltered in C3 deficient mice

Nathalie D. van Burgel[1]
Nathalie C.M. Balmus[2]
Erol. Fikrig[3]
Alje. P. van Dam[4]

Ticks Tick Borne Dis. 2011 Mar;2(1):20-6.

[1] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Pathology, Leiden University Medical Center, Leiden
[3] Yale University School of Medicine, Department of Internal Medicine, New Haven,
[4] Department of Medical Microbiology, Onze Lieve Vrouwe Gasthuis, Amsterdam, the Netherlands

Abstract

B. burgdorferi, *B. afzelii* and *B. bavariensis* show resistance to mouse and human complement. *B. garinii* and *B. valaisiana* are sensitive to mouse and human complement. We evaluated whether the absence of C3 in mice influenced infectivity and pathogenicity of different *Borrelia* species.
C3 knockout mice (C3 -/-) and syngeneic C57Bl/6 wild type (WT) mice were challenged with five different *Borrelia* species. After two weeks, quantitative PCR (qPCR), culture, histopathology and immunofluorescence was performed on heart, joint, brain, bladder and skin.
Spirochetes were detected by qPCR after infection with *B. burgdorferi, B. afzelii* or *B. bavariensis* strains. In joints of C3 -/-, but not WT mice challenged with *B. burgdorferi*, spirochetes were detected by qPCR. No other significant differences between C3 -/- and WT mice were seen. Histopathology demonstrated concordance between borrelia load and inflammation score. Only after *B. burgdorferi* and *B. afzelii* infection spirochetes were detected by immunofluorescence microscopy.
B. burgdorferi was cultured from heart, joint, bladder and skin from all mice within 2 weeks. *B. afzelii* and *B. bavariensis* grew only from heart tissue from both C3 -/- and WT mice after 2-6 weeks.
The infectivity and pathogenicity of complement-resistant *Borrelia* strains is unchanged in complement-deficient mice. Complement-susceptible strains do not become infectious in the absence of C3.

Introduction

Lyme disease is a multiorgan tick-borne infection caused by spirochetes belonging to *Borrelia burgdorferi* sensu lato (sl) complex. Several species have currently been identified: *B. burgdorferi* sensu stricto (ss) is present in North America and in Europe, whereas *B. afzelii, B. bavariensis* and *B. garinii* are present only in Eurasia. In Eurasia other *Borrelia* species that have been associated with Lyme disease such as *B. valaisiana and B. spielmanii* have been isolated from ticks [532]. The pathogenicity of *B. valaisiana* in humans is still under discussion [143, 533, 534]. Ticks can carry many other borrelia species of which pathogenicity in humans is not yet described like *B. lusitaniae, B. tanukii, B. japonicum, B. turdi and B. andersonii. B. bavariensis* has recently been proposed to be delineated as a separate species. This species was previously known as *B. garinii* OspA serotype 4 [512].

Infectivity and pathogenicity of the different strains of the *B. burgdorferi* sl complex varies greatly. In mammals for instance the infectious dose of a *B burgdorferi* ss strain was much lower in comparison to that of a reference *B. afzelii* or *B garinii* strain [535]. Moreover, after infection with the *B. burgdorferi* ss strain, arthritis and carditis were much more severe [536]. Pachner et al. also showed that after infections with *B. garinii* or *B. afzelii* spirochete load was lower than in *B. burgdorferi* ss infected mice and non-human primates. Furthermore many *B. garinii* strains were not infective at all in mice [537].

Complement plays an important role in killing of bacteria. Deposition of complement on the membrane of bacteria can lead to opsonization and lysis of the bacteria, but also influences adaptive immune responses directly. Borrelia spirochetes differ in their susceptibility to complement [64]. In vitro, *B. afzelii*, *B. burgdorferi* ss and *B. bavariensis* strains are highly resistant to human and mouse serum, whereas *B. valaisiana* and *B. garinii* strains are readily killed by human and mouse serum [64, 65]. *B. burgdorferi* sl is capable of evading innate immunity by binding of host factor H and factor H-like protein 1 (FHL-1) [66-68]. Borrelia bind factor H/FHL-1 through expression of several proteins such as CspA, CspZ and OspE proteins, also designated Complement Regulatory Acquiring Proteins (CRASPs) on the spirochetal membrane. These CRASPs are upregulated during various parts of the mammalian tick transmission cycle but it is not always clear what roles they play in the protection to complement mediated killing [95, 538, 539]. Primarily *B. burgdorferi* and *B. afzelii*, but also *B. bavariensis* have the ability to bind factor H by CRASPs [77, 84, 540-542].

The role of complement in murine Lyme borreliosis has been addressed by Bockenstedt et al, who found that C5-deficient mice infected with *B. burgdorferi ss* ran a similar clinical course as wild type mice [543]. Lawrenz et al. demonstrated that infection of C3 deficient mice with *Borrelia burgdorferi* ss resulted in a higher spirochete load and higher histopathology scores in ear, ankles and heart, compared to wildtype (WT) mice, but only early in infection [191]. This was confirmed for skin tissue in another study [98]. Very little research has been done on the effect of C3-deficiency on the infectivity of other *Borrelia* spp. Lawrenz et al. described that all three C3 -/- mice infected with 10^4 spirochetes of a *B. garinii* strain developed a positive PCR on bladder tissue, whereas this was the case in only 1/3 WT mice. However, they found no infection in WT and C3 -/- mice after an inoculum of 10^3 spirochetes versus detectable infection of all mice after an inoculum of 10^5 spirochetes.
In the present study, WT and C3 -/- mice were injected with spirochetes belonging to different *B. burgdorferi* sl species to determine whether complement deficiency in mice leads to increased susceptibility to Lyme borreliosis. The infectivity and pathogenicity of the different species was assessed by culture, quantitative PCR and histology.

Materials and methods

Bacterial and mouse strains

The experimental protocol was approved by the Animal Ethical Committee of the Leiden University Medical Center. Mice were housed in an animal facility, placed in groups of 2 to 5 in polycarbonate cages with wide bar lids and micro-isolator tops and provided with food and water ad libitum.
C57Bl/6 C3 deficient mice were generated by replacing the 5'-flanking region of the C3 gene with a neomycin resistance marker [544]. These C3 (-/-) mice produce no detectable C3 protein and have no complement activity.
For infectivity testing *B. burgdorferi* ss N40 was used, for all other species a mix of 5 different *Borrelia* strains per species was used in order to correct for the per strain pathogenicity: for *B. afzelii* A17S, A20S, A57T, A63T, pKo; for *B. garinii* A77S, PBr, 20047, A87S, VSBM; for *B. bavariensis* A01C, A19S, A91S, A94S, PBi; for *B. valaisiana* IDP3, M19, M49, M53 and UK2 (Table1). The origin of the strains is shown in table 1. An uninfected C57Bl/6 mouse was also sacrificed to serve as a negative control. Before injection in the mice, fresh medium was added to the cultures and cultures were incubated at 37°C for 4 hours. Borrelia were counted and rinsed with 37°C PBS directly before injection. All mice were injected with $2*10^5$ spirochetes per strain in a total volume of 100 µl PBS, intradermally in the

scapular region of 6 weeks old mice [535, 545]. The high load was chosen due to the previous report that *B. garinii* have low infectivity rates under a needle inoculation dose of 10^5 [191]. Each group consisted of three mice. Mice were sacrificed two weeks post inoculation by asphyxiation. Heart, kidney, bladder, brain, joint and skin were collected. The period of two weeks was elected due to previous studies where the peak of the infection takes place two weeks after syringe inoculation [191, 459, 546].

Strain	Species	Biological source	Geographical origin	Serum resistance*
N40	*B. burgdorferi* ss	*I. scapularis*	US	Resistant
A17S	*B. afzelii*	Human Skin	The Netherlands	Resistant
A20S	*B. afzelii*	Human Skin	The Netherlands	Resistant
A57T	*B. afzelii*	*I. ricinus*	The Netherlands	Resistant
A63T	*B. afzelii*	*I. ricinus*	The Netherlands	Resistant
PKo	*B. afzelii*	Human Skin	Germany	Resistant
20047	*B. garinii*	*I. ricinus*	France	Sensitive
A77S	*B. garinii*	Human Skin	The Netherlands	Sensitive
A87S	*B. garinii*	Human Skin	The Netherlands	Sensitive
PBr	*B. garinii*	Human CSF	Germany	Sensitive
VSBM	*B. garinii*	Human CSF	Switzerland	Sensitive
A01C	*B. bavariensis*	Human CSF	The Netherlands	Resistant
A19S	*B. bavariensis*	Human Skin	The Netherlands	Resistant
A91S	*B. bavariensis*	Human Skin	The Netherlands	Resistant
A94S	*B. bavariensis*	Human Skin	The Netherlands	Intermediate
PBi	*B. bavariensis*	Human CSF	Germany	Intermediate
IDP3	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
M19	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
M49	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
M53	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
UK2	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive

Table 1: Strains used in this study. *Human and mouse serum sensitivity as determined in literature and remaining strains (B. valaisiana) were determined as described [64, 66, 513].

Real-time quantitative PCR

For quantification of bacterial loads a 5x5 mm biopsy of abdominal wall skin, one third of the heart, one third of the bladder, one quarter of the brain, and half of the tibiotarsal joint were collected and stored at –80 °C. Nucleic acids from organs were extracted with a QiaAmp *Mini Blood DNA* kit (Qiagen, Hilden, Germany) All samples were extracted according to the manufacturer's instructions, resulting in 200 µl of purified nucleic acids, which were stored at –20°C. Quantitative DNA analysis was performed using the iCycler PCR system. The relative starting copy number was determined by cycle threshold detection

using iCycler relative quantification software (Roche). Primers and probe for FlaB were designed from an interspecies conserved region of *FlaB* using the Beacondesigner software. Sequences used for the PCR are FlaB forward 5'-GCT TCT GAT GAT GCT GCTG-3', FlaB reverse 5'-TCG TCT GTA AGT TGC TCT ATT TC-3' and FlaBProbe 5'-GAATTRGCAGTAACGG-FAM 3'. The assay was optimized using a TA vector into which the complete FlaB gene from strain B31 had been cloned and had an analytical sensitivity of 1 copy per PCR in saline 0.9% *w/v*. The oligonucleotide primers used to quantify murine β -actin were β -act forward (5'-CAA TAG TGA TGA CCT GGC CGT-3') and β -act reverse (5'-GA GGG AAA TCG TGC GTG AC -3'). Amplification reactions were performed in a 50-µL final volume, containing 25 µL IQ Supermix (Bio-Rad, Veenendaal, the netherlands), 15 pmol forward primer, 15 pmol reverse primer, 2.5 mM $MgCl_2$, 0.3 µM probe or Sybrgreen (Molecular Probes inc.), and 10 µL DNA extract. Following an enzyme activation step for 3 min at 95°C, amplification comprised 50 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C in an iCycler IQ real-time detection system (Bio-Rad). Total load was expressed in copies FlaB/1000 copies β -actin. Amplification of joint tissue initially resulted in negative results of the β -actin PCR due to the presence of inhibitory substances, therefore all joint samples were additionally tested in a 1/10 dilution. Minimal detection limit per PCR reaction for spirochete load was 0.1 copies FlaB/1000 copies β-actin.

Pathology

Histopathology was performed on multiple sections of all organs fixed using 4% neutral buffered formalin, embedded in paraffin, sectioned at 3 µm thickness and stained with hematoxylin/eosin (HE). Joints were decalcified before embedding. Sections were evaluated in a blinded fashion. Inflammation was scored from zero to three, zero denoting normal tissue and three being severe inflammation, based on infiltration of granulocytes, exudate and synovial proliferation for joints [547]. Inflammation in the other organs was based on infiltration of lymphocytes, macrophages, plasma cells and necrosis [548]. Immunofluorescence (IF) staining was performed on multiple sections of all organs fixed using 4 % neutral buffered formalin, embedded in paraffin and sectioned at 3 µm thickness. Sections were dried on Starfrost (Klinipath) slides, deparaffined, dehydrated, and trypsinized for 20 minutes at 37 °C. Subsequently slides were rinsed with demineralised water, washed three times in sterile PBS and incubated with 1:100 dilution of a rabbit anti-*B. burgdorferi* serum (ImmunoLogic). Slides were washed three times with PBS and incubated with a 1:50 dilution of FITC-conjugated swine anti-rabbit Ig (DakoCytomation), washed again three times in sterile PBS, and coverslips were mounted using Vectorshield mounting medium (Vector Laboratories, Burlingame, USA). Recordings were made using an Axioplan 2 microscope. The assay was

validated with clinical materials from patients with an EM, borrelia culture and borrelia negative tissue samples and used in a clinical setting. Spirochete load was scored according to the amount of spirochetes per low power field (20x), from zero (none), one (1-10), two (10-50) and three (>50). This was performed in a blinded fashion.

Culture

For borrelia culture a 5x5mm biopsy of abdominal wall skin, one third of the heart, one third of the bladder, one quarter of the brain, half a kidney and half of the tibiotarsal joint were harvested and incubated in BSK-H medium (Sigma) supplemented with fosfomycin (100ng/ml) and rifampicin (500ng/ml). Cultures were kept at 33 ˚C and checked under darkfield microscopy weekly for six weeks. All cultures were passaged into fresh medium after two weeks. Visual confirmation of live motile spirochetes was considered a positive culture.

Arthritis score

After 10 days arthritis score was done by visual confirmation of swelling of the right ankle. Swelling was scored zero to three, zero being no swelling and three being severe swelling of the ankle.

Statistical analysis

All statistical analyses were done using SPSS 16.0 and Microsoft Excel software. The two-tailed Student t-test was used to analyze quantitative PCR results. Values of $p < 0.05$ were considered to be significant.

Results

Culture

Spirochetes grew from all organs from mice injected with *B. burgdorferi* ss within 2 weeks (Table 2). From the animals injected with *B. afzelii*, spirochetes could be cultured from the heart of 2/3 WT and 1/3 C3-/- mice after 4 weeks. All other cultures from these mice remained negative. From mice injected with *B. bavariensis*, spirochetes grew only from the heart of 1/3 C3-/- mice and 1/3 WT mice both after two weeks. None of the cultures of mice challenged with *B. garinii* and *B. valaisiana* strains became positive.

Real time PCR

At two weeks of infection *B. burgdorferi* ss N40 was detectable by qPCR in bladder, heart, skin and ankles (Table 2).The average number of *B. burgdorferi* ss spirochetes was only significantly higher in the ankles of C3 -/- mice ($p<0.05$),

since spirochete DNA was not detectable in the ankles of WT mice. Spirochetes were not detected by qPCR in the brain of WT or C3 -/- mice.
Regarding the mice injected with *B. afzelii* strains; bladder, heart, skin and ankle tissue had a detectable load of spirochetes in all mice. Loads of spirochetes were slightly, but not significantly higher in bladder, heart and skin of C3 -/- mice. No effect was seen on the load of *B. afzelii* in the ankles. Only one mouse in the wild type group had a weakly positive qPCR signal in brain tissue.
In heart, skin and ankle tissue of mice injected with *B. bavariensis* strains a low, but detectable load of spirochetes was found. Loads were comparable in the WT and C3-/- mice. All mice injected with *B. bavariensis* were infected at two weeks, because at least one of the organs of all mice was positive by PCR. However, all bladder samples and six of nine other organ samples (joint, skin and heart) from C3 -/- mice and three of nine organ samples from WT mice were negative in the PCR. None of the mice injected with *B. garinii* or *B. valaisiana* strains had detectable spirochete DNA in any of the organs.

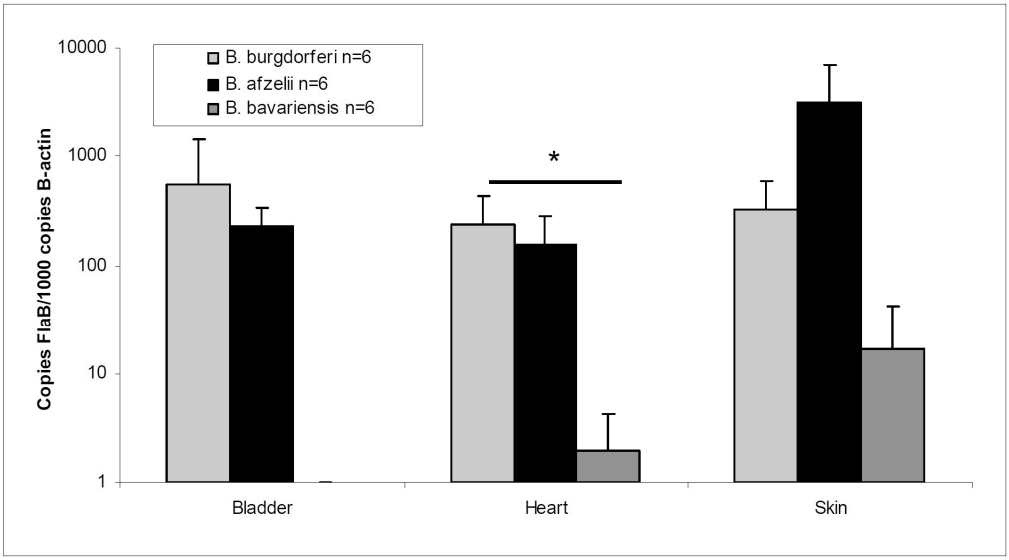

Figure 1: Loads of Borrelia/1000 copies β-actin in tissues from infected mice. Real time PCR results grouped by species and per organ, expressed in copies FlaB/1000 copies β-actin. Borrelia loads are significantly higher in heart B. burgdorferi compared to B. bavariensis strains. Each group consisted of n=6 mice. (* $p<0.05$)

Since no significant difference in spirochete DNA loads between C3 -/- and WT mice was found in heart, skin and bladder tissue and only in the mice infected with strains that are already serum resistant we pooled the data to increase power and compared DNA loads in different tissues for all mice infected with *B. burgdorferi* ss, *B. afzelii* or *B. bavariensis* (Figure 1). It is clear that loads in mice

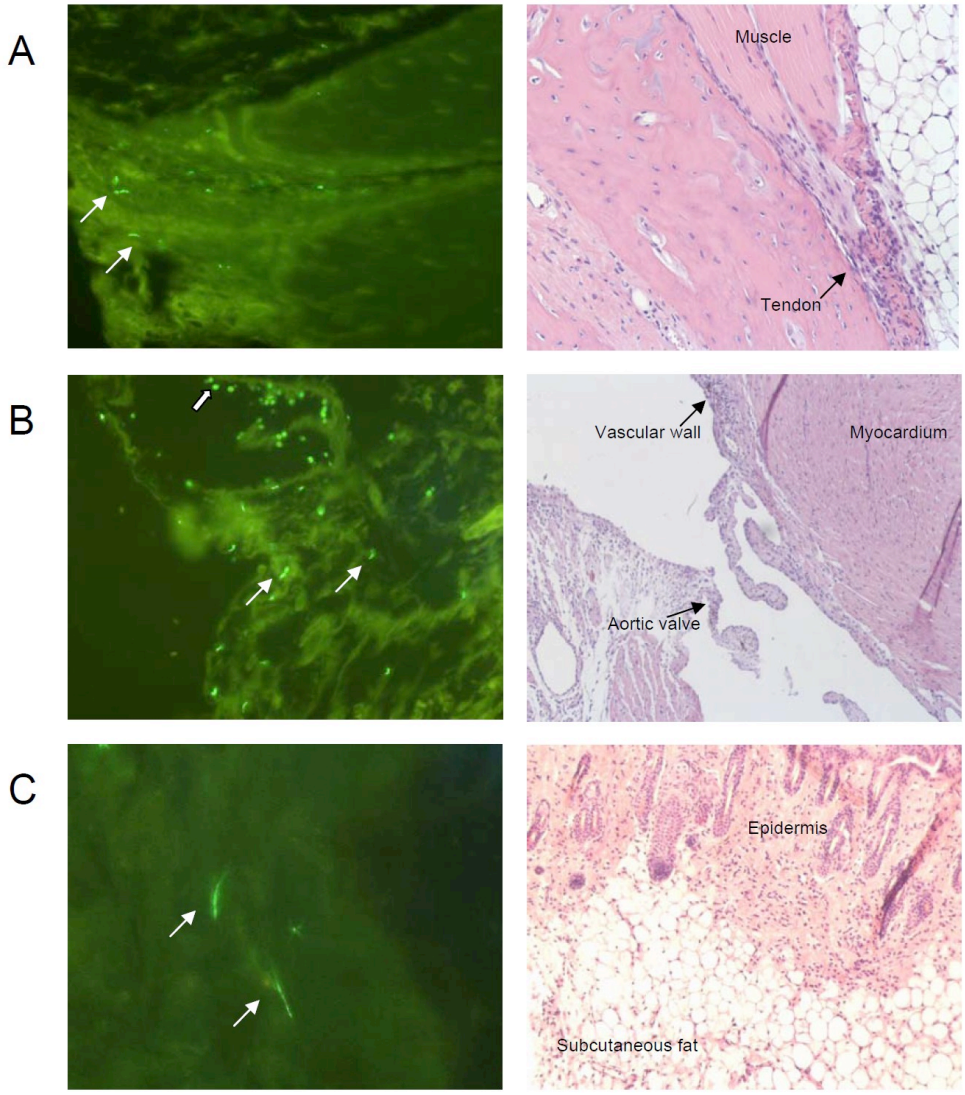

Figure 2: Pathology and IF results. IF and HE slides of organs from a C3 -/- mouse inoculated with B. afzelii. A: Joint: IF (40x), spirochetes in the tendon connected to the tibia (white arrow). HE (20x), Infiltration of plasma cells, lymphocytes and sporadically a granulocyte in the tendon. B: Heart: IF (63x), spirochetes located in the epicardium (white arrow), intravascular erythrocytes also fluoresce (fat arrow). HE (10x), aortal base with valve. Infiltration of macrophages in the adventitia, vascular wall and in a lesser amount in the myocardium. C: Skin: IF (63 x): spirochetes mainly localized on the lining of the dermis with the epidermis (white arrow). HE (10x), Infiltration of plasma cells, lymphocytes and histiocytes in the dermis and subcutaneous fat. As control an uninfected mouse was sacrificed. No spirochetes were detected in the tissue of the control mouse.

infected with *B. burgdorferi* ss are comparable in all organs and are high, ranging from a mean of 240 copies to 850 copies FlaB/1000 copies β-actin. *B. afzelii* infected mice showed a wider range of loads, ranging from 150 to 3000

copies FlaB/1000 copies β-actin. Highest loads of *B. afzelii* were reached in skin. There were no significant differences in loads between *B. burgdorferi* and *B. afzelii*. Loads in mice infected with *B. bavariensis* strains differ little between organs and are low, ranging from 2 to 20 copies FlaB/1000 copies β-actin. Spirochete loads in heart tissue of *B. bavariensis* infected mice were significantly lower than in *B. burgdorferi* ss infected mice.

Pathology

By IF staining, spirochetes were only demonstrated in tissue samples from C3 -/- and WT mice injected with *B. burgdorferi* ss or *B. afzelii* (Table 2).
In *B. burgdorferi* ss injected mice the highest numbers of spirochetes were seen in heart and skin tissues. Bacteria were visually more abundant in the C3 -/- mice. In the C3 -/-, as well as the WT mice injected with *B. burgdorferi ss*, lower numbers of spirochetes were seen in the bladder and in the kidney.
In mice injected with *B. afzelii* spirochetes were seen in joint, skin, heart, kidney and bladder. No differences were seen between WT and C3 -/- mice.
In the heart, most spirochetes were seen in the epicardium surrounding the base of the vessels of the truncus arteriosus. In ankle tissues spirochetes were most abundant in the tendons and in the skin along the dermal epidermal junction. In organs from mice injected with *B. bavariensis*, *B. garinii* or *B. valaisiana,* no spirochetes were detected by IF staining.
Inflammation was present in the heart and skin of *B. burgdorferi* ss injected mice and *B. afzelii* injected mice (Figure 2). Tissue inflammation was related to the amount of spirochetes seen after IF staining. There was no difference in inflammation scores between WT and C3-/- mice.
In general IF microscopy had a very low sensitivity for detecting infection with borrelia. Borrelia were only visualized and detected by IF when the quantified load in the real time PCR was more than 100 borrelia/1000 copies β-actin

Arthritis score

After 10 days inflammation scores of the ankle joints were evaluated. None of the *B. burgdorferi* ss injected mice showed thickening of the ankle joint, regardless of the presence of C3 in the mice.

Table 2: Results from the real-time qPCR, pathology scores and culture. *Real time PCR results expressed in copies FlaB/1000 copies β-actin (Standard deviation). Pathology scores performed as described. Immunofluorescence (IF) score is a score based on the amount of visible spirochetes by IF. The hematoxylin eosin (HE) staining is scored on amount of inflammation as described. No spirochetes were seen by IF in any of *the B. garinii* and *B. valaisiana* infected mice. No inflammation was seen in *B. garinii* and *B. valaisiana* infected mice except for an inflammation score of 1 in the bladder of one WT mouse infected with *B. garinii* (mean score 0.3). None of the cultures, or qPCRs from *B. garinii* or B. valaisiana was positive.

	B. burgdorferi		*B. afzelii*		*B. bavariensis*		*B. garinii*		*B. valaisiana*	
	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)
Bladder										
Real time PCR*	10 (17)	1102 (1101)	100 (82)	357 (377)	-	-	-	-	-	-
Pathology IF	0	1,3	0,7	1,3	0	0	0	0	0	0
Pathology HE	0,5	0	0,7	1,3	0	0	0,3	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Heart										
PCR*	137 (109)	345 (215)	77 (54)	226 (138)	3 (3)	1 (1)	-	-	-	-
Pathology IF	0,7	2,3	1,7	2,0	0	0	0	0	0	0
Pathology HE	0,7	2,7	2,0	2,3	0	0	0	0	0	0
Culture	3/3	3/3	2/3	1/3	1/3	1/3	-	-	-	-
Brain										
PCR*	-	-	6 (11)	-	-	-	-	-	-	-
Pathology IF	0	0	0	0	0	0	0	0	0	0
Pathology HE	0	0	0	0	0	0	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Skin										
PCR*	304(405)	341 (107)	1454 (1222)	4687 (5473)	28 (32)	6 (11)	-	-	-	-
Pathology IF	1,0	2,3	2,7	3,0	0	0	0	0	0	0
Pathology HE	2,0	2,3	2,3	3,0	1,0	0,7	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Ankle										
Real time PCR*	-	844 (523)	1918 (1248)	1880 (1563)	6 (3)	3 (4)	-	-	-	-
Pathology IF	0	0	0,7	1,3	0	0	0	0	0	0
Pathology HE	0	0	0	0,3	0	0	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Kidney										
Pathology IF	0,7	1,0	0,7	1,3	0	0	0	0	0	0
Pathology HE	0,3	0	0	0	0	0	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-

Among B. afzelii injected mice, 2/3 mice in the C3-/- group showed a slight swelling of the ankles. None of the three mice in the WT group demonstrated any swelling. In the mice injected with B. garinii 1/3 mice from the WT and 1/3 mice from the C3-/- group had slight swelling of the ankle joints.In B. bavariensis injected mice slight swelling was seen in 1/3 C3-/- mice and in 3/3 WT mice; two of those mice had slightly and one moderately swollen ankle joints. A slight swelling in the ankles of 3/3 WT mice infected with B. valaisiana was also seen whereas ankles of C3 -/- mice injected with this species appeared normal.

Discussion and conclusions

In vitro, some *Borrelia* species can evade lysis by complement components. In this study we evaluated the capacity of different *B. burgdorferi* sl species with different levels of complement resistance to induce infection and inflammation in WT and complement-deficient mice in vivo. Infection was not detected in mice after challenge with complement-susceptible spirochetes belonging to *B. garinii* or *B. valaisiana* species. The absence of complement in C3 -/- mice did not enhance the infectivity of complement-susceptible strains.
All cultures from mice inoculated with the *B. burgdorferi* strain N40 were positive, in contrast only a few heart samples from mice infected with the *B. afzelii* strains, despite the fact that spirochete loads in tissue were comparable between mice infected with either of these species. A limited amount of culture positivity in *B. afzelii* infections in mice has previously been reported [537]. This suggests that *B. burgdorferi* ss strains are much easier to culture in vitro and that the results of spirochete cultures from infected humans or mice is dependent on the spirochete genetic background.

Since the infectivity and pathogenicity of the individual isolates that were used in this study was unknown, mixtures of different isolates belonging to the same *Borrelia* species for experiments were applied. Infection could be detected after infection of WT and C3 -/- mice with *B. burgdorferi* ss, *B. afzelii* and *B. bavariensis;* in contrast none of the mice injected with complement sensitive strains became infected. In mice infected with complement-susceptible *B. garinii* strains none of the strains were able to sustain infection. Four of the five strains of the complement sensitive *B. garinii* that we used had been isolated from either human skin or human CSF and have been able to sustain infections in humans. Of the strains used in this study, *B. garinii* PBr has been used in mouse experiments before, but was unable to establish an infection in wild type Swiss Webb mice [537]. In the same study, it was shown that only 4/9 *B. garinii* strains were infectious for mice; complement-sensitivity of the infectious

strains was not assessed. Escudero et al. found that 8 out of 9 tested *B. garinii* strains from well-characterized different genetic backgrounds were infectious for mice; these 9 strains were all non-OspA serotype 4 strains [549]. A possible explanation of the lack of infectivity of the non-OspA serotype 4 *B. garinii* in WT and C3-/- mice could be that they have lost virulence factors, such as plasmids, by previous excessive in vitro culture. However, most strains had been passaged in vitro less than 8 times before inoculation, which renders loss of plasmids less likely. Apparently, absence of the complement-mediated killing pathway in the C3 -/- mice did not result in increased infectivity of these strains in mice.
The five *B. valaisiana* strains had all been obtained from ticks, since this species has never been cultured from human patients and only once from laboratory mice [549]. Therefore, a low virulence of *B. valaisiana* strains was expected and confirmed by this study.

In nature, *B. garinii* and *B. valaisiana* are more often cultured from birds [507, 550-555]. In concordance with this finding some *B. garinii* are resistant to killing by pheasant serum in vitro, but not to mammalian sera [65]. Removing complement activity did not alter infectivity, thus this study underscores that infectivity is not dependent on complement resistance. Other factors must be more crucial for infectivity of non-OspA serotype 4 *B. garinii* and *B. valaisiana*.

Although C3 -/- mice generally had higher spirochete loads than WT mice by qPCR, this was not statistically significant. The only exception was the ankle joints from mice infected with *B. burgdorferi* ss, where C3 -/- mice contained significantly more spirochetes, in comparison to the negative qPCR results for joints from the WT mice. The low load of spirochetes in *B. burgdorferi* ss infected WT mice can be due to the C57/Bl6 mouse model. It has been described that *B. burgdorferi* ss in C57/Bl6 mice can give low loads of spirochetes in comparison to C3Hen mice [556]. These reports also describe that a low load of spirochetes was accompanied by a relatively low rate of inflammation in the C57/Bl6 mice.

When grouping the WT and the C3 -/- mice together we can conclude that *B. burgdorferi* and *B. afzelii* show comparable loads, while loads in mice infected with *B. bavariensis* strains were much lower. This is in concordance to what has been reported previously using different *B. burgdorferi* ss, *B afzelii* and *B. garinii* strains, where *B. burgdorferi* and *B. afzelii* frequently were strongly PCR positive, while *B. garinii* infected mice differ in infectivity and generally are present with lower loads [537].

We can generally conclude that the absence of C3 does not lead to major differences in infectivity of spirochetes in C57/Bl6 mice. This is in agreement with findings of Woodman et al., who found no major effects of the infectivity of *B. burgdorferi* ss injected in factor H deficient mice concluded that mice lacking factor H were as efficiently infected by *B. burgdorferi* as WT mice [98]. A potential problem with that model is the fact that factor H deficient mice practically do not have C3, compensating for their factor H deficiency and can not kill invading spirochetes by complement activation [99]. Knocking out CRASP2 in a *B. burgdorferi* ss strain did not alter infectivity in a mouse model, though in that model one can still argue that other CRASPs can complement factor H binding [86].
An additional explanation for the apparent lack of involvement of complement-mediated killing in this study is the use of syringe inoculation. Syringe inoculation is not a natural route of infection and tick inoculation is a more efficient method for infection. In the process of transmission from tick to host many proteins are involved. Borrelia proteins but also tick salivary proteins are of importance; SALP 15 binding to OspC can aid *B. burgdorferi* ss to enter the mammalian host, but also other SALPs might be crucial in efficient transmission [46, 48, 557]. In the transmission cycle from tick to host a meticulous up and down regulation of several proteins takes place [79, 558]. Syringe inoculation might bypass essential steps necessary for efficient borrelia infection. For the complement resistant strains however needle inoculation did not seem to influence infectivity in C3 -/- nor in WT mice. It is doubtful whether tick inoculation of spirochetes would make the complement sensitive strains more infectious.

The results from this study suggest that complement evasion strategies are not critical for effective infection and dissemination. Other components play a crucial role especially in infection with *B. garinii*. Developing a model that can properly mimic natural entry of spirochetes in the mammalian host will aid in future research. More studies on *B. burgdorferi* sl, the hosts and the European tick *I. ricinus* are required to fully understand the complex interaction involved in transmission, infection and disease.

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Chapter 4

High sensitivity and specificity of C6-peptide ELISA on CSF in Lyme neuroborreliosis patients

Nathalie D. van Burgel[1]
Afke Brandenburg[2]
Hendrik-Jan Gerritsen[1]
Aloys C.M. Kroes[1]
Alje P. van Dam[3]

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[1] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[2] IZORE, Center for Infectious Diseases, Leeuwarden, the Netherlands
[3] Department of Medical Microbiology, Onze Lieve Vrouwe Gasthuis, Amsterdam, the Netherlands

Abstract

LNB is a serious but treatable disease. Diagnosing LNB poses a challenge to clinicians and improved tests are needed. C6-peptide ELISA is frequently used on serum but not CSF. Data about sensitivity of C6-peptide ELISA in CSF in patients suffering from LNB has been conflicting.
Serum-CSF pairs from 59 LNB patients, 36 Lyme non-neuroborreliosis cases, 69 infectious meningitis/encephalitis controls and 74 neurological controls were tested in a C6-peptide ELISA.
Using the optimal cut-off of 1.1, sensitivity of the C6-peptide ELISA for LNB patients in CSF was 95% and specificity was 83% in the Lyme non-neuroborreliosis patients, 96% in the infectious controls and 97% in the neurologic controls
These results suggest that C6-peptide ELISA has a high sensitivity and good specificity for diagnosing Lyme neuroborreliosis patients in CSF. The C6-peptide ELISA can be used on CSF in a clinical setting to screen for LNB.

Introduction

Lyme neuroborreliosis (LNB) is the neurologic manifestation of an infection with the tick-borne spirochete *B. burgdorferi* sensu lato (sl). LNB can present with many neurological signs, varying from facial nerve paralysis and Bannwarth's syndrome to a range of neurological disorders [114, 559]. Diagnosing Lyme neuroborreliosis poses a challenge to clinicians. Detecting *B. burgdorferi* sl directly by culture or by PCR from cerebrospinal fluid only yields a maximum sensitivity of about 50% [235]. A standard for diagnosing LNB is determining intrathecal specific antibody index (AI), despite the fact that the sensitivity of AI has been reported to vary from 48-92% [354, 356].
A peptide of interest for diagnosing LB has been the immunoreactive peptide C6 (IR6), a highly conserved peptide among different *B. burgdorferi* sl [339].
C6-peptide is the sixth invariable region of the VlsE protein. The vls locus consists of 15 silent vls cassettes and the gene for the VlsE lipoprotein. By application of unidirectional recombination events VlsE can display antigenic variation [57]. The C6-peptide has been shown to be an immunodominant peptide [341]. IgG antibodies to C6-peptide have been shown to be detectable as early as two weeks post-infection and antibodies wane over time after treatment [339, 347]. Sensitivity and specificity of C6 ELISA in serum has been reported to be equal, if not superior, to 2-tier testing in North American patients [234, 310]. C6-peptide serology has been implicated to have a high sensitivity in LNB patients, varying from 67 to 100% [345, 560]. The commercially available C6-peptide ELISA has only been validated for serum samples. Data on performance of the C6-peptide ELISA performed on CSF for diagnosing LNB is limited and conflicting [360-362]. The aim of this study was to determine whether a C6-peptide based ELISA can be used on CSF samples to diagnose early and late LNB patients, using a large cohort of well-defined patients and controls.

Materials and methods

Selection of clinical specimens and control samples

Patients and controls from the time period between January 2004 and October 2009 were identified retrospectively using the laboratory information management system from the Leiden University Medical Center (Leiden), OLVG Hospital (Amsterdam), IZORE Center for Infectious Diseases (Leeuwarden), Academic Medical Center Amsterdam (Amsterdam), and the Isala clinic (Zwolle). Cerebrospinal fluid (CSF)-serum pairs from 59 LNB patients were included. Criteria for diagnosing LNB patients were four of the following five

LNB criteria: 1; detection of *B. burgdorferi* antibodies in serum, 2; CSF pleocytosis (>5/µl), 3; absence of other evident cause of meningitis, 4; evidence of intrathecal production of specific *B. burgdorferi* antibodies 5; objective neurological complaints with favorable outcome after treatment [414]. Thirty-six CSF-serum samples were available from Lyme borreliosis (LB) patients that did not have LNB according to the applied algorithm. The LB patients' group consisted of 12 recent erythema migrans (EM) patients, 21 Lyme arthritis patients and 3 acrodermatitis chronica atrophicans (ACA) patients. CSF and serum samples were available from 69 patients with other infectious diseases, 62 CSF-serum pairs were collected from neurological inflammatory diseases and 12 CSF-serum samples were collected from patients with neurological complaints including dizziness, headache and fatigue without evident diagnosis and trauma patients (See table 1). Additional data was collected for all patient groups; age at presentation, sex, duration of illness (>6 months was classified as late LNB), CSF findings at diagnosis (intrathecal leukocytes and erythrocytes/µl, percentage mononuclear cells, glucose level, total protein, IgG and albumin). For LNB patients the clinical presentation, duration of complaints and report of an EM was documented.

C6-peptide ELISA

All sera and CSF samples were tested in the C6 Lyme ELISA Kit (Immunetics, Boston USA). Preliminary results showed good performance of a 1:5 dilution for CSF. Therefore and for practical reasons all CSF samples were tested in a 1:5 dilution continuing thereafter with the manufacturer's protocol for serum. C6-peptide ELISA was performed on sera according to manufacturer's protocol. The Lyme Index value (LI) was calculated according to the manufacturers' protocol: $Absorbance_{450\text{-}650nm}$ sample/ (($Absorbance_{450\text{-}650nm}$ calibrator) plus 0.3). Samples with LI values < 0.9 are considered negative, 0.9-1.1 equivocal and values≥ 1.1 positive for antibodies against C6-peptide in serum.

Antibody index

All sera and CSF samples were tested with the IDEIA^{TM} Lyme Neuroborreliosis kit according to manufacturer's protocol (OXOID, Cambridgeshire, UK). Antibody index (AI) was calculated as (OD_{csf}/OD_{serum}) * (OD_{csf}-OD_{serum}). The CSF contained IgG or IgM if the OD_{csf} IgG or IgM is > 0.150. The AI was positive when the CSF was positive and the AI_{IgG} or AI_{IgM}≥ 0.3.

Statistical analysis

Statistical analysis was performed using a statistical software package (SPSS for windows, version 17.0). The Student's t-test was used to compare levels of C6-peptide LI between groups. P-values <0.05 were considered significant.

Results

Patient characteristics

All patient serum and CSF samples were tested according to protocol. Patient epidemiologic data are represented in Table 1. The group of LB and LNB patients showed a bimodal distribution of age with a peak in childhood and a peak at 55 years. Of the 59 LNB cases 20% reported an EM at presentation.

		Male/Female (%	Mean age yrs (SD)	Mean CSF leukocyte count (/µl CSF) (SD)
Lyme neuroborreliosis (LNB)	59	60/40	39 (24)	135 (159)
Lyme borreliosis (LB)	36	50/50	51 (17)	1(1)
Infectious meningitis/encephalitis controls	69			
T. pallidum	12	83/17	40 (8)	40 (79)
C. neoformans	2	50/50	52 (6)	94 (89)
Bacterial meningitis				
S. pneumoniae	2	50/50	41 (6)	337 (99)
L. monocytogenes	1	0/100	61	1280
M. tuberculosis	1	0/100	4	25
Viral meningitis/encephalitis				
HIV	6	50/50	43 (8)	51 (45)
VZV	11	45/55	51 (23)	130(173)
HSV1	6	33/67	55 (30)	46 (51)
Enterovirus	23	61/39	13 (17)	271 (381)
Parechovirus	3	0/100	0 (0)	1 (1)
TBE	2	50/50	37 (4)	59 (12)
Neurologic controls	74			
Facial nerve paralysis eci	19	66/34	48 (18)	40 (145)
Multiple sclerosis	26	35/65	35 (14)	15 (17)
Polyneuritis/polyneuropathy	16	56/44	45 (17)	17 (22)
ADEM	1	0/100	21	266
Neurologic non-inflammatory controls	12	25/75	47 (13)	4 (6)

Table 1: Epidemiological characteristics of patients and baseline cerebrospinal fluid (CSF) leukocyte count (per µl).

Clinical presentation consisted most frequently of facial nerve paralysis (58%) and meningoradiculitis (27%), the remainder of the cases presented with malaise and headache (10%), meningoencephalitis and a sensation of altered vision with papilloedema. Most patients had an early disseminated LNB (53/59). Four of the six patients with a late LNB presented with a meningoradiculitis with duration between 6 months and 2 years. Two patients had complaints for 6 to 18 months of an altered gait with MRI abnormalities. Ninety-five percent of patients presented with pleocytosis. Only two patients presenting with early LNB, one facial nerve paralysis and one meningoradiculitis, and one patient presenting with late LNB, meningoradiculitis, did not have pleocytosis. These three patients eventually all had antibodies against *B. burgdorferi* in serum and CSF and the AI was positive. Furthermore, they all responded favorably to treatment.
The AI in the IDEIA neuroborreliosis detected anti-Borrelia IgG or IgM in 78% of the LNB patients. IgG AI was positive in 75% and IgM AI in 49% of LNB patients (Table 2).

	[illegible]-tive (%)	**IgM +**	**IgG +**	**[illegible] -index +**	**[illegible]-index +**	**Ig + (%)**	**[illegible]-index + (%)**	**Tot[illegible] n**
Lyme neuroborreliosis	1 (2)	52	49	29	44	58 (98)	46 (78)	59
Lyme borreliosis	26 (72)	3	8			10 (38)		36
Infectious controls	61 (88)	7	1	2		8 (12)	2 (3)	69
Neurologic controls	70 (95)	3	1	1		4 (5)	1 (1)	74

Table 2: Results of the antibody index from the IDEIA. Represented are the number of samples that are negative or positive for IgM and IgG against flagellin in CSF and the index calculated as described. Ig and Ig index are the results of the IgM and IgG results combined per patient.

C6-peptide ELISA results on serum

The results for the C6-peptide ELISA are shown in figure 1. C6-peptide antibodies were detected in serum in 98% of the LNB patients with a mean LI value of 8.4 (95% CI 7.7-9.1). The one C6-peptide ELISA negative patient was a young child with an early LNB presenting with a facial paralysis with an elevated CSF leukocyte count (236/µl). In this patient the CSF showed detectable antibodies in the C6-peptide ELISA (LI=8.7) as well as a positive AI in the IDEIA for IgG and IgM. The patients that presented with an early LNB had a comparable LI value in serum as the patients that presented with a late LNB with a respective mean LI 8.3 and mean LI 9.1 (p=0.5). In the non-

neuroborreliosis LB patient group the sensitivity was 97%, mean value LI=6.9 (95% CI 5.6-8.2). In all other controls the C6-peptide seroprevalence was 5%.

C6-peptide ELISA results on CSF

Sensitivity and specificity for the C6-peptide ELISA on CSF are shown in table 3. C6-peptide ELISA on CSF detected antibodies in 95% (56/59) of the LNB patients.

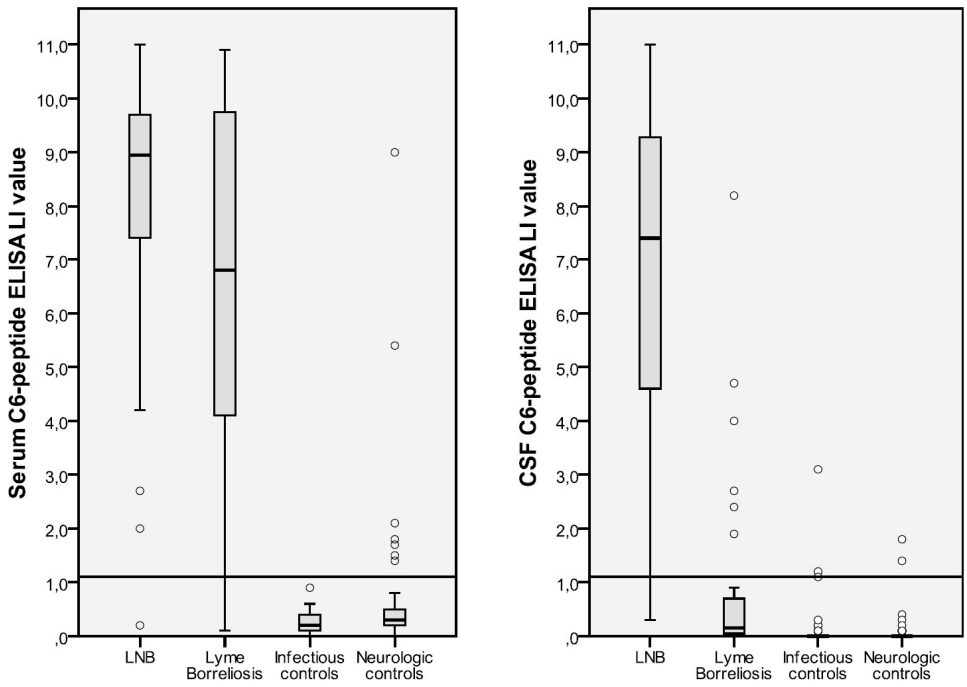

Figure 1: Values for C6 ELISA in serum and CSF. Horizontal lines indicate medians, bars represent interquartile ranges, and lines represent 95% confidence interval and bullets represent outliers. The reference line is located at the cut-off for detection of antibodies (LI=1,1).

The patients that presented with an early LNB had a lower LI value in CSF than the patients that presented with a late LNB with a respective mean of LI 6.6 (95% CI 5.6-7.3) and mean LI 8.6 (95%CI 7.3-10.3) ($p<0.01$). Two patients did not have detectable antibodies in the CSF, a child and an adult. Both had early LNB with facial nerve paralysis at presentation. The adult patient presented with a right facial paralysis but did not have pleocytosis at presentation and a negative AI. Antibodies against C6-peptide were already present in the serum at presentation (LI=9.2). Diagnosis was later substantiated when he presented with bilateral facial paralysis and conclusive CSF serology. The child had

antibodies against C6-peptide (LI=9.4) and IgM was detected in the IDEIA, but both AI were negative in the CSF. At presentation she had a pleocytosis of 56/µl CSF and she responded rapidly to treatment. The third patient, a borderline (LI=0.9) positive patient had a pleocytosis of 31 leukocytes/µl and complaints of dysarthria with a high IgM AI and an elevated IgG AI. Antibodies against C6-peptide in serum were detectable (LI=6.8).

	Anti-C6-peptide negative (%)	**Equivocal (%)**	**Anti-C6-peptide positive (%)**	**Total**
Lyme neuroborreliosis	2 (3)	1 (2)	56 (95)	59
Lyme borreliosis	29 (81)	1 (3)	6 (17)	36
Infectious controls	66 (96)		3 (4)	69
Neurologic controls	72 (97)		2 (3)	74

Table 3: LI values of C6-peptide ELISA in CSF. Samples with LI values < 0.9 are considered negative for antibodies against C6-peptide, 0.9-1.1 equivocal and values≥ 1.1 positive for antibodies against C6-peptide.

Specificity in all controls varied from 83-97% (See table 3). Specificity was high in the infectious and neurologic control group (96% and 97% respectively). In the infectious control group there were no controls with detectable antibodies against C6-peptide in the serum, but three controls had detectable levels in the CSF. These controls were an enterovirus meningitis, a neurosyphilis and an HIV meningitis patient. In the neurologic control group seven patients had detectable antibodies against C6-peptide in serum; these were five MS patients and 2 Guillain-Barré patients. In the CSF two MS patients had low detectable antibodies against C6-peptide (LI=1.4-1.8). In the LB group alone the specificity was 83% (30/36). Values of the C6-peptide ELISA in CSF were significantly higher in the LNB than in the LB cases (mean LI= 6.7 (95% CI 6.0-7.6) and LI=0.8 (95% CI 0.3-1.4) respectively, $p<0.05$). Lowering the LI threshold to 0.5 would enhance sensitivity to 97%, but lower specificity to 63% in LB patients. No effect was seen on the specificity in the other controls.

Discussion and conclusions

In this study we evaluated the C6-peptide ELISA on CSF for diagnosing a LNB infection. We found a high sensitivity as well as a good specificity of the C6-peptide ELISA.

We chose an algorithm defining LNB patients where a LNB patient could either have absence of pleocytosis or absence of intrathecal antibody production in an abundance of other criteria which made LNB evident. C6-peptide serology has a good sensitivity in the Lyme neuroborreliosis patients, varying from 67 to 100% in serum [314, 345, 360, 560]. The lower sensitivities were mainly reported in very early LNB when duration of symptoms was less than 8 days. The sensitivity of C6-peptide ELISA on serum of LNB patients was 98% in this study. Serum from one child with early LNB was negative for anti-C6-peptide antibodies in the ELISA. In previous studies it was demonstrated that patients with LNB can have an early response to the flagellin antigen which can be detectable earlier intrathecally than in serum, leading to reports of seronegative LNB [359, 366]. This finding had not been substantiated for the C6-peptide ELISA until now.
In this study we found 95% sensitivity of the C6-peptide ELISA on CSF for diagnosing LNB. Previously two European publications using the C6-peptide ELISA have determined the sensitivity of the C6-peptide ELISA on CSF and the data has been conflicting. Skarpaas et al used undiluted CSF and a cut-off of OD=0.5, which is comparable to the LI value of 0.9 compared to OD/cut-off standard used in the present kit. This cut-off is comparable to the borderline cut-off in our study. Prospectively, sixty adult LNB patients, defined as clinical LNB, pleocytosis and evidence of intrathecal anti-Borrelia IgG production by ELISA, were tested in the C6-peptide ELISA and a sensitivity of 98% on CSF was found. The C6-peptide ELISA was also performed on CSF from 42 controls in whom the specificity was 88% [360].
Another study used diluted and undiluted CSF with a LI cut-off of 0.5 and 1 [362]. Retrospectively 31 tentative LNB were identified by evidence for intrathecal antibody production by western blot. Twenty-eight LNB patients were identified according to clinical presentation and concurrent clinical response to antibiotic treatment. Sensitivity of the C6-peptide ELISA in these patients was only 61%, which is lower than the previously reported sensitivity and our findings. The low sensitivity found in that study may be explained by inclusion of non-LNB patients in the study group. Clinical data, including CSF findings, were not provided. Furthermore the use of immunoblots to determine intrathecal antibody responses is problematic and can lead to overdiagnosis [300, 376, 378]. In addition, it has been reported that up to 20 percent of patients that have detectable antibodies against *B. burgdorferi* sl and respond to treatment do not have LNB but have other self-limiting conditions [370, 561]. It is likely that the low sensitivity of C6-peptide ELISA that has been reported results from a poorly defined LNB patient group.

Specificity of the C6-peptide ELISA on CSF for detecting LNB was 88% in previously reported studies. In the current control group the specificity varied

from 83-97% with lowest specificity in the LB patient group (83%). In the infectious and neurological control group the specificity was 96% and 97% respectively. Passively acquired antibodies from the serum can be an explanation for the detectable anti-C6-peptide antibodies in the CSF. However, in the infectious control group none of the analyzed controls with antibodies in the CSF had anti-C6-peptide antibodies in the serum. In the neurologic control group only two MS patients had detectable antibodies in the CSF. Production of polyclonal Ig in the CSF due to MS might also be an explanation for the false positive result in these patients. Calculating the C6-peptide AI with the IgM/IgG C6-peptide ELISA was not possible because it was a combined IgM and IgG ELISA. Because no actual AI could be calculated, specificity of the C6-peptide ELISA with CSF will by definition be suboptimal in patients with detectable anti-Borrelia antibodies in the serum because no correction is made for passively acquired antibodies in the CSF.

A shortcoming of this study is that it is a retrospective study which might have accounted for a selection bias. The strength of this study however is the number and wide variety of the controls. Many samples were selected from patient groups from whom the clinical presentation could be mistaken for LNB. The LNB and control groups also included patients from all age groups. Based on the previous publications, the specificity of the C6-peptide ELISA for diagnosing LNB on CSF was insufficiently investigated.
In interpreting serology results a combination of duration of complaints, patient history and knowledge of laboratory parameters, as for instance pleocytosis in LNB, is essential to reach correct diagnosis. In a Lyme endemic region antibodies can be detected in patients who do not suffer from neuroborreliosis. When faced with the clinical situation wherein diagnosis of a LNB seems less probable, with detectable anti-C6-peptide in the CSF, it can be useful to use a more specific assay like calculating a specific AI.
In conclusion, we show a good sensitivity and specificity of the C6-peptide ELISA on CSF. The C6-peptide ELISA is a reliable screening test that can be used in serum and CSF to assist in the diagnosis of LNB.

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Chapter 5

Discriminating Lyme neuroborreliosis from other neuro-inflammatory diseases using levels of CXCL13 in cerebrospinal fluid

Nathalie D. van Burgel[1]
Floor Bakels[2]
Aloys C.M. Kroes
Alje P. van Dam[3]

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[1] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[3] Department of Medical Microbiology, Onze Lieve Vrouwe Gasthuis, Amsterdam, the Netherlands

Abstract

Lyme neuroborreliosis is a severe but treatable disease. Intrathecal production of chemoattractant CXCL13 has been suggested to be a good biomarker for diagnosing LNB. Our aim was to determine levels of the CXCL13 biomarker in cerebrospinal fluid (CSF) in LNB and several groups of patients with inflammatory neurological diseases, in order to evaluate performance of a CXCL13 ELISA for diagnosing LNB.

Fifty-eight adult and pediatric LNB patients, 36 Lyme non-neuroborreliosis cases, 93 infectious meningitis/ encephalitis controls and 74 neurological controls were tested for levels of CXCL13 in CSF.
Levels of CXCL13 were highly elevated in the patients who presented with LNB. Sensitivity using an optimal cut-off of 250pg/ml CSF was 88%. Children (n=24) had lower levels of CXCL13 intrathecally than the adult population (n=35), this difference was not significant (median=932 compared to median 1678; p=0.4).
In the controls elevated levels of CXCL13 in CSF were seen in several groups of patients. Overall specificity was 89%; this was lowest in the HIV positive population where it was 77%.
After treatment there was a rapid decline in CXCL13 levels in CSF of LNB patients. Determining CSF CXCL13 as a marker for follow up after adequate treatment seemed promising.

Determining levels of CXCL13 as a marker for LNB can be useful, but should be interpreted with care especially in the immunocompromised patient and in the patient with an autoimmune disorder. HIV infection should be excluded in individuals with elevated levels of CXCL13 in CSF.

Introduction

Lyme neuroborreliosis (LNB) is the neurologic manifestation of an infection with the tick-borne spirochete *B. burgdorferi* sensu lato. LNB can clinically present with many symptoms varying from classical presentations like facial nerve paralysis and Bannwarth's syndrome to a range of neurological disorders [111]. Diagnosing Lyme neuroborreliosis is a challenge for the clinician. Calculating the *B. burgdorferi* specific antibody index (AI) is the preferable method, though the sensitivity can be variable, ranging from 66-79% [354, 356, 562].

Measuring intrathecal levels of CXCL13 has been suggested as a potential biomarker for diagnosing LNB. CXCL13 is produced by antigen presenting cells and is a selective chemoattractant for B-cells and B-helper T-cells. High levels of CXCL13 have been detected in muscle tissue in chronically *B. burgdorferi* infected rhesus macaques [563]. In vitro dendritic cells were able to produce high amounts of CXCL13 when they were exposed to *B. burgdorferi* antigens [564]. In vivo production of CXCL13 could be confirmed in non-human primates infected with *B. burgdorferi* intrathecally, where ectopic germinal centers were formed in the brain when high levels of CXCL13 were detected [564]. CXCL13 has been found to be expressed at high levels in pooled cerebrospinal fluid (CSF) from human LNB patients (219ng/g total protein) by a cytokine array, while in pooled CSF from subjects with non-inflammatory neurological disease levels were barely detectable (<1.7ng/g protein) [380]. In a patient cohort of acute LNB patients CXCL 13 levels were highly elevated in all 37 definite LNB cases and no or minimal elevation was seen in the non LNB controls (n=8) [381]. In another study 28 LNB cases had significantly elevated levels of CXCL13 compared to neurological and infectious controls. Some infectious controls had high levels of CXCL13 intrathecally, but overall sensitivity and specificity for LNB using a cut off of 337ng/g total protein were 96% and 97% respectively [382]. Case reports describing early diagnosis of LNB using CXCL13 levels in CSF have already been reported [565, 566]

Our aim was to determine the diagnostic potential of levels of intrathecal CXCL13 to distinguish acute and late LNB from other central nervous system diseases in the pediatric and adult population.

Materials and methods

Clinical samples

Subjects from June 2004 to May 2010 were identified retrospectively using the laboratory information management system from the Leiden University

Medical Center (Leiden), OLVG Hospital (Amsterdam), IZORE Center for Infectious Diseases (Leeuwarden), Academic Medical Center (Amsterdam) and the Isala clinic (Zwolle).
Cerebrospinal fluid (CSF) samples from 58 LNB patients before treatment were included. Criteria for diagnosing LNB patients were; no other cause of meningitis and three of the following four characteristics: positive serology at presentation, pleocytosis, evidence of intrathecally produced specific *B. burgdorferi* antibodies with IDEIA™ Lyme Neuroborreliosis (OXOID, Cambridge, UK) and objective neurological complaints with favorable outcome after treatment according to the EUCALB guideline [415]. Definite LNB were patients from this group who had a pleocytosis and a positive antibody index. Probable LNB were patients who either had pleocytosis or a positive antibody index [414].
Ninety-three CSF samples from subjects with an infectious cause of meningitis/ encephalitis which consisted of cases with; neurosyphilis (n=12), tuberculosis (TBC) meningitis (n=1), pneumococcal meningitis (n=2), Listeria meningitis (n=1), cryptococcal meningitis (n=8), toxoplasma encephalitis (n=14), intrathecal aspergilloma (n=4), human immunodeficiency virus (HIV) meningitis (n=6), varicella zoster virus (VZV) Bell's Palsy and encephalitis (n=11), herpes simplex virus-1 (HSV-1) encephalitis (n=6), enterovirus meningitis (n=23), parechovirus meningitis (n=3) and tick borne encephalitis (TBE) (n=2). Sixty-two CSF samples were collected from neurological inflammatory diseases including; multiple sclerosis (MS) (n=27), polyneuritis (n=16), idiopathic facial nerve paralysis (FNP) (n=18), acute disseminated encephalomyelitis (ADEM) (n=1). Thirty-six CSF samples were collected from Lyme borreliosis subjects that did not have LNB defined as absence of pleocytosis and lack of objective neurological complaints. Twelve CSF samples were collected from subjects with non inflammatory neurological complaints consisting of trauma patients, dizziness and headache without evident diagnosis. Additionally seven CSF samples from HIV patients that had no neurological complaints or evidence of an intrathecal infection were collected. Demographic data was collected for all patient groups; age at diagnosis, sex, CSF findings at diagnosis (intrathecal leukocyte count, percentage lymphocytes, erythrocytes, glucose and total protein levels). For LNB patients the clinical presentation, duration of symptoms at presentation and report of an erythema migrans (EM) were documented.

CXCL13 ELISA

CSF samples were tested in the Quantikine Human CXCL13/ BLC/ BCA-1 Immunoassay (R&D systems, Minneapolis, USA) according to manufacturer's protocols. Briefly, 50 µl of CSF was diluted with 100µl of Assay Diluent RD1S, transferred to a monoclonal anti-CXCL13 precoated microtiterplate, and

incubated for 2 hours at room temperature (RT). Each well was aspirated and washed four times with 400µl wash buffer. Two hundred microliters of a horseradishperoxidase conjugated mouse monoclonal anti-human CXCL13 was added to each well and incubated for 2 hours at RT. Each well was aspirated and washed four times with 400µl wash buffer. Two hundred microliters of 3,3',5,5'-tetramethylbenzidine was added to each well for 30 minutes at RT in the dark, then 50µl of 1M sulphuric acid was added to each well and absorption was read at 450nm within 30 minutes. CXCL13 concentration was calculated with a standard curve, included in each set of samples assayed. Samples that were outside the linear range of measurement and standard curve were diluted with Assay Diluent and retested accordingly. Analysis was performed with samples in pg CXCL13/ml CSF.

Antibody index

Sera and CSF samples were tested in the IDEIA™ Lyme Neuroborreliosis kit according to manufacturer's protocol (OXOID, Cambridgeshire, UK). Briefly, serum samples were diluted 1:200, CSF samples were diluted 1:4 and both tested in a total volume of 100 µl in the ELISA. Samples were incubated for 60 minutes and subsequently rinsed four times with 350 µl of wash buffer and 100 µl of flagellum conjugate was incubated for one hour. Wells were aspirated and rinsed four times with 350 µl of wash buffer and 100 µl of substrate was added and incubated for 10 minutes after which 100 µl stop solution is added and absorbance is measured within 30 minutes at 450nm. Antibody index (AI) is calculated as (OD_{csf}/OD_{serum}) * $(OD_{csf}-OD_{serum})$. The CSF is positive for IgG or IgM if the OD_{csf} IgG or IgM is > 0.150. The AI is positive when the CSF is positive and the AI_{IgG} or $AI_{IgM} \geq 0.3$.

Statistical analysis

Statistical analysis was performed using SPSS (version 17.0). The Pearson bivariate correlation algorithm was used to calculate correlation. The Mann-Whitney U test was used to compare levels of CXCL13 between groups. P values <0.05 were considered significant. A receiver operating characteristic (ROC) curve was used to calculate discriminatory capacity of CXCL13 levels. The maximal value of the Youden index was applied to choose an optimal cut-off (J_{max}= (sensitivity + specificity)-1).

Results

Clinical findings

Patient characteristics are depicted in table 1. Of the 58 LNB patients 91% of the LNB patients presented within 6 months of the start of complaints; the range was 7 days to 48 months. Forty-one percent of LNB patients were children. Most LNB patients presented with a facial nerve paralysis (60%) or meningoradiculitis (26%). Seventeen percent of LNB patients reported experiencing an EM before or at presentation. In patients with LNB 98% had pleocytosis and 79% was positive for intrathecal production of borrelia specific IgG and / or IgM. Overall 45 patients could be classified as definite LNB and 13 as probable LNB.

	n	Male/Female (%)	Mean age yrs (SD)	Mean leukocyte count /µl CSF (SD)
Lyme neuroborreliosis	58	65/35	38 (25)	138 (159)
Lyme borreliosis	36	47/53	51 (17)	1 (1)
Infectious meningitis/ encephalitis controls	93			
T. pallidum	12	83/17	40 (8)	40 (80)
C. neoformans	8	37/63	55 (14)	81 (69)
Aspergillosis	4	25/75	41 (8)	2 (3)
Toxoplasmosis	14	43/57	35 (20)	1 (0)
Bacterial meningitis				
S. pneumoniae	2	50/50	41 (6)	3032 (893)
L. monocytogenes	1	0/100	61	1280
M. tuberculosis	1	0/100	4	25
Viral meningitis/encephalitis				
HIV	6	50/50	43 (8)	51 (45)
VZV	11	45/55	51 (23)	130 (174)
HSV1	6	33/67	55 (30)	46 (51)
Enterovirus	23	61/39	13 (17)	217 (381)
Parechovirus	3	0/100	0	1 (1)
TBE	2	50/50	37 (4)	59 (12)
Neurologic controls	74			
Facial nerve paralysis	18	56/44	48 (19)	42 (150)
Multiple sclerosis	27	33/67	35 (13)	14 (17)
Polyneuritis	16	56/44	45 (17)	17 (22)
ADEM	1	0/100	21	266
Neurologic non-inflammatory controls	12	25/75	50 (15)	4 (7)
HIV controls	7	86/14	50 (8)	1 (1)

Table 1: Demographic data and CSF leukocyte count per patient group

CXCL13 levels in CSF at diagnostic lumbar puncture

CXCL-13 ELISA on all samples was performed as indicated. CSF CXCL13 levels are depicted in figure 1. Median levels of CXCL13 were significantly elevated in LNB patients compared to the Lyme non-neuroborreliosis controls (median 1183 and median 3pg/ml; p<0.001). CSF levels of CXCL13 were lower in children than in adults with LNB, but this difference was not significant (median 932 pg/ml compared to median 1678 pg/ml; p=0.4).

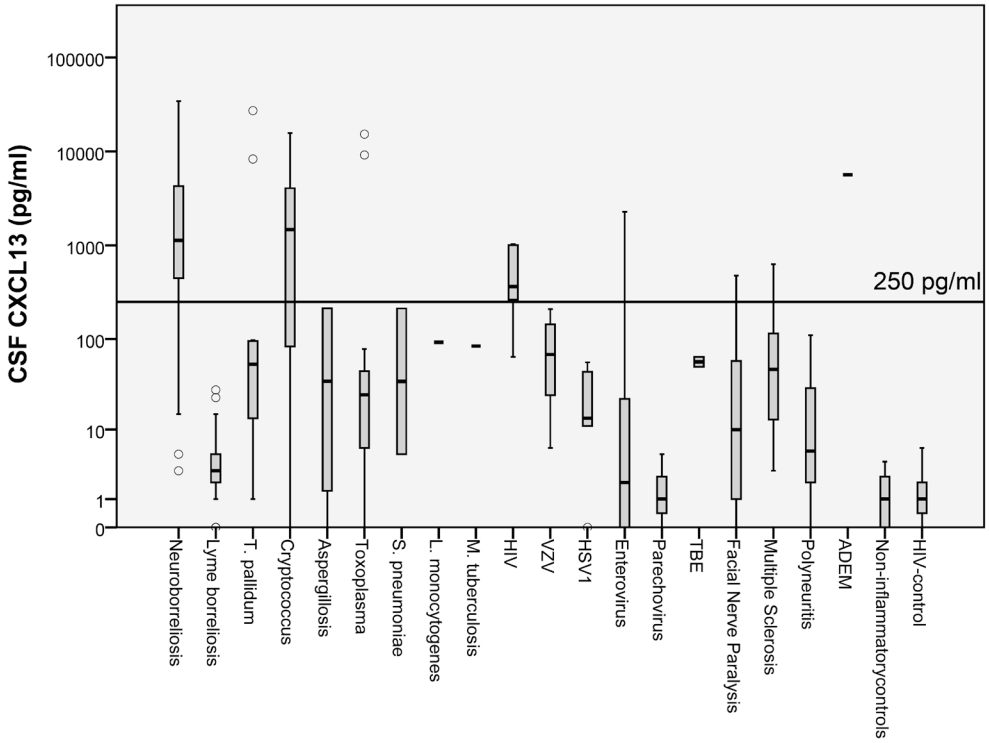

Figure 1: Levels of CXCL13 in CSF of LNB patients and controls. Horizontal lines indicate medians, bars represent interquartile ranges, lines represent 95% confidence interval and bullets represent outliers. Reference line is located at cut-off 250pg/ml as was determined by ROC curve analysis for optimal sensitivity and specificity for discriminating LNB patients from controls.

In figure 2 it is shown that in LNB patients CSF levels of CXCL13 correlated with the amount of leukocytes in the CSF at presentation (R^2=0.172; p<0.01). No correlation was found between duration of complaints to levels of CXCL13 at presentation in early and late LNB (R^2=0.11; p=0.4). This was also true when only the adult population with early LNB were analyzed (R^2=0.13; p=0.6). The

duration of storage of a sample before testing at -20°C did not result in lower levels of CXCL13 in the LNB group (R^2=0.015; p=0.4).

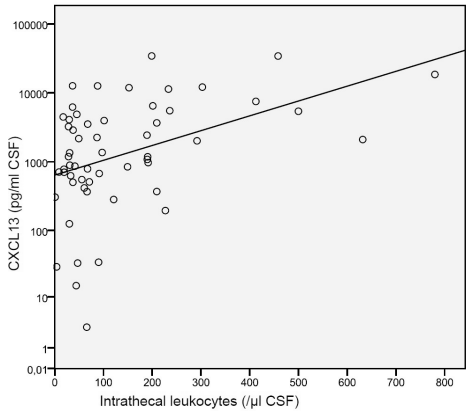

Figure 2: Correlation of intrathecal leukocytes with the level of CXCL13 measured intrathecally in LNB patients. Reference line is a regression line, the correlation is significant (R2=0.172, p=0.001)

Sensitivity of CXCL13 for diagnosing LNB

ROC analysis revealed an optimal cut-off of 250pg/ml which resulted in 88% sensitivity and 89% specificity (figure 3). Results for positive and negative levels of CXCL13 using the cut-off of 250pg/ml in LNB patients and controls are shown in table 2. Seven LNB patients had CXCL13 levels under 250pg/ml CSF. This group consisted of 3 children en 4 adults, of them 5 had early LNB and 2 had late LNB. Lowering the cut-off to 30pg/ml would result in a sensitivity of 97%, but in a reduction in specificity to 65% in the overall population. In the neurologic non-inflammatory controls and the LB controls none of the subjects had levels of CXCL13 over 30pg/ml.

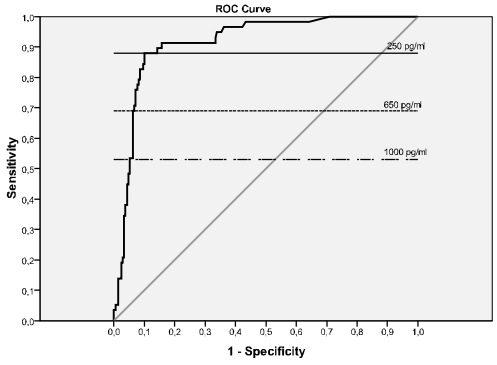

Figure 3: ROC curve analysis using levels of CXCL13 to discriminate between LNB and all controls. Horizontal lines show three different cut-off values for levels of CXCL13. Optimal cut-off was located at 250pg/ml.

Specificity of CXCL13 in the controls

Eleven percent of the controls had CXCL13 levels over 250pg/ml CSF (table 2). None of the CXCL13 positive controls had positive serology for Lyme. All positive controls are specified in table 3. A remarkable result is the elevated levels of CXCL13 in CSF for some HIV positive patients. As is shown in table 3 the specificity of the assay is higher in the HIV negative controls; overall specificity in the HIV negative population is 92%(153/166) while the specificity in the HIV positive controls is only 77% (34/44). The control group of HIV patients without signs of intrathecal infection did not show elevated CXCL13 levels. As is shown in figure 3 elevation of the cut-off to two randomly chosen cut-offs led to rapid decline of sensitivity.

		LNB (n=58) CXCL13 ≥250pg/ml (%)	**<250pg/ml (%)**	**Controls (n=210) CXCL13 ≥250pg/ml (%)**	**<250pg/ml (%)**
AI	positive	42 (72)	4 (7)	0 (0)	4 (2)
AI	negative	9 (16)	3 (5)	23 (11)	183 (87)
Pleocytosis		50 (86)	7 (12)	19 (9)	76 (36)
No pleocytosis		1 (2)	0 (0)	4 (2)	111 (53)
Definite LNB		41 (71)	4 (7)		
Probable LNB		10 (17)	3 (5)		
No LNB				23 (11)*	187 (89)

Table 2: CXCL13 positivity in patients and controls using a cut-off of 250pg/ml. *No controls with elevated levels of CXCL13 had serum serology positive for Lyme.

Discussion and conclusions

We confirm high levels of intrathecal CXCL13 expression in adult and pediatric patients with LNB; however in contrary to previous publications we also identify other groups of subjects with clinically similar presentation with high levels of CXCL13 in CSF.

Identification of LNB is difficult due to the clinical presentation which has much similarity to other diseases. The current definite diagnosis of LNB consists of a positive AI and elevated CSF leukocytes [414]. However, many patients with LNB present with absence of one of those parameters. This can vary from a very early presentation with negative AI to late presentation with few leukocytes intrathecally [111, 112, 356].

This study is the first to include children in the analysis for CXCL13 levels as a diagnostic marker in LNB. Furthermore it is the first study that studied a large adult and pediatric population of neuro-inflammatory patients as controls.

In this study high levels of CXCL13 were found in LNB patients in 88% of LNB patients. This is a lower sensitivity than what was found in previous studies (96-100%) [380-382]. One possible explanation could be that storing samples for a prolonged time would decrease levels of CXCL13, however in this study no effect on median levels of CXCL13 was found for up to five years of storage.
Another explanation for the lower sensitivity found here could be the different study populations. As is shown in table 2, sensitivity is 91% (41/45) in definite LNB cases where correct diagnosis is imminent due to presence of pleocytosis in combination with a positive AI. In probable LNB cases, where either pleocytosis or a positive AI is absent, the sensitivity was 77% (10/13). Previously these probable cases were not well investigated though the role of CXCL13 as an additional marker in these probable cases specifically could be very important.
Previous studies expressed CXCL13 levels in ng CXCL13 /g total protein in CSF [382]. ROC curve analysis for amount of CXCL13 per milliliter compared to amount CXCL13 per gram of total protein showed a similar AUC in this population (0.91 to 0.90 respectively). Applying the previously proposed cut-off of 337ng/g for identifying LNB patients in this study led to a slightly lower sensitivity and slightly lower specificity of 82% and 88% respectively. One study defined a cutoff of 142 pg/ml of CSF. In our study, such a cutoff led to a sensitivity of 90% and a specificity of 84% [372].

	≥250 pg/ml/ total n	**≥250pg/ml/ total n (HIV+)**	**≥250pg/ml/ total n (HIV-)**
T. pallidum	2/12	2/8	0/4
C. neoformans	6/8	2/4	4/4
Toxoplasma encephalitis	2/14	0/12	2/2*
HIV meningitis	5/6	5/6	-
Enterovirus meningitis	1/23	0/1	1/22**
Facial nerve paralysis	1/18	1/5	0/13
Multiple Sclerosis	5/27	0/0	5/27
ADEM	1/1	0/0	1/1

Table 3: High CXCL13 levels in controls (pg CXCL13/ml CSF). * both subjects were congenital toxoplasmosis patients; **positive is in a subject suffering from hypogammaglobulinemia.

A high prevalence of elevated levels of CXCL13 levels in a number of groups of patients with diseases other than LNB was found in this study. Diseases with

elevated expression of CXCL13 intrathecally have been described previously; patients with MS showed marginally elevated CXCL13 levels [380, 385], bacterial and viral causes of meningitis also resulted in moderate elevation of intrathecal CXCL13 [382]. CXCL13 levels were comparable to what was found in this report. This study reports an elevated expression of CXCL13 intrathecally in several other infections. One of the most remarkable observations is that in HIV positive patients levels of CXCL13 are elevated in several central nervous system infections. Elevated expression of CXCL13 in serum of HIV patients has been reported previously, as well as the combination of altered expression of the receptor and elevated production of the CXCL13 cytokine in vitro [384, 567]. Dysfunction of B cells in HIV patients may lead to high levels of CXCL13, independent of the cause of the meningitis or encephalitis.
Neonates with congenital toxoplasmosis and subjects with cryptococcal meningitis showed very high levels of CXCL13, both most likely due to the prolonged infection and inability to clear the infection. Clinically a cryptococcal infection could resemble a neuroborreliosis, but cryptococcal meningitis will often be found only in immunocompromised individuals. One patient that suffered from hypogammaglobulinemia and chronic enterovirus meningitis had CSF CXCL13 levels of 2278pg/ml. Inability of B-cells to respond adequately to the presented antigen will likely lead to continuous production of CXCL13 and high levels intrathecally in hypogammaglobulinemia patients. In the neurological controls the slightly elevated level of CXCL13 was confirmed in MS subjects. Furthermore the patient with an ADEM had very high levels of CXCL13, most likely due to high dysfunctional activity of B-cells intrathecally. One other patient is of interest; a child with coxsackie B3 enterovirus meningitis with subsequent presentation with Henoch-Schonlein purpura (HSP) displayed above average CXCL13 levels of 249pg/ml. HSP and systemic lupus erythematosis (SLE) are autoimmune disorders that both display B-cell dysfunction. Elevated levels of CXCL13 in serum of SLE patients have been described previously [387]. These results suggest that in subjects with autoimmune disorders involving B-cell dysfunction intrathecal CXCL13 levels can be elevated in the absence of LNB.

Treatment of LNB leads to vast reduction in CXCL13 CSF levels, which makes it a potential marker for studying disease activity and effective clearance after treatment [381, 382]. This application seemed very promising. Further studies including follow-up of patients with objective and subjective symptoms of LNB should be performed.

Determining levels of CXCL13 as a marker for LNB can be useful, but should be interpreted with care because it is not a specific marker, especially in the

immunocompromised patient and in the patient with an autoimmune disorder. HIV infection should be excluded in individuals with elevated levels of CXCL13 in CSF. Determining CSF CXCL13 as a marker for disease activity seems promising, but further research for this application is necessary.

Acknowledgements
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Chapter 6

Prevalence and clinical presentation of Lyme arthritis in a large cohort of patients with recent-onset arthritis

Nathalie D. van Burgel[1#]
Jeska K. de Vries-Bouwstra[2#]
Tom Vreeswijk[1]
Aloys C.M. Kroes[1]
Tom W.J. Huizinga[2]
Annette H.M. van der Helm-van Mil[2]

#Both authors contributed equally
Manuscript in preparation

[1] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands

Abstract

As the value of performing *Borrelia burgdorferi* serology in recent-onset arthritis patients is unknown, we aimed to evaluate the prevalence of positive Lyme serology among patients with recent-onset arthritis in relation to their long-term disease outcome.
Serologic testing for *B. burgdorferi* was performed in 1180 consecutive patients presenting with recent-onset arthritis. Serology was performed using an Enzyme Immune Essay (EIA) and confirmation was done by an immunoblot. The clinical diagnosis at presentation and follow up during the disease course was studied in medical files of the patients with positive or unequivocal lab results. Subsequently, the proportion of patients with Lyme arthritis was determined, as well as the diagnostic accuracy of *B. burgdorferi* serology.

Of 1180 patients with recent-onset arthritis, 53 patients had positive EIA which was confirmed by a positive or equivocal immunoblot, indicating a seroprevalence of of 2.0- 4.5%. Nine cases of definite LA were identified, indicating a prevalence of 0.8% within a recent-onset arthritis cohort. These patients were characterized by younger age, and typically presented with involvement of at least one knee. Four patients were *B. burgdorferi* seropositive and did not have a clear other diagnosis. However, there was no indication that LA presented with other primary clinical manifestations than arthritis with involvement of large joints. The remaining *B. burgdorferi* seropositive patients had a clear different clinical diagnosis. Based on the low prevalence of LA, positive predictive value (PPV) of serologic testing was low; however by selecting patients with a high clinical suspicion, eg in patients presenting with an oligo- or monoarthritis of large joints, the PPV increased to 67%.
The present data advocates performing *B. burgdorferi* serology testing only in patients with an increased clinical suspicion, and not in all patients presenting with recent-onset arthritis.

Introduction

Early identification and prognostication of early arthritis patients has been shown relevant and is associated with an improved disease outcome as treatment strategies can be initiated early. Lyme arthritis is an uncommon form of early arthritis that is curable.

Lyme arthritis is one of the clinical manifestations of Lyme Borreliosis (LB), caused by *Borrelia burgdorferi* sensu lato (sl). There are several species of *Borrelia burgdorferi* sl that can cause disease in humans: *B. burgdorferi* ss, *B. afzelli*, *B. garinii*, *B. bavariensis* and *B. spielmanii* [568]. *B. burgdorferi* ss is the only species prevalent in North America, while in Eurasia all abovementioned species can be prevalent [35]. Different species are associated with different clinical manifestations: *B. burgdorferi* ss in particular is associated with Lyme arthritis (LA), but the species prevalent in Eurasia can also cause an arthritis. A report on 1471 patients in Sweden with a definite diagnose of Lyme disease showed that 7% of all patients had arthritis with arthritis being the sole manifestation in 4.4%, [430] while data from North-America indicate that up to 60% of untreated infected patients develop arthritis [150]. LA can present 12-50 weeks after a recorded tick bite and is considered a late manifestation of LB. The classical clinical manifestation of LA comprises mono- or oligo-artritis of the large joints, most often the knee, sometimes typically preceded by an erythema migrans (EM). Nevertheless, also elbows, ankles and hips can be affected and polyarthritis is described in up to10 % of cases complicating the differentiation from other forms of recent-onset arthritis [569].

Cultivation of *B. burgdorferi* from a patient's skin or blood is the gold standard for demonstration of active infection, but it is time consuming and lacks clinical sensitivity [235]. Serological testing is the most commonly applied technique to support of the diagnosis of LB. However serological tests for *B. burgdorferi* can not distinguish past from present infection. For diagnosis of Lyme arthritis guidelines recommend a 2-tier testing approach starting with IgG enzyme immune assay (EIA), followed by IgG immunoblot to confirm the result [109, 416, 570]. As LA is a late manifestation and a symptom of disseminated disease, sensitivity of serologic testing for anti-borrelia IgG is generally very high in patients with LA. However, in areas endemic for Lyme disease the combination of C6 EIA and immunoblot can be positive as well in 64-79% of patients with previous Lyme disease and in 1-3% of healthy subjects. Experts underline the importance of taking into account the a priori chance of active *B. burgdorferi* infection and the estimated specificity of *B. burgdorferi* serology in the subject of interest [234, 571].

It is unclear whether *B. burgdorferi* serological testing should be applied to all patients with an recent-onset arthritis. A Swedish study indicated that positive serological results, though helpful in diagnosing Lyme arthritis, can also be found in patients with definite other rheumatological diagnoses and thus can be false positive. Furthermore, we hypothesized that the clinical presentation of Lyme arthritis in Europe may be less characteristic as several species other than *B. burgdorferi* ss are prevalent [35]. Previous studies generally diagnosed Lyme based on the classical presentation in combination with positive Lyme serology [112, 116, 117]. By this approach patients with an atypical presentation of LA may have been missed.

With the current study we aimed to evaluate the prevalence of positive *B. burgdorferi* serology among patients with recent- arthritis and to appreciate the value of serological testing in these patients by studying the relation between the test results and the disease course over a follow-up of several years to determine the final diagnosis.

Our second aim was to evaluate the contribution of serologic testing to the clinical diagnosis of LA. We studied the Leiden Early Arthritis Clinic (EAC), a large inception cohort enrolling all consecutive patients presenting with recent-onset arthritis. In 1180 early arthritis patients' clinical evaluation and serologic testing for *B. burgdorferi* was performed.

Patients and methods

Study population

Patients were included in the Leiden Early Arthritis Clinic cohort, a large inception cohort initiated in 1993 that enrolled consecutive patients presenting with arthritis to the Leiden Rheumatologic outpatient clinic and that had symptom duration less than 2 years. A detailed description is provided in previous reports [572]. At the first visit, the rheumatologist completed a questionnaire regarding the presenting symptoms, symptoms, this included information on skin manifestations and tick bites. Physical examination was performed and blood samples were taken for routine diagnostic laboratory screening. Follow-up visits were performed on a yearly basis. Written informed consent was obtained from all participants. This study was approved by the local Medical Ethics Committee.

The present study included all patients who presented to the EAC during the period of February 1993 to April 1997 (cohort 1) and the period July 2003 to June 2008 (cohort 2). For patients of cohort 1 serology for common infectious causes of arthritis including *B. burgdorferi* was performed routinely at inclusion;

for patients in cohort 2 *B. burgdorferi* serology was performed on prospectively stored serum samples.

B. burgdorferi serology

In sera from cohort 1, antibodies against *Borreliae* were detected by the IgG and IgM flagellin-EIA (Dako Cytomation, Glostrup, Denmark), using the procedure recommended by the manufacturer. All available positive samples were retrospectively retested with the IgG/IgM C6 Lyme EIA Kit (Immunetics, Boston, USA) according to manufacturers' protocol. In cohort 2, the C6 Lyme EIA Kit was used on prospectively stored serum samples taken for the EAC cohort. All positive enzyme immunoassay (EIA) results from both cohorts were confirmed by the *Borrelia* Europe LINE blot (Virotech GmbH, Rüsselsheim, Germany) and a second EIA, the Liaison® *Borrelia burgdorferi* IgG (VlsE) and IgM (VlsE+OspC) (Diasorin, Italy), both according to the manufacturers' protocols.

Evaluation of serologic test results

The results of current *B. burgdorferi* serologic testing strategies among recent-onset arthritis patients were evaluated in cohort 2. First, serologic results were interpreted by an experienced microbiologist, unaware of clinical presentation and final diagnosis. Serologic test results were classified as: A. Positive, very suspect for active or recent infection, defined as a C6 Lyme index (IgG/IgM) > 5 and a Liaison VlsE IgG value > 40, combined with a positive IgG immunoblot, B: Positive, all other confirmed immunoblot positive serologic results not fitting the abovementioned criteria most likely fitting an old, cleared infection, C: Inconclusive/equivocal immunoblot, combined with a positive/equivocal EIA; a consecutive serum would be necessary to make an adequate serological diagnosis. However, as LA is a late manifestation this serologic classification is most likely not compatible with LA and category D: Negative EIA screening serology.

Clinical diagnosis of Lyme arthritis

Clinical records of all patients with positive or inconclusive serology were traced and the data obtained at the time of the initial diagnosis and during the disease course were thoroughly studied by two experienced rheumatologists (JdVB, AHM). The final diagnosis was classified in two groups; if another clinical diagnosis was certain this was classified as "No LA" and all other cases were classified as "Possible LA".

Based on the combination of clinical data and serologic results patients were reclassified into three LA categories. The first category were patients with a

"definite LA", the second category of patients were patients with a "possible LA", and the third category were patients with "No LA".
For further analyses on the prevalence and clinical characteristics of Lyme arthritis only patients with positive C6-peptide EIA confirmed by blot and a clinical diagnosis of Lyme arthritis were considered.

Statistical analysis

Statistical analysis was performed using a statistical software package (SPSS for windows, version 17.0). Baseline characteristics were compared between patients with and those without definite diagnosis of LA as determined prospectively by the treating physician. Positive predictive value and negative predictive value of *B. burgdorferi* serology was determined for patients in the EAC.

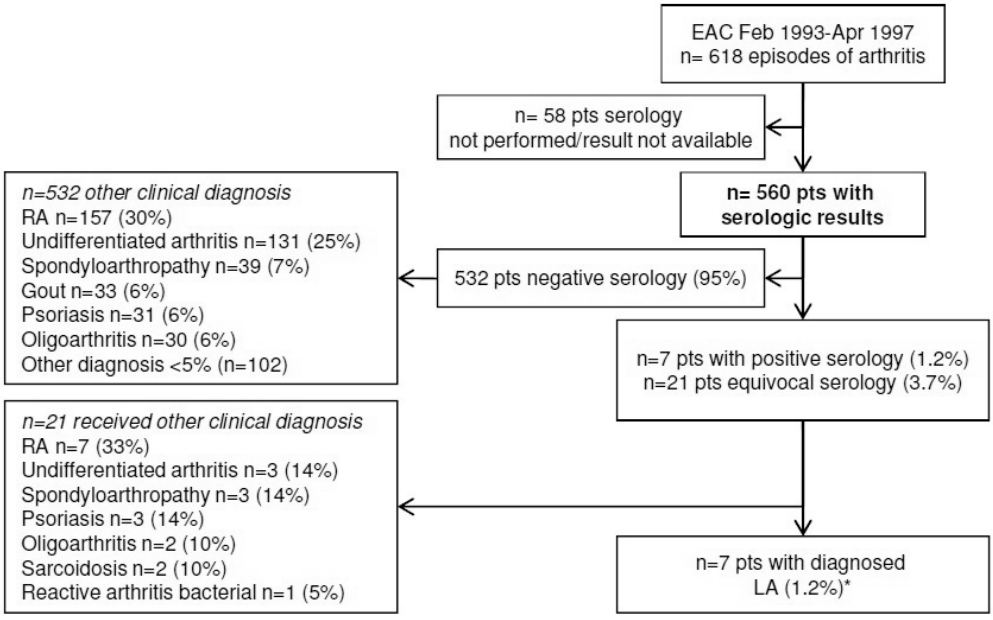

Figure 1: Serological results from cohort 1. * 6 pts could be confirmed with the C6-peptide EIA, the C6-negative patient had an equivocal immunoblot.

Results

Prevalence of positive *B. burgdorferi* serology in early arthritis patients

Cohort 1 consisted of 618 patients consecutively included between 1993 and 1997. Serology results were available for 560 patients. Seven patients had a positive EIA confirmed by a positive immunoblot (1.2%), twenty-one patients

had a positive flagellin EIA with a inconclusive immunoblot (3.7%). Seven of the 28 serologically positive or equivocal patients were diagnosed as Lyme arthritis by their treating rheumatologist (Figure 1). Twenty-three of the 28 serologically positive or equivocal patients could be retrospectively retested in the C6-peptide EIA. In six patients (1.1%) the C6-peptide EIA was also positive; these patients were all diagnosed with Lyme arthritis with a positive immunoblot. The one patient that was diagnosed as LA but had a negative C6-peptide EIA had an equivocal immunoblot. She presented with arthritis of the elbow that resolved after antibiotic treatment. This could have been a LA, but because of the absence of sufficient IgG in the immunoblot it is unlikely, after treatment there was no serological follow up. One patient had a positive immunoblot, but a negative C6-peptide EIA, clinically she was diagnosed as an spondylarthopathy. Serology for this patient can be consistent with a previously cleared infection [591].

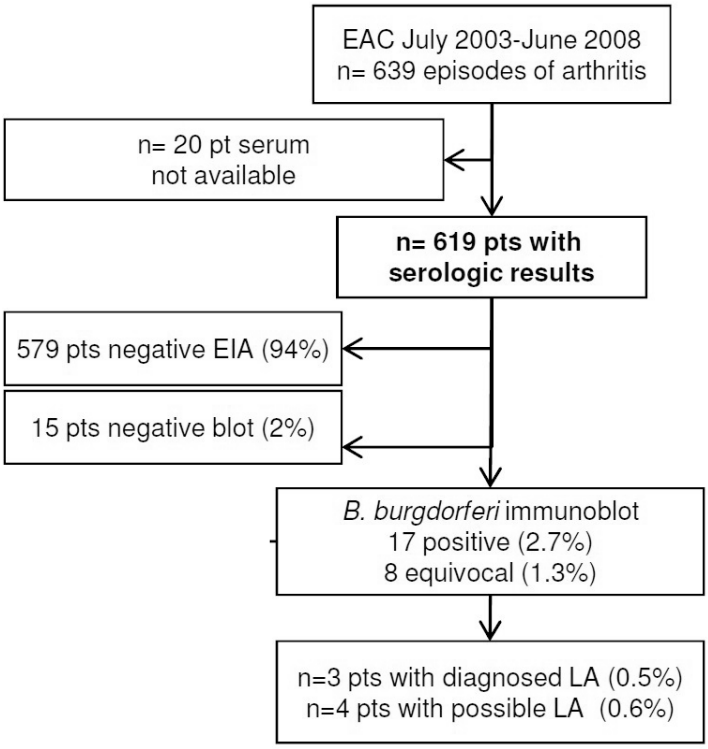

Figure 2: Serological results from cohort 2.

Cohort 2 consisted of 639 patients included between 2003 and 2008. Serum was available of 619 patients. Of these, 40 were reactive in the C6-peptide EIA, which was confirmed by a positive immunoblot in 17 patients, yielding a

seroprevalence of 2.7 %. (Figure 2). The immunoblot was equivocal in 8 patients (1.3%).

Prevalence of Lyme arthritis

The prevalence of definite LA in cohort 1 was 6 of 560 included patients (1.1%). Characteristics and outcome of the 25 patients from cohort 2 with positive C6-peptide EIA and positive or equivocal immunoblot are shown in table 1. Three patients (12%) were diagnosed at presentation with LA and treated with antibiotics, which had resulted in resolution of arthritis. One patient with positive serology was treated with antibiotics without any improvement of the MCP's; afterwards the clinical diagnosis was set at RA and he achieved remission on DMARD therapy. In the remaining 21 patients the diagnoses were recorded by the treating rheumatologist (See table 1). In four patients with other recorded diagnosis and positive or equivocal serology for Lyme, LA could have been considered. These patients were RF/ACPA seronegative and did not have any other typical symptoms like psoriasis, dactylitis or gut disease. One (case 4) of four also had erosive damage on X-ray. These four patients achieved remission and were discharged without any antibiotic treatment. In the natural course of LA natural remission, but also progression to erosive disease has been described [155]. Case 7 responded to treatment with prednisone for arthritis of the hands. There was no progression of disease, making LA unlikely. The prevalence of LA in cohort 2 was 0.5% of definite LA to 1.1% when combined with the possible LA cases.

Diagnostic performance of B. burgdorferi serology

Diagnostic performance of third generation EIA and immunoblot was only studied in cohort 2 because in this cohort all patients were tested with a C6-peptide EIA and immunoblot. Taking into account that in 3 cases a definite diagnosis of LA and 4 cases a possible diagnosis of LA was made, overall specificity of *B. burgdorferi* serology for the clinical diagnosis was 96-97%, due to the low prevalence of Lyme disease. LA is considered a late manifestation of an infection with *B. burgdorferi* and serology should be unequivocally positive. If patients with equivocal blood results (serologic category C) were regarded as negative for active *B. burgdorferi* infection, then serologic specificity increased to 98%. Given the prevalence of LA among this second cohort (0.5-1.1%), the positive predictive value of positive *B. burgdorferi* serology was very low (12-28%).

Pt	Clinical category	Serol. categ.	Lyme category	Diag-nosis	Tick bite	Month started	RF pos.	ACPA pos.	#swollen joints	Involved joints	Current status
1	Possible	A	Definite	LA	no	okt	no	NA	1	knee	minimal hydrops after AB po and iv
2	Possible	A	Definite	LA	no	okt	no	no	1	knees, alternating	remission after AB
3	No LA*	A	Definite	RA	yes	okt	yes	yes	9	knee, MCPs	remission after AB of knee; treatment for RA
4	Possible	A	Possible	OA	no	jan	yes	NA	1	knee, ankle	persistent OA hands and hydrops knee; after first visit no ankle complaints anymore
5	Possible	B	Possible	OA	NA	okt	no	NA	1	knee	OA with hydrops one knee
6	Possible	C	Possible	OA	NA	jan	no	NA	1	knee	OA one knee
7	Possible	A	Possible	UA	no	jul	no	no	9	MCP, PIP,MTP	remission after prednisone (SAVE-trial)
8	No LA	A	No LA	UA	no	jun	yes	no	10	MCPs PIPs	no FU after 2005
9	No LA	A	No LA	RA	no	UK	yes	no	3	MCP,sc	remission with methotrexate
10	No LA	A	No LA	PA	no	sep	no	no	2	MCP, PIP	remission without medication
11	No LA	A	No LA	Gout	NA	okt	yes	no	14	MCP, PIP	gout; remission with allopurinol
12	No LA	A	No LA	RA	no	apr	no	no	12	wrists, MCP, PIP	remission
13	No LA	A	No LA	UA	NA	UK	no	no	6	PIP, MCP	Psoriasis
14	No LA	B	No LA	PA	UK	okt	yes	NA	6	MCP	Psoriasis
15	No LA	B	No LA	SLE	no	jan	no	NA	3	MCP, MTP	SLE with nefritis; currently in remission
16	No LA	B	No LA	RA	no	jan	yes	yes	14	MCP, wrist MTP	RA with methotrexate
17	No LA	B	No LA	RA	NA	dec	yes	NA	10	PIP,MTP,MCP	active RA
18	No LA	B	No LA	RA	yes	jan	no	no	6	PIPs	UCTD/SLE overlap with leucopenia, elevated liverenzymes, livido reticularis and ANF+
19	No LA	C	No LA	SA	NA	mar	no	NA	7	DIP, PIP, MTP	IBD
20	No LA	C	No LA	RA	NA	may	yes	yes			remission with methotrexate
21	No LA	C	No LA	RA	NA	sept	yes	no	8	PIP, MCP	RA with methotrexate
22	No LA	C	No LA	RA	NA	UK	no	NA	14	elbows, wrists, knees, MCP, MTP	remission
23	No LA	C	No LA	OA	NA	jan	no	NA	1	PIP	no FU
24	No LA	C	No LA	RA	NA	mar	yes	yes	4	MCP, PIP	moderately active RA
25	No LA	C	No LA	RA	NA	sep	yes	yes	10	MCP, MTP	no FU after 2007

Table 1: Clinical characteristics of *B. burgdorferi* serologically positive/equivocal patients. Lyme arthritis (LA) rheumatoid arthritis (RA), oligoarthritis (OA), undifferentiated arthritis (UA), psoriatic arthritis (PA), spondylarthropathy (SA), undifferentiated connective tissue disease (UCTD), inflammatory bowel disease (IBD). UK: unknown, NA: not applicable, FU; follow-up. * patient presented with arthritis of the knee and hands and was treated as LA accordingly; the symptoms of the knee disappeared but the second diagnosis RA remained.

Lyme arthritis: clinical presentation in an early arthritis cohort

Among the total number of 1180 patients studied, a definite diagnosis of Lyme arthritis was made in 9 patients (0.8%). Of 1180 patients with recent-onset arthritis, 53 patients had a positive EIA which was confirmed by a positive (24pts) or equivocal (29pts) blot, indicating a seroprevalence of 2.0- 4.5%. Characteristics of the patients with and without Lyme arthritis are presented in Table 2. As compared to other arthritis patients, patients with Lyme arthritis were younger, and had lower swollen joint count. The distribution of inflamed joints was more often asymmetric including almost always one (n=8) or both (n=1) knee(s). In one patient both wrists, MCPs and PIPs of both hands were also affected in addition to arthritis of one knee. Interestingly, this patient was initially diagnosed as Lyme arthritis and treated with antibiotics which resulted in improvement of the knee arthritis, but not of that of the other joints. Because of persisting arthritis of the wrists and small hand joints during several months, an additional diagnosis of rheumatoid arthritis was made and currently he is still being treated with DMARDs with good clinical response.

	Patients with Lyme arthritis (n=9)	**Patients with other diagnoses (n= 1170)**	**-value**
Female, n (%)	5 (56)	691 (59)	0.834
Age, median (IQR)	26 (22-48)	52 (38-65)	**0.002**
Rheumatoid factor positive, n (%)•	1 (11)	299 (27)	0.274
ACPA positive, n (%)	1 (13)	235 (28)	
Additional symptoms			
none, n (%)	5 (56)	475 (43)	0.441
systemic, n (%)	3 (33)	417 (38)	0.794
Swollen joint count, median (IQR)	1 (1-2)	4 (1-8)	0.066
Affected joints at start of symptoms#			
Large joints only	8 (89)	365 (32)	**0.004**
Symmetric distribution	1 (11)	552 (50)	0.115
Lower extremities only	7 (78)	303 (27)	**0.015**
Upper extremities only	0 (0)	403 (36)	
CRP, median (IQR)β	12 (5-25)	13 (4-33)	0.374
ESR, median (IQR)	18(14-30)	26 (11-46)	0.198
Duration of symptoms in weeks, median (IQR)	9 (4-21)	13(5-29)	0.637
VAS morning stiffness, median (IQR)	10 (4-31)	50 (20-73)	0.009
VAS pain, median (IQR)	12 (7-52)	48 (28-64)	0.018

Table 2: Baseline characteristics of patients with definite diagnosis of Lyme arthritis (n=9) compared to patients with other diagnosis

By selecting only patients with high clinical suspicion for LA (oligoarthritis including a knee and < 50 years) from all tested patients form cohort 1 and 2, the a priori chance of LA increased from 0.8% to 6.4% (n = 125 out of n = 1180) with improvement of PPV (42-85%). Of all patients with definite LA (n=9), two reported skin abnormalities: one an erythema, possibly erythema migrans (not confirmed by a physician) and one nodulosis. Of all patients included in the study 35 (3%) reported a tick bite in the past, in two of these patients a clinical diagnosis of Lyme arthritis was made. Hence the majority of patients with definite diagnosis of LA (n=7 of 9 pts) did not remember any tick bite. Selecting patients with a tick bite in the past and oligoarthritis increased a priori chance of LA to 10%; however, the majority of patients with a diagnosis of LA did not remember any tick bite, therefore this is not a sensitive marker.

Discussion

Lyme arthritis (LA) is relevant to identify because the disease should be treated with antibiotic therapy. *B. burgdorferi* serologic testing is available to aid in the diagnosis, but interpretation of serology can be difficult as also patients with previous Lyme disease can have positive serology. Among this large cohort of patients with recent-onset arthritis seroprevalence of Lyme antibodies was 2.0-4.5%; incidence of definite Lyme arthritis was 0.8%. The positive predictive value for LA of *B. burgdorferi* seropositivity was very low in the recent-onset arthritis population (≤28%). Due to the low prevalence of Lyme arthritis serologic test performance improves markedly by preselecting patients with high clinical suspicion.

Most studies concerning Lyme disease have focused on describing the full clinical spectrum of the disease by selecting all patients with typical presentation and positive serology. By this approach, theoretically, patients with atypical presentation could have been overlooked. In our study, serology for *B. burgdorferi* antibodies was performed on all patients presenting with recent-onset arthritis. Patients with definite diagnosis of LA, defined by positive serology and clinical diagnosis of LA, were characterized by younger age, and typically had arthritis of one or both knees. Four *B. burgdorferi* seropositive patients did not have a clear other diagnosis. The clinician did not perform serology at the first visit of the outpatient clinic. All patients were discharged without antibiotic treatment and complaints resolved spontaneously. In the natural course of LA natural remission, but also progression to erosive disease has been described [155]. It is also possible that these patients recently suffered from LA that had naturally resolved when the visit to the outpatient clinic took place. Two patients had hydrops of the knee with a low cell count that resolved

over time. From this cohort of 1180 patients of early onset arthritis we conclude that it is unlikely that LA presents with other symptoms than arthritis with involvement of the large joints. Thorough examination of clinical charts of patients with positive serology but no other clinical diagnosis did not give arguments for atypical patterns of Lyme arthritis. Only one patient in the possible LA group did not have arthritis of the large joints, and this patient responded well to prednisone treatment, without progression of disease making a LA less likely.
Main drawback of this study is the lack of a gold standard to confirm the diagnosis Lyme arthritis. However, this problem is inherent to the diagnostic testing of Lyme in general as also culturing of *B. burgdorferi* species and PCR on synovial fluid lack sufficient sensitivity and specificity respectively. In fact, this advocates evaluation of performance of serologic testing in patients with recent-onset arthritis. By using the Leiden EAC cohort we had the possibility to check clinical records for a long follow-up period, confirming our data on final clinical diagnosis.

The frequency of LA is comparable between the first cohort (1993-1997; 1.1%) and the second cohort (2003-2008; 0.5-1.1%). It is striking though that the incidence of LB in Europe seems to be gradually increasing the last decades, while we find a stabile of decreasing amount of LA presenting to rheumatologists over the years [118-120]. The higher seroprevalence in the absence of LA in the second cohort can be due to more old *B. burgdorferi* infections. Possibly, growing awareness of Lyme disease resulted in more frequent antibiotic treatment in case of tick bites or reported skin abnormalities.
For the Netherlands, no exact data on seroprevalence among the population are known. The CBO guidelines refer to data from personal communication indicating that seroprevalence varies between 4 and 8%, but seroprevalence is highly influenced by geographic area and population of interest. For example, among Dutch foresters seroprevalence was as high as 20% [322]. In our population the seroprevalence was 2.0-4.5%.
Frequency of LA was low in our cohorts. As Lyme arthritis is considered a manifestation of late, disseminated Lyme disease sensitivity of a serologic test approaches 100%. Based on the low frequency of Lyme arthritis, negative predictive value (NPV) is consistently high, but PPV only contributes significantly for patients with high clinical suspicion of Lyme disease. Most patients in whom Lyme arthritis was confirmed did not remember any tick bite nor a skin lesion, making these characteristics less useful for determination of a priori chance of Lyme disease. The classical clinical manifestation of LA comprises mono- or oligo-artritis of the large joints, most often the knee, but also elbows, ankles and hips can be affected and polyarthritis is described in up

to10 % of cases of recent-onset arthritis [569]. Selecting patients with oligoarthritis including a knee resulted in 226 patients out of 1180, including 8 out of 9 definite LA cases. The one case missed by this selection was the patient who was additionally diagnosed with RA and presented with polyarthritis. Of n=226 patients, n=12 had positive *B. burgdorferi* serology making antibiotic treatment a worthwhile consideration for he benefit of these patients. Based on these data serologic testing for Lyme disease should always be considered in patients with oligoarthritis including a knee, even in the absence of a tick bite or erythema migrans.

In conclusion, our data show that amongst patients presenting with early-onset arthritis LA is diagnosed in 0.8%. Given the very low prevalence of LA, preselection of patients with a suspect clinical presentation is necessary to increase the positive predictive value to acceptable levels.

Acknowledgements

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Chapter 7

Severe course of Lyme neuroborreliosis in an HIV-1 positive patient; case report and review of the literature

Nathalie D. van Burgel[1]
Mayke Oosterloo[2]
Frank P. Kroon[3]
Alje P. van Dam[4]

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[1] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands.
[2] Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[3] Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands
[4] Department of Clinical Microbiology, Onze Lieve Vrouwe Gasthuis, Amsterdam, the Netherlands

Abstract

Lyme Neuroborreliosis (LNB) in a human immunodeficiency virus (HIV) positive patient is a rare co-infection and has only been reported four times in literature. No case of an HIV patient with a meningoencephalitis due to LNB in combination with HIV has been described to date.

A 51 year old woman previously diagnosed with HIV presented with an atypical and severe LNB. Diagnosis was made evident by several microbiological techniques. Biochemical and microbiological recovery during treatment was rapid, however after treatment the patient suffered from severe and persistent sequelae.

A clinician should consider LNB when being confronted with an HIV patient with focal encephalitis, without any history of Lyme disease or tick bites, in an endemic area. Rapid diagnosis and treatment is necessary in order to minimize severe sequelae.

Introduction

Lyme Neuroborreliosis (LNB) in a human immunodeficiency virus (HIV) positive patient is a rare co-infection and has only been reported four times [573-576]. All published cases are early presentations of Lyme disease and no report of a meningoencephalitis due to *B. burgdorferi* in an HIV patient has been made to date. We present a case of an HIV positive patient that presented with a severe LNB, without any previous sign of Lyme disease.

Case presentation

A 51-year-old woman, diagnosed with HIV 10 years before, presented early spring 2006 at the outpatient clinic in the west of the Netherlands. She had noticed an altered gait that was progressive since three months. Strength and sensibility were unaltered, but there was paresthesia in both legs. She also had problems unbuttoning clothing with both hands. There was no complaint of headache, photophobia or visual changes. The medical history showed an anxiety disorder, hypertension and glaucoma. At presentation, the patient had been using HAART for six years (zidovudine, lamivudine and nevirapine) in combination with antihypertensive medication and a selective serotonin reuptake inhibitor. There was no indication for antibiotic prophylaxis. She had no history of tick bites, rash, erythema migrans or other signs of early or late-stage Lyme borreliosis. However, she frequently worked in her garden in an area where *B. burgdorferi* is endemic. Neurological examination revealed a bipyramidal walking pattern, an intention tremor of the posture and the hands, bilateral hyperreflexia in her legs and arms, a positive Hoffman-Trömner and a bilateral Babinski. There were no meningeal signs and all cranial nerve function was intact. RR was 190/113 and temperature was normal. Routine laboratory tests showed no signs of infection; blood leukocytes level was 5,9*109/l. Plasma HIV RNA load was undetectable, CD4+ T lymphocyte count was 501/µl. A lumbar puncture was performed; the opening pressure was 28cm H_2O. The cerebrospinal fluid (CSF) showed a leucocytosis of 201/µl, 70% T-lymphocytes, 6% NK-cells, and 6% B-lymphocytes, glucose 2 mmol/l (serum glucose 6,2 mmol/l), protein 1,26g/L. By isoelectric focusing oligoclonal IgG was detected intrathecally, but there was no evidence of a monoclonal B-cell population. Cytology and immunophenotyping of the intrathecal leukocytes were negative for hematological malignancies.

An MRI of the spine as well as brain was performed. No abnormalities were detected in the spine, but in the midline of the pontine region a hyperintense

signal was seen on DUAL and FLAIR view (Figure 1-2). This lesion did not enhance under gadolinium.

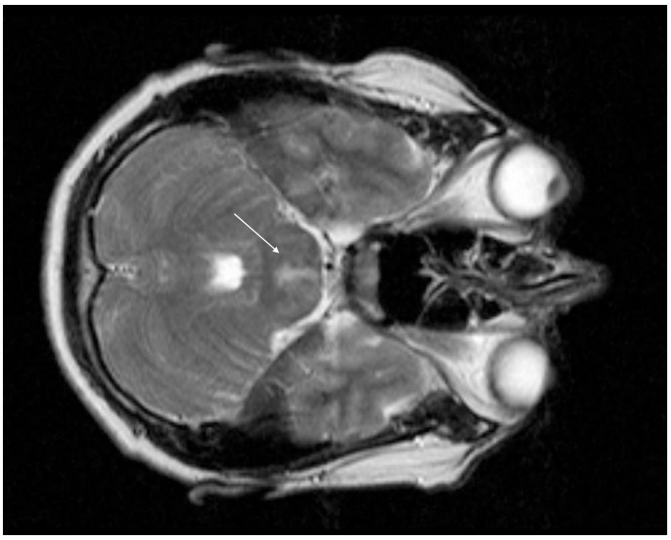

Figure 1: DUAL TSE at presentation with a diffuse lesion located centrally in the pons

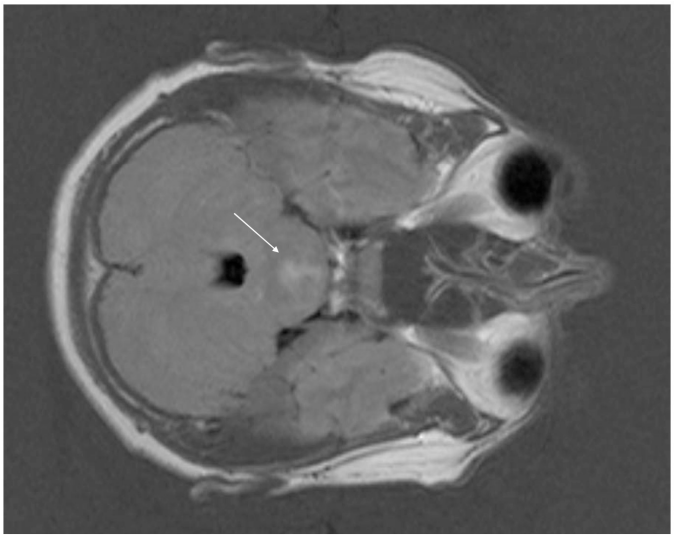

Figure 2: FLAIR at presentation with a diffuse lesion located centrally in the pons

CSF was examined and found PCR negative for HIV RNA, neurotropic viruses (Cytomegalovirus, Epstein Barr, Varicella zoster virus, Herpes simplex virus, and JC virus), tuberculosis, toxoplasmosis, Bartonella and *Treponema pallidum*. CSF and serum serology was negative for *T. pallidum* and cryptococcal antigens.

Serology for Bartonella, Babesia, Anaplasma and Ehrlichia showed no indication of an active infection. Culture was negative for cryptococcosis, tuberculosis and other common bacteria. Results for Lyme disease showed specific intrathecal IgG antibodies against *B. burgdorferi* in ELISA, no additional bands on blot were seen in CSF compared to serum. The intrathecal antibody index (AI) was positive (AI 19; cut off 0.3), indicating a specific production of antibodies to *Borrelia* in the CSF (IDEIA, Oxiod, UK). Also real-time PCR for *Borrelia burgdorferi* OspA conducted on the CSF was positive [259, 574]. Serum antibodies against *Borrelia* were detected with the QuickELISA Borrelia C6 assay (Lyme index >10) (Immunetics, Boston, USA), and their presence was confirmed by a positive band for p100/83, VlsE, p41(i), p39 and DbpA on the RecomBlot Borrelia IgG assay (Mikrogen, Martinsreid, Germany). A serum from three years preceding this clinical presentation tested completely negative for antibodies against *B. burgdorferi*.

The diagnosis of a Lyme meningoencephalitis was made. The patient was treated intravenously with ceftriaxone 2 g/day for 1 month according to the EUCALB guideline. During the first week of treatment her clinical condition worsened. She was no longer able to walk independently and was forced to use a wheelchair. The cerebral MRI however showed decline of the hyperintense region at the end of intravenous treatment. An MRI performed one month later showed no abnormalities at all (Figure 3).

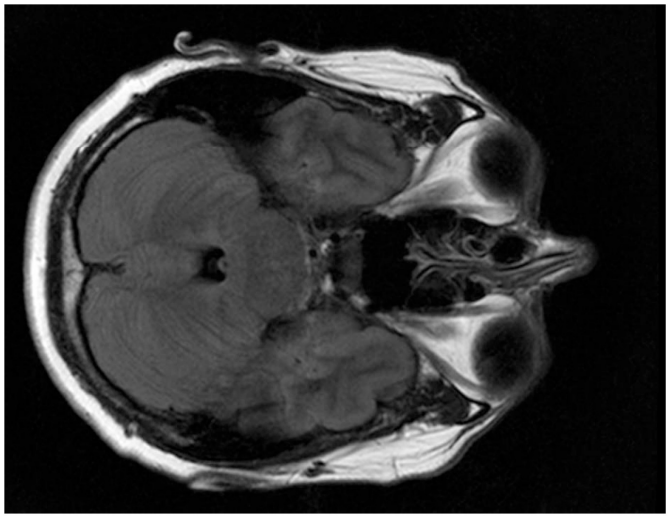

Figure 3: FLAIR several months after treatment, the lesion completely resolved.

In addition, post treatment CSF showed a reduction in pleocytosis (7/µl), an absence of intrathecal specific antibodies against Borrelia and the real-time PCR for *OspA* on the CSF was negative. After a year of regular check up the patients'

physical examination showed paraparesis of the right m. iliopsoas grade 4, right hamstrings grade 3, right footlifters degree 4 and hyperreflexia of both legs with Babinski signs. The bipyramidal walking pattern was still present. The patient was able to stand alone and walk with a cane. No further clinical improvement was detected at regular check up for four years.

Discussion and conclusions

We describe a case of a patient with an HIV infection and severe neurology and MRI lesions due to a *B. burgdorferi* central nervous system (CNS) infection. After treatment with ceftriaxone the laboratory parameters of infection disappeared along with the abnormalities on MRI, however the patient persistently suffered from severe verifiable sequelae.
Diagnosing Lyme disease using serology in patients with HIV is complicated due to reduced positive predictive value of serology in HIV patients [577]. Also, false positive serologic findings are described in patients with neurological infections with other spirochetes such as *Treponema pallidum* [578]. Present patients' clinical presentation was compatible with a localized cerebral infection with *B. burgdorferi*, confirmed by the positive serology, intrathecal specific anti-*Borrelia* antibody production and a positive real-time PCR for *B. burgdorferi OspA* on the CSF. The sensitivity of the *OspA* real-time PCR on CSF is only about 50% but the specificity is very high >99% [259]. In HIV positive patients where diagnosis of LNB by an indirect method such as serology is already compromised, a method where the microorganism is detected directly is of great value.

Little is known about the course of LNB in HIV patients. Animal models have shown that CD4 + T-cells facilitate clearance of *B. burgdorferi* [189]. In recent studies the importance of CXCL13 in B-cell recruitment in patients with LNB has been suggested [382]. In HIV infection the levels of CXCL13 in serum are elevated and the receptor CXCR5 on B-cells is down regulated causing impaired trafficking of B-cells [384]. Inadequate humoral response could lead to accelerated progression of LNB. In animal experiments immunodeficiency leads to higher spirochete burdens and higher infectivity of *B. burgdorferi* [547]. In contrast, reports from a study where Lyme borreliosis patients were treated with immunosuppressive agents, no significant effect on clinical course and response to treatment was observed [193]. *T. pallidum* is a spirochete and more is known about disease course in syphilis HIV co-infection. Disease course is altered in *T. pallidum* and HIV co-infections; there is a higher rate of

asymptomatic infection, a faster progression to secondary disease which is often more aggressive with a significant predisposition for the development of neurological complaints. However, after treatment HIV positive patients recover as well as the HIV negative population [579, 580]. In co-infections of HIV and *Leptospira* species. a more fulminant disease course has also been suggested [581]. For LB and HIV co-infections no such synergistic complications have been described in patients to date.

Case no.*	Anti-retroviral	Antibiotic rophyl.	Skin lesions	Presentation	Treatment	Clinical recovery
1	none	none	Annular erythema tous lesion	Several weeks later: fever, bilateral facial palsy	IV ceftriaxone 2g/day 14 days	After treatment vast improvement 2 months, complete recovery
2	Zidovudine Saquinavir Zalcitabine	none reported	Erythema	Headache, painful limbs, weight loss, pneumonia 2 weeks later: fever, diplopia	Primarily: PO Azithromycin 1 day 500mg, 4 days 250mg IV ceftriaxone 2g/day 14 days	Progression to neuroborreliosis Improved rapidly
3	Zidovudine Didanosine	none reported	Erythema tous lesion	4 weeks later:: radiculitis	IV ceftriaxone 2g/day 15 days	Complete recovery 18 months, no relapse
4	none	none reported	Multiple Maculous erythema s	Fever, chills, arthralgias 2 weeks later left facial palsy	IV cefotaxime 2g TID 21 days	After treatment mild facial palsy 6 months, slight hypokinesia face
5	Zidovudine Lamivudine Nevirapine	none	None	Altered gait for months	IV ceftriaxone 2g/day 1 month	After treatment severe sequelae 3 years, no relapse

Table 1: Clinical data from all patients with LNB and HIV reported in literature. *Case 1[573], 2 [575], 3 [576], 4 [574], 5 this report.

Despite the high incidence of HIV, co-infection with *B. burgdorferi* is not reported very often [573-576]. The low incidence of HIV LNB co-infections can not be easily explained. In HIV infection antibiotic prophylaxis is sometimes prescribed but none of the reported cases of HIV patients with LNB used antibiotic prophylaxis. Prophylaxis usually consists of cotrimoxazole, which is ineffective against *B. burgdorferi* which renders a positive effect of antibiotic prophylaxis on the development of Lyme disease unlikely [582]. There is no data about anti-borrelia activity for anti-retroviral medication but an association seems unlikely because three of the five described patients were using anti-retroviral medication. All reported LNB HIV patients' results have been summarized in table 1 and table 2.

Case no.*	Ag	x	Yrs HI	Blood CD4+ count (/µl)*	CSF cell count (/µl)	Protei (g/l)	CT/MRI	Serum Serology	CSF Serology
1	39	M	0	386	30	1,02	nd	IgG + ELISA /WB	IgG + ELISA /WB
2	39	M	1	250	496	3,62	nd	IgM + / IgG -	IgG +
3	50	M	10	70	'aseptic meningitis'		nd	ELISA IgG + (month 4) WB IgG + IF Negative >4 months	ELISA IgG + (month 4) WB IgG +
4	46	M	16	426	416	3,02	normal	IgM + / IgG + ELISA /WB	IgM + / IgG + ELISA /WB
5	51	F	11	501	200	1,26	abnormal	IgM - / IgG + ELISA /WB	IgM - / IgG + ELISA /WB

Table 2: Laboratory data from all patients with LNB and HIV reported in literature.
Case 1[573], 2 [575], 3 [576], 4 [574], 5 this report.. Serology was performed by ELISA, Western blot (WB), or indirect immunofluorescence (IF). (nd= not done) normal range peripheral blood; case 1: 580-1570 /µl, case 3: 600-1000/µl, case 5: 560-1490 / µl

Case 1 had had an EM and presented with a rather classical course of a bilateral facial palsy shortly after noticing the EM, rapidly improving on IV ceftriaxone treatment. HIV serology was found positive during workup for the cause of his bilateral facial palsy, CD4+ count was decreased at that time. Case 2 had a low CD4+ count with progression to neuroborreliosis, despite treatment for his recent early LB. Serology in CSF and serum was clearly positive for an early LNB. After IV treatment with ceftriaxone he recovered completely. Case 3 had a very low CD4+ count and was the only case that met the criteria for AIDS. He primarily showed a slow seroconversion although this was only determined by indirect immunofluorescence and not by ELISA or Western Blot. Four months after presentation with a painful radiculitis he had low detectable Ig titers against *Borrelia* by ELISA and Western Blot. He recovered completely. Case 4 had a moderately low CD4+ count and had a classical course of EM, followed by malaise and a facial palsy with signs of early LNB in serology of CSF and serum. He responded well to treatment with IV cefotaxime. Case 5 the present case had a moderately low CD4+ T-lymphocyte count and had rapid progression to a meningoencephalitis. This is the only case described to date of an HIV patient with a Lyme meningoencephalitis. The course of disease was rapid and atypical with a primary presentation of altered gait due to Lyme meningoencephalitis, which is an uncommon presentation of neuroborreliosis, found in only 3-5 % of patients with LNB [114]. Diagnosis was made obvious due to positive PCR combined with positive IgG serology in CSF and serum. After treatment microbiological response was rapid. AI returned negative after two months,

which is a rapid decline but not uncommon in literature [368]. Despite this rapid improvement biochemically and microbiologically severe sequelae remained. Post treatment sequelae are rare; patients with post treatment sequelae have complaints of fatigue, cognitive impairment and paresthesia but sequelae are rarely as severe after a relatively short duration of illness as in this case [114, 447, 583]. Although this patient had complaints for only three months, it is likely that earlier recognition and treatment of LNB in this patient would have led to less neurological damage and therefore to better recovery.

A clinician should consider LNB as a rare possible cause of focal encephalomyelitis in an HIV patient, without any history of Lyme disease or tick bites in an area endemic for Lyme disease. Diagnosis of LNB can be compromised in HIV co-infected patients, however when applying optimal serological and molecular diagnostic techniques confirming the presence of LNB is imminent. This report raises the possibility that LNB might take a more severe course in immunocompromised patients, such as those with HIV infection.

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Chapter 8

Summarizing discussion

This thesis covers research regarding pathogenesis as well as diagnosis and clinical presentation of an infection with *B. burgdorferi*. *B. burgdorferi* is an extracellular pathogen. It is therefore plausible that the humoral immune response in the form of complement-mediated bactericidal activity is of importance in the defense against infection and disease. It has previously been shown that distinct *B. burgdorferi* strains are able to withstand complement mediated killing in vitro.

Complement and other immune evasion strategies

The mammalian-vector cycle that Borrelia use to replicate is an elaborate lifecycle with many potential hosts. Not only is *B. burgdorferi* able to maintain itself in the vector at very low temperatures, but it is also able to withstand the relatively high temperatures of the mammalian host. Furthermore *B. burgdorferi* can infect a wide range of hosts, while all hosts have their own specific set of receptors and proteins to withstand infections. One of the proteins that *B. burgdorferi* can bind in the mammalian and avian host is complement factor H (CFH) and in humans specifically factor H like protein-1 (FHL-1). In the mammalian and avian host these proteins are responsible for inactivating the complement cascade at the C3 level *B. burgdorferi* possesses several proteins to bind CFH and FHL-1, these were previously designated as Complement Regulator-Acquiring Surface Proteins (CRASP) [74]. Several CRASPs from several species from the *B. burgdorferi* sl complex have been described and every CRASP is responsible for a specific time point in the infection cycle where complement is inactivated. CRASP-1 is thought to be responsible for inactivation of CFH and FHL-1 early in the infection [539].

In **chapter 2** of this thesis we describe two new CRASP-1 proteins of *B. bavariensis*, BGA66 and BGA71, that can bind human CFH and/or FHL-1, but we also showed that several other paralogues located on the same plasmid, and very likely under the same promoter can bind to CFH from several other mammalian and avian species. All the genes from the gbb54 paralogous family are upregulated at the same moment, namely at the time of tick feeding on the host. This means that independent of the type of host, different CFH-binding-proteins are expressed that are able to bind CFH and deactivate MAC formation on the borrelial surface in the tick midgut. Later in the infection other CFH-binding proteins are upregulated and CRASP-1 proteins are downregulated [79]. These proteins able to bind CFH later in infection, namely the Erp proteins, are a large family of similar proteins located on the cp32 plasmid of which borrelia can bear several non-similar copies [584]. It is likely that these proteins are also able to bind fluid phase complement regulators of a wide range of hosts. In

order to understand more about the mammalian-tick-lifecycle these CRASPs should be studied for host specificity.

In the **chapter 3** we studied whether infectivity from several species of *B. burgdorferi* that have been reported to be pathogenic in the human host would be altered in the absence of an effective complement system. In this study we showed that absence of complement did not lead to increased infectivity of complement sensitive strains. Studies on the role of complement in infectivity of *B. burgdorferi* have been conflicting, finding no or only a minimal effect on burden or disease severity [98, 191]. A study that used a CRASP-1 knockout *B. burgdorferi* ss strain for detecting complement resistance has shown a large negative effect on the binding of CFH and a decline in serum resistance in vitro. This strain could not be tested in vivo, because the strain itself was unable to give infection in the mammalian host [526]. In a CRASP-2 knockout *B. burgdorferi* ss strain no alteration in serum resistance was found in vitro and no effect was seen on infectivity in a mouse model [86]. Complementation of a CRASP-4 gene in a serum sensitive strain did not alter serum sensitivity [88].
An additional hurdle in unraveling the role of complement-binding proteins in *B. burgdorferi* infection is caused by differences in virulence among the strains. Reference *B. burgdorferi* ss strains are virulent as well as pathogenic in mice, in our study displaying a positive PCR as well as rapid positive cultures. On the other hand we found that both *B. afzelii and B. bavariensis* strains had positive PCR results from many organs, while we were only able to culture from heart tissue. Apparently *B. afzelii* and *B. bavariensis* were more difficult to reculture spirochetes from organs in BSK-H medium. The used *B. garinii* and *B. valaisiana* strains failed to cause murine infection altogether. A possible explanation is that these strains have lost essential plasmids and essential virulence factors during in vitro culture. The low virulence in mammals of *B. garinii* and *B. valaisiana* strains has been described before; it is likely the lack of infectivity in mice is due to the preference of these species for infection of avian hosts [24, 585].

Genetic tools for transformation of strains of the *B. burgdorferi* sl complex have been described, but transformation of low passage strains has proven to be tedious. Transformation of higher passage plasmids is possible, but these strains often lack plasmids that are essential for natural infection [586, 587]. This complicates the ability to study the effect of alteration of CRASPs on the infectivity in vivo. Knock-in of genes in *B. garinii* strains has been successfully performed, albeit not in an infective strain [88]. We were not successful in producing knockout CRASP-1 *B. afzelii*, *B. bavariensis* or *B. garinii*. Therefore it was impossible to do additional studies on knockout mutants of *B. afzelii*, *B. bavariensis* and *B. garinii* strains.

In our model we studied complement resistance by syringe inoculating the cultured *B. burgdorferi*. We now know that the system of up and downregulating proteins in the tick-host cycle is a complex and meticulous system involving many proteins that are able to alter serum sensitivity. The CRASPs for inactivating complement by binding to CFH, but also OspC and very likely many other outer surface proteins can bind tick derived salivary proteins that can aid entry and persistence of *B. burgdorferi* in the vertebrate host. Furthermore the wide scala of mammalian hosts and the specificity of specific complement altering proteins for specific hosts further complicate studying these proteins. This complex combination of expression of proteins makes it difficult to study the individual roles of the different proteins in the entire infective process. One can argue that perhaps specific proteins in the cycle are not essential but all contribute small pieces that cumulatively ensure adequate transmission of *B. burgdorferi* in the large group of potential hosts.

Is the laboratory and clinical diagnosis of Lyme disease really so complicated?

The difficulties observed in the laboratory diagnosis of *B. burgdorferi* strikingly contrast with the broad introduction of many rapidly evolving techniques for detection of microorganisms in modern clinical practice. Until now no good gold standard for detection of active infection with *B. burgdorferi* sl has been developed. What makes *B. burgdorferi* sl so special that the laboratory diagnosis seems to be so difficult?

Direct detection of *B. burgdorferi*

The most reliable way of diagnosing an infection with a microorganism is cultivating the organism which causes the disease from blood or the site of infection. Cultivating the pathogenic organism from a diseased individual proves that viable organisms can replicate and can cause infection. Bacterial culture is widely applied in the field of diagnostic microbiology. *B. burgdorferi* sl is a highly fastidious organism, some species of *B. burgdorferi* sl are more difficult to culture, which we have shown in **chapter 3** in an in vivo experiment. *B. burgdorferi* sl culture is laborious and can take up to several weeks and the sensitivity of culture in an active infection is too low to use it in a routine diagnostic setting [235].

Other ways of direct detection of a pathogenic microorganism can be direct visualization, for example by IFA. This generally has a very low sensitivity due to

the low load of Borrelia in the host [235]. This has also been demonstrated in **chapter 3** of this thesis.
Nucleic acid amplification techniques have been widely applied during the last two decades for detection of pathogens. In general they are considered to be very sensitive diagnostic tools for diagnosing infection with fastidious or uncultivable organisms. Most infections have a high load of microorganisms in tissue or blood (up to 10^9 copies/ml blood), but *B. burgdorferi*, specifically in disseminated infection, are present only at very low levels. The low load of *B. burgdorferi* in tissue may lead to false negative PCR results during active infection. However, as is demonstrated in **chapter 3** of this thesis, PCR on infected tissue does give the highest sensitivity of the abovementioned techniques. Direct detection of *B. burgdorferi* can have an additive effect in a specific individual, but the general conclusions is that currently available direct detection methods for *B. burgdorferi* are insufficiently sensitive to in- or exclude active *B. burgdorferi* infection [257].

Indirect detection of *B. burgdorferi*

Indirect detection of recent or past infection by serology is a common diagnostic tool for fastidious or uncultivable microorganisms. In the diagnosis of *B. burgdorferi* infection it is the most frequently used technique, because of the abovementioned reasons. In the development of serological tests it is logical to start with whole cell lysates. Development of serologic tests of bacteria has some major complications, because bacteria share many homologous proteins with other bacteria. Antibodies induced against major structural proteins can cross react with antibodies against other, similar, bacteria. Using either a whole bacterium in an immunofluorescent assay (IFA) or a whole-cell bacterial sonicate in an enzyme immunoassay (EIA) can easily give false positive results because of the abovementioned reasons. Serology for bacterial diseases can therefore be more compromised than for many clinically relevant viruses that contain a relatively unique and more limited amount of different proteins.

In viral serology often virus-specific conserved proteins have been isolated and used for specific serology, like gp41 or gp120 in HIV infection [588]. Nowadays, in virological serology many virus EIAs are built with recombinant antigens. In *B. burgdorferi* sl some specific recombinant antigens have been identified that elicit good antibody responses in many infected individuals, but are uncommon in other bacteria. Examples of these are VlsE (or C6-peptide), OspA, OspC, DbpA and flagellin internal fragment. The setback of using these antigens is that the species in the *B. burgdorferi* sl complex that are able to cause disease

in humans are so different that sometimes only a mix of an antigen like OspC from several species is warranted to obtain positive serology in all affected individuals. As the *B. burgdorferi* sl complex in North America only consists of *B. burgdorferi* ss this is mainly a problem in the Eurasian continent [589].

In **chapter 4** we show a high sensitivity of anti-C6-peptide serology in serum and CSF in the manifestation of Lyme neuroborreliosis in Dutch patients. VlsE or C6-peptide based assays are now the most commonly used commercial diagnostic strategy for screening in the Netherlands. It can be debated whether a screening assay solely based on one peptide will detect all different species of *B. burgdorferi* sl. If we compare this to commercial EIAs for other pathogens often at least two different antigens are used in the screening EIA. Primarily C6-peptide is conserved between species, while VlsE is less conserved between species. From literature however there is little reason to presume that the VlsE or C6-peptide based assays lack sensitivity to detect patients with later manifestations of Lyme borreliosis [314, 345, 590, 591]. It has been demonstrated however that species specific C6-peptide has a higher sensitivity especially in early manifestations of LB [345]. For early manifestations of the illness additional antigens can improve sensitivity, for instance OspC, being a good candidate in the IgM EIA [592].

A more specific way to look at antibody responses is a (native) western immunoblot. Antibodies against a specific subset of bacterial proteins can be measured, but the interpretation of the immunoblot is highly dependent on the algorithm that is used for determining positivity. It was previously shown that the different species throughout Europe of *B. burgdorferi* sl can have different serological profiles. A previously mentioned suggestion was that perhaps different algorithms for interpretation of the immunoblot for different region with prevalence of other *B. burgdorferi* sl species should be used [303]. In this setting and considering that a specific species of the *B. burgdorferi* sl complex can cause specific clinical manifestations it could be useful to define different interpretation algorithms for specific clinical manifestations. In a routine laboratory setting this is difficult to implement, because the clinical microbiologist does not always have the clinical data at hand.

The presence of anti-pathogen IgM generally is an indicator of early or active infection. However, in Lyme disease it has already been demonstrated that IgM can stay positive after effective treatment for many years [317, 326-330]. Furthermore, IgM is not an indicator of active disease. In late manifestations of Lyme disease IgM can often be absent, while there is still an active infection. This combination can easily lead to misinterpretation of serologic results. For

late manifestations of Lyme disease anti-Borrelia IgM results should play no role in the interpretation of serological results. An example of a proposed algorithm to interpret *B. burgdorferi* serology is shown in figure 1.

For some microorganisms antibodies against early and late expressed proteins are detected in combination to distinguish past from recent infection. In this case Epstein Barr Virus (EBV) would be an excellent example. Anti-EBV-Viral Capsid Antigen (EBV-VCA) IgG is used as a marker for EBV infection, and presence of EBV-Early Antigen (EBV-EA) IgG and absence of EBV-Nuclear Antigen (EBV-NA) IgG generally indicate recent infection [593]. In the case of EBV-infection the most common clinical manifestation is a recent primary infection. It is known that in *B. burgdorferi* infection certain proteins are expressed early during transmission to the mammalian hosts. Examples are OspC, OspA and several CRASPs. Although anti-OspC is a good marker for early infection, antibodies against OspC can not distinguish past from present infection. To date no conserved *B. burgdorferi* antigen has been described that can distinguish cleared from active infection. The conserved C6-peptide seemed to be a promising antigen for detecting active infection because antibody titers are highly elevated and can drop after treatment. The seroprevalence of anti-C6-peptide antibodies is lower than for VlsE, making it likely that anti-C6-peptide would be a more suitable marker of active disease [591]. This is also clearly demonstrated in figure 1 of **chapter 4** of this thesis; the patients with recent or active LB have higher anti-C6-peptide values than the controls. However, this phenomenon is clearly measurable on a population level, but will often not lead to a solid conclusion in an individual case in a single serum sample. An infection with *B. burgdorferi* is dependent on many factors, including host response. The combination of the heterogeneity of *B. burgdorferi* sl, with the heterogeneity in clinical presentation and the duration from infection to clinical presentation make the search for biomarkers, antigens or serological profiles that can distinguish cleared infection from active present infection an almost impossible quest.

Other surrogate markers in serum are sometimes used for detecting infection with spirochetes. One example is the anti-cardiolipin antibodies that are found during active *T. pallidum* infections [594, 595]. It is well known that anti-cardiolipin are not specific and also detectable in a number of other inflammatory diseases but for example also during pregnancy [596]. Detection of these antibodies has a solid status in the detection of clinical response to treatment for syphilis. The test strategy consists of detecting of specific anti-*T. pallidum* antibodies together with anti-cardiolipin antibodies. The titers against anti-cardiolipin are followed over prolonged time intervals to detect treatment response, failure

and perhaps reinfection. During infection with *B. burgdorferi* anti-cardiolipin (IgM) antibodies can be positive but they are usually negative in patients with active infection [597]. Screening for anti-cardiolipin as it is used in *T. pallidum* serology is not a suitable option in Lyme patients.

One of the additional problems in Lyme disease serology is that residual complaints after diagnosis and treatment of Lyme disease have been described, but that no serological test is able to discriminate between persistent infection and residual antibody response after successful treatment. Again, anti-C6-peptide antibodies in many patients decline over time. Although it will not be a marker that can give a black or white answer, a significant decline in titers, or even becoming anti-C6 seronegative after treatment is a strong clue that the original antigen has been eliminated. In specific cases this can perhaps aid the clinician in the choice between treatment directed on reducing symptoms instead of an antimicrobial treatment. Other markers of infection have been studied; a biomarker of recent interest is CXCL13. In serum this is not a good predictor of active *B. burgdorferi* infection [383, 390]. A complicating factor is that treatment failure measurable by objective parameters is extremely rare, so serological follow up of patients with persistent complaints can never be compared to a worthy gold standard.

Diagnosing Lyme neuroborreliosis

For the diagnosis of Lyme neuroborreliosis (LNB) the gold standard is the antibody index for anti-Borrelia antibodies. The problem with this assay is that not all routine clinical laboratories have the capacity to perform this assay. Also there is a lack of expertise in interpreting the results as LNB is a relatively rare diagnosis, while the request for LNB diagnostics is fairly high. There is a need for a screenings assay on CSF without having to implement new assays in routine laboratories.

In **chapter 4** we developed and tested a protocol for CSF using a commonly used antigen for the screening EIA, namely C6-peptide. We found a high sensitivity of the anti-C6-peptide serology for detection of antibodies in the CSF in case of a LNB. Therefore the anti-C6 peptide assays can be used to screen for LNB. Complete absence of anti-C6-peptide antibodies in the CSF makes a LNB highly unlikely. As is often the case with a screening test the specificity of the C6-peptide total Ig assay is not so high, but 93% of all non-neuroborreliosis patients could be excluded with just one test on the CSF. Confirmation with the gold standard for detecting LNB, calculating the AI, is less sensitive, but has a higher specificity. It is advisable that the confirmation assay is performed in a

laboratory that has clinical expertise in the field of LNB and interpreting the results from the calculated Ig-index assays. This means also taking into account the duration of illness, (multiple) treatment before the lumbar puncture and cell count together with other abnormalities in the CSF.

It is clear that in the case of LNB gold standard for diagnosis should be comprised of multiple parameters. All parameters that are being used to diagnose acute active disease have exceptions that have been elaborately described. The clinical manifestation is often typical, but some anecdotal atypical manifestations have been described [188, 598]. Serum serology is usually positive, but especially in early LNB this can be negative. The latter is infrequently seen in children who present with a facial nerve paralysis (FNP) very early after infection [599]. The a priori chance that a FNP in children is caused by *B. burgdorferi* is high in endemic countries, so a clinician should always perform follow up serology or do a lumbar puncture at presentation. The intrathecal white blood cell count is usually elevated, but in anecdotal cases no elevation has been found [114]. The anti-Borrelia antibody index is specific, but not very sensitive, especially in early LNB [366, 368, 369]. PCR on CSF can help confirm the diagnosis, but is not sensitive enough and certainly cannot exclude LNB.

Furthermore, after treatment the abovementioned parameters can all stay positive after adequate antimicrobial treatment. Many patients however suffer from residual complaints where the abovementioned parameters can not beyond a doubt demonstrate or disprove persistent infection in all individuals (See table 1). There is a need for a marker that can differentiate between persistent active and past infection.

In **chapter 5** we show a high sensitivity and reasonable specificity of intrathecal CXCL13 levels in LNB patients compared to patients with other inflammatory and infectious neurologic illnesses. It was shown in this study that most inflammatory illnesses have no or only minimal amounts of intrathecal CXCL13. In particular, longstanding infections like cryptococcal meningitis or *T. pallidum* especially during HIV co-infection, also resulted in elevated levels of intrathecal CXCL13. These specific groups of patients can usually be eliminated in the differential diagnosis. Furthermore, in our study several autoimmune diseases, where B-cell dysregulation or dysfunction plays a role, can also present with increased levels of intrathecal CXCL13. These findings underscore the nature of the chemokine CXCL13: CXCL13 is only a marker of inflammation and merely semi-specific for LNB due to the slow, protracted infection *B. burgdorferi* can cause [600]. It is another marker in the already widespread field of *B. burgdorferi* diagnostics that cannot be interpreted on its own, like serology, medical history

and clinical presentation. In a diagnostic strategy for LNB it can take a strong position, because elevated levels of CXCL13 are highly sensitive compared to the AI and far more specific than intrathecal leucocytosis. An algorithm in which the parameters from table 1 are classified into major and minor criteria should be studied prospectively in LNB patients presenting pre-treatment on a regional basis in order to develop two diagnostic algorithms; one algorithm with a high sensitivity in order to diagnose all individual LNB patients and start antimicrobial treatment, and one algorithm for European international multicenter studies with high specificity in order to select patients for new diagnostic or treatment studies on Lyme disease. The latter is important in future research to reduce the background noise of inclusion of patients without an active infection with *B. burgdorferi*.

Parameter	Pre-treatment	Post-treatment
Clinical presentation	Typical presentation to anecdotal atypical reports	Often: residual neurological damage or aspecific complaints
Blood serology (Several Ags)	Early LNB: can be negative	Early treatment: serology can stay negative, or become positive
	Late LNB: positive	Late treatment: serology can stay positive or become negative.
CSF white blood cell count	Usually elevated	Decline over weeks
Antibody-index CSF	~75% positive AI	Can stay positive for years, Can become negative,
	~25% negative AI*	Can stay negative.
PCR	Early: low sensitivity Late: low sensitivity	Usually negative, but anecdotal reports describe a positive PCR without residual complaints**.
CXCL13	Usually highly elevated	Rapid decline after treatment

Table 1: Parameters for diagnosing Lyme neuroborreliosis pre-treatment and post-treatment. (Ags: Antigens, CSF: cerebrospinal fluid, AI: antibody index) * Mostly early, but also late infections; **Reference [259]

It has also been shown that CXCL13 shows a rapid decline after start of treatment in literature and in **chapter 5** of this thesis [382]. This is most likely due to the rapid deterioration of antigen and the subsequent lack of production of CXCL13 in antigen presenting cells. This potentially makes CXCL13 an interesting marker in the follow up of patients with residual complaints after treatment for a LNB. If CXCL13 levels drop and residual complaints are present a treatment strategy focused on symptom reduction by anti-inflammatory agents, pain relief medication or cognitive behavioral therapy instead of antimicrobial

therapy are more warranted. In this sense intrathecal CXCL13 can be used comparably to the serum anti-cardiolipin antibodies in *T. pallidum* infection where a non-specific marker is followed over time to have insight in the treatment response. This specific application for routine diagnostics of LNB will very likely play an important role in a diagnostic algorithm in the near future.

Preselection of patients eligible for Lyme disease testing.

In clinical practice it is important to select patients eligible for Lyme disease testing in order to have a higher pretest probability. In the general healthy population in the Netherlands the seroprevalence of antibodies against *B. burgdorferi* sl is about 5-10%, depending on the assay used and the region [111, 319-321, 599]. The seroprevalence can even be up to 45% in persons working in professions where they are at risk for contracting a tick bite [320, 322, 322, 324, 601, 602].
In **chapter 6** of this thesis we show in a large early arthritis cohort that testing for Lyme disease should only be done in a selected population with a clinical presentation that is compatible with Lyme disease. The pretest probability for Lyme disease in this cohort was only 0.5-1.1%. The positive predictive value of randomly performed *B. burgdorferi* serology in patients presenting with arthritis is disturbingly low. Clinicians should never underestimate the influence of positive serology on the mental state of the patient. Doctors might be able to see the relativity of positive serology, but to the patient a positive serology can be equal to actually having the active disease. This psychological burden underscores the importance of clinically preselecting patients eligible for *B. burgdorferi* serologic testing.

On the other hand testing for Lyme disease should be performed in patients with a clinical picture that is compatible with *B. burgdorferi* infection. One problem is the many clinical pictures Lyme disease can mimic, especially in immunocompromised patients that already have a more elaborate differential diagnosis.
In **chapter 7** we describe a case of an HIV patient with a meningoencephalitis caused by *B. burgdorferi*. The progression of disease was unusually fast and after treatment there were elaborate sequelae. We hypothesized that *B. burgdorferi* can have a more severe course in HIV co-infected patients. This has previously been described for *T. pallidum* and *Leptospira* spp. infection [579-581].
The increased pathogenesis of spirochetes in HIV infected patients is not well understood. In **chapter 5** we found highly elevated levels of intrathecal CXCL13 in LNB patients. It is known that B-cells of HIV infected patients have downregulated CXCR5 and inadequately respond to CXCL13 produced by antigen presenting cells [384, 567]. This specific dysfunction might be a first clue in

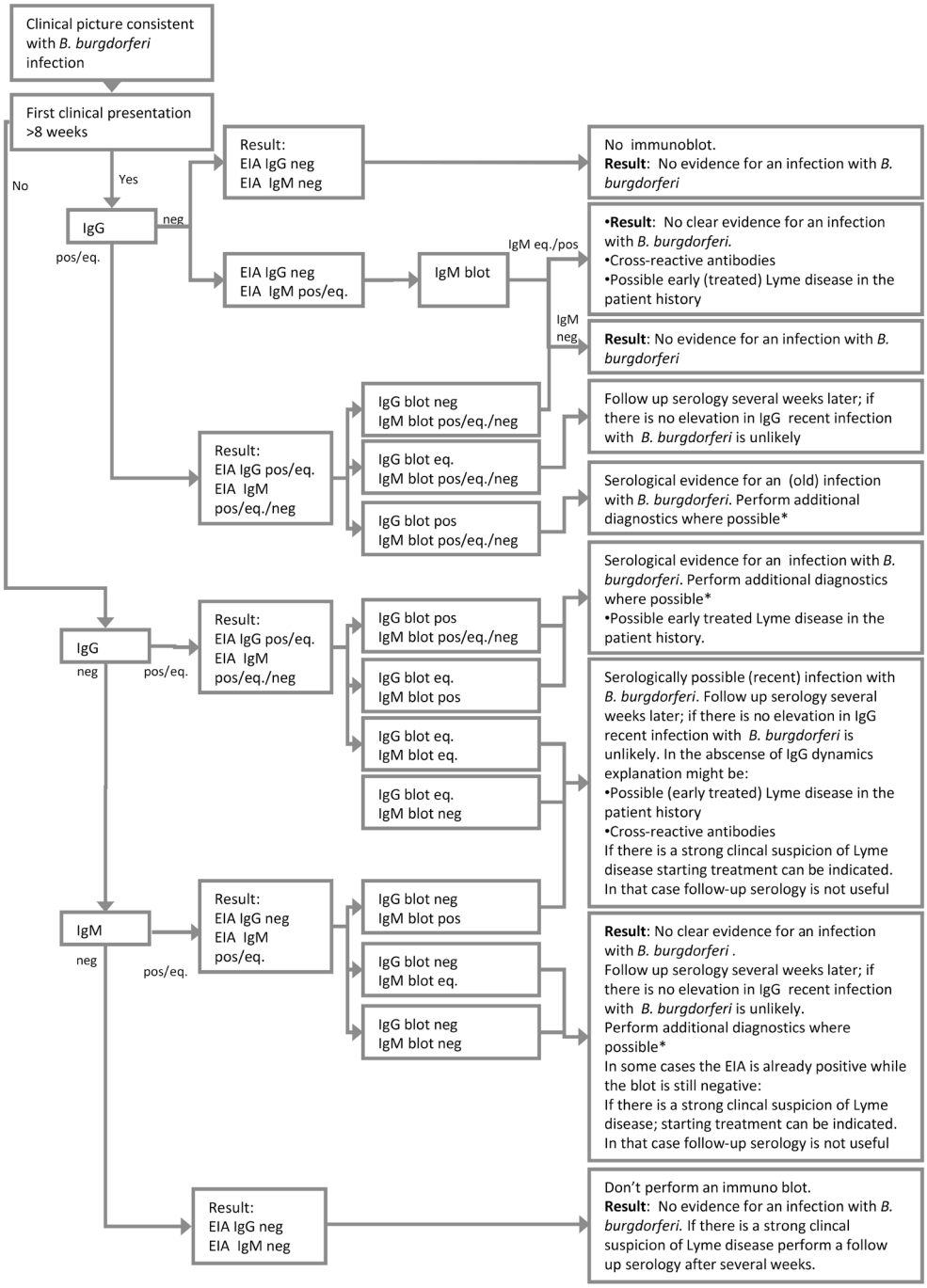
unraveling the altered and more severe pathogenic course in spirochetal infections in HIV patients.

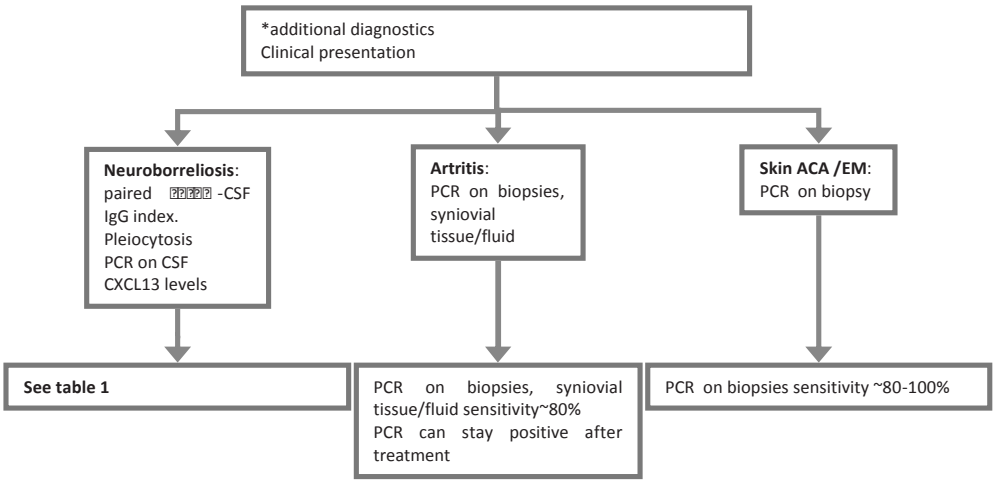
Concluding remarks

Lyme disease can have a significant impact on a patient's quality of life and correctly making the diagnosis is essential for proper treatment. It is important to test for *B. burgdorferi* infection in the presence of a clinical picture compatible with Lyme disease.
As serological tests are only surrogate markers of a disease, no single marker can prove or disprove active infection. It is of the highest importance to take into account all available clinical data from a patient in order to correctly interpret serological results in combination with non-specific biomarkers such as CSF white blood cell count and CXCL13 levels in CSF. For *B. burgdorferi* infection this is becoming a rapidly expanding field of tests and tests strategies with all their own specific pitfalls, leading to confusing discrepancies of test results for clinicians as well as patients. Identifying the optimal test strategy in an international setting, combined with quality control and recognition of the available commercial assays by a European panel of experts is desirable.

Perhaps *B. burgdorferi* sl is not so special in the world of diagnostics, as was stated earlier in this chapter. However, *B. burgdorferi* sl has an unfortunate combination of factors which makes it an infection that needs some special attention in correctly interpreting the tests at hand. The question at the start was: Is the laboratory and clinical diagnosis of Lyme disease really so complicated? The correct answer is that it is difficult, but not more complicated than in other infectious diseases involving uncultivable or fastidious organisms. In a field where one is forced to use an indirect strategy such as serology, the interpretation of the tests should be an interdisciplinary discussion between a treating physician and a medical microbiologist. If the medical microbiologist and clinician can properly utilize their expertise simultaneously for the benefit of the patient then perhaps the diagnosis is not so difficult after all.

Figure 1: An algorithm to interpret *B. burgdorferi* serology and an indication of when to perform additional diagnostics with their specific pitfalls. (Published in revised version [603]).




*additional diagnostics
Clinical presentation
Neuroborreliosis:
paired -CSF
IgG index.
Pleiocytosis
PCR on CSF
CXCL13 levels
Artritis:
PCR on biopsies, syniovial tissue/fluid
Skin ACA /EM:
PCR on biopsy
See table 1
PCR on biopsies, syniovial tissue/fluid sensitivity~80%
PCR can stay positive after treatment
PCR on biopsies sensitivity ~80-100%

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List of abbreviations

Ab	Antibodies
ACA	Acrodermatitis Chronica Atroficans
ADEM	Acute disseminated encephalomyelitis
Ag	Antigens
ALS	Amyotrophic lateral sclerosis
AP	Alternative pathway
BD	Healthy Blood Donors
BL	Borrelia lymphocytoma
CFH	Complement factor H
CFHR	Complement factor H related proteins
CMV	Cytomegalo Virus
CNS	Central nervous system
CP	Classical pathway
CRASP	Complement regulatory acquired surface protein
CT	Computed tomography (scan)
EBV	Epstein Barr Virus
EBV-EA	Epstein Barr Virus early antigen
EBV-NA	Epstein Barr Virus nuclear antigen
EBV-VCA	Epstein Barr Virus viral capsid antigen
EEG	Electroencephalogram
EIA	Enzyme immunoassay
ELB	Early Lyme Borreliosis
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema Migrans
FHL-1	Factor H-like protein 1
FNP	Facial Nerve Palsy
HiNHS	Heat inactivated non-immune human serum
HIV	Human immunodeficiency virus 1/2
HSV	Herpes simplex virus
IR	Invariable region
iv	intravenous
LA	Lyme Arthritis
LB	Lyme Borreliosis
LLB	Late Lyme Borreliosis
LNB	Lyme Neuroborreliosis
LP	Lectin pathway
MBC	Minimal Bactericidal Concentration
MBL	Mannose binding lectin
MIC	Minimal inhibitory concentration
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
LNB	Lyme Neuroborreliosis
NHS	Non-immune human serum
NPV	Negative predictive value
PCR	Polymerase chain reaction
PNS	Peripheral nerve system
po	per os
PTLDS	Post treatment Lyme disease syndrome
PPV	Positive predictive value
RF	Reuma factor
SALP	Tick salivary protein
SCR	Short consensus repeats
sl	sensu lato
ss	sensu stricto
ST4	OspA serotype 4
TP	*Treponema pallidum*
US	United States
VR	Variable region
VZV	Varicella Zoster Virus

Samenvatting voor de niet medisch onderlegde lezer

De ziekte van Lyme is een infectie van de mens met de bacterie *Borrelia burgdorferi*. Deze bacterie wordt door middel van een tekenbeet overgedragen. Teken nemen normaal een aantal maal in hun leven een bloedmaaltijd, meestal op (kleine) knaagdieren of vogels, maar soms ook op de mens. Tijdens het leven van de teek kan deze bij een bloedmaaltijd besmet worden met *B. burgdorferi*. Gedurende de volgende bloedmaaltijd van de besmette teek kan *B. burgdorferi* worden overgedragen op een gastheer. Na overdracht van de bacterie kan een mens na enkele weken ziek worden, maar dat hoeft niet noodzakelijk zo te zijn. Als iemand wel een infectie krijgt dan is de meest bekende uiting een erythema migrans; een vaak typische rode vlek die er uit kan zien als een bull's eye (zie fig. 1, pag. 19). Deze kan uitbreiden, maar uiteindelijk ook weer verdwijnen. Sommige mensen met de infectie hebben deze vlek nooit gezien of überhaupt nooit gekregen. Latere uitingen van de ziekte kunnen onder meer een verlamming van het gezicht (facialis parese), een pijnlijke huid op een gedeelte van het lichaam (radiculitis), een ontsteking aan een groot gewricht (artritis), of een lang bestaande infectie van de huid (acrodermatitis chronicum atrophicans) zijn. De ziekte kan in principe goed worden behandeld met antibiotica. Helaas zijn er na de behandeling soms restverschijnselen, waarvan lastig kan worden onderscheiden of dit een restverschijnsel, een recidief van de infectie of een nieuwe infectie is.

B. burgdorferi is een spirocheet; een kurketrekker-vormige bacterie. Het gehele complex, *B. burgdorferi* sensu lato (sl; in brede zin), bestaat uit enkele subspecies, waaronder *B. burgdorferi* sensu stricto (ss), *B. garinii*, *B. afzelii*, *B. bavariensis*, *B. spielmanii*, *B. valaisiana,* maar ook nog vele andere soorten. De eerste vijf soorten zijn duidelijk geassocieerd met ziekte in mensen. In de VS komt alleen *B. burgdorferi* ss voor, terwijl in Europa en Azië meerdere soorten naast elkaar kunnen voorkomen. Verschillende soorten zijn geassocieerd met verschillende ziektebeelden, maar dit is geen zwart-wit verdeling. *B. burgdorferi* ss is vooral geassocieerd met het erythema migrans en de artritis klachten; *B. garinii* en *B. bavariensis* met de neurologische klachten en *B. afzelii* met de uitingen in de huid.

Als de teek een gastheer bijt en begint met een bloedmaaltijd dan krijgt *B. burgdorferi* een signaal waardoor hij zich gaat klaarmaken voor overdracht. Op het buitenmembraan van de bacterie komen eiwitten tot uiting die de bacterie helpen met de overdracht naar de gastheer. De bacterie verplaatst zich vanuit de darm naar de speekselklieren van de teek, om daarna via het speeksel in de huid of bloedbaan van de gastheer terecht te komen. Eenmaal in de mens

probeert de bacterie een infectie te veroorzaken. Eén van de eerste hordes die de bacterie moet overwinnen is het complementsysteem van de gastheer.

Het complementsysteem is de eerste afweer tegen binnendringende micro-organismen. Het complementsysteem bestaat uit een aantal eiwitten die kunnen binden aan het membraan van cellen en bacteriën. Deze eiwitten kunnen een complex op het membraan vormen waardoor de bacterie zal sterven. Praktisch alle gewervelde dieren bezitten een complementsysteem. Het is het zogenaamde aangeboren (innate) afweersysteem. Dit type afweer hoeft niet speciaal de bacterie eerder gezien te hebben om het membraan van de bacterie te kunnen herkennen en te doden. Het menselijk lichaam bezit eiwitten, onder andere factor H en factor H-like eiwit, om de lichaamseigen cellen te beschermen tegen de vernietigende eiwitten van het complementsysteem.
Sommige *B. burgdorferi* soorten kunnen binden aan factor H en factor H-like eiwit uit het bloed van de gastheer om zichzelf tegen activatie van het complementsysteem te beschermen. De eiwitten van de bacterie die kunnen binden aan factor H en factor H-like eiwit heten Complement Regulatory Acquired Surface Proteins (CRASP) 1 t/m 5. De verschillende CRASPs komen ieder op een ander moment tijdens de infectie tot uiting. Niet elke soort *B. burgdorferi* is even goed in het omzeilen van het complementsysteem. Zo is bekend dat *B. burgdorferi* ss en *B. afzelii* dit goed kunnen en dus ongevoelig is voor menselijk complement zijn. *B. garinii* is echter redelijk gevoelig, terwijl *B. bavariensis* (eerder bekend als *B. garinii* OspA serotype 4) redelijk ongevoelig is. CRASP-1 is een eiwit wat heel vroeg in de infectie een rol speelt. CRASP-1 komt tot uiting op het membraan van de bacterie net voor tot kort na de overdracht vanuit de teek. CRASP-1 beschermt dus de bacterie in een heel vroeg stadium van de infectie tegen het complement van de mens. Van *B. burgdorferi* ss, *B. afzelii* en *B garinii* waren de CRASP-1 eiwitten al ontdekt en beschreven. Van *B. bavariensis* die relatief ongevoelig voor complement is, waren nog geen CRASP eiwitten beschreven.

In **Hoofdstuk 2** wordt aangetoond dat *B. bavariensis* complement ongevoelig is en dat de bacterie kan voorkomen dat complement wordt geactiveerd op het buitenmembraan van de bacterie. Van *B. bavariensis* kloneren en zuiveren we enkele van de mogelijk geïdentificeerde CRASP-1 eiwitten van het lineaire plasmide 54. Deze eiwitten heten BGA66, 67, 68 en 71 en hadden een, tot dusver, onduidelijke functie. We tonen via verschillende technieken aan dat twee van deze eiwitten, BGA 66 en BGA 71, kunnen binden aan factor H aan factor H-like eiwit van de mens. Daarnaast laten we zien dat bij binding het actieve deel van factor H en factor H-like eiwit nog steeds beschikbaar is.

Hierdoor kan bij binding van factor H en factor H-like eiwit, aan BGA 66 en BGA71 op het celmembraan van de bacterie, geen complement worden geactiveerd op het celmembraan van de bacterie en kan de bacterie niet door het complement worden gedood.

Naast het feit dat verschillende *B. burgdorferi* soorten verschillen in gevoeligheid voor menselijk complement verschilt deze gevoeligheid ook tussen diersoorten. Zo is *B. garinii* bijvoorbeeld juist minder gevoelig voor complement van vogelsoorten. We vermoedden dat de familie van eiwitten die we hebben gekloneerd niet alleen menselijk factor H kunnen binden, maar ook factor H van andere diersoorten. Van de vier eiwitten die gezuiverd zijn werd bepaald of ze aan factor H van een muis, koe, paard, hond, poes, varken of vogel konden binden. Hierbij werd duidelijk dat de verschillende geteste BGA eiwitten kunnen binden aan factor H van verschillende diersoorten. Dit verklaart voor een gedeelte mogelijk de grote verscheidenheid aan gastheren die *B. burgdorferi* kan infecteren. De volgende stap is kijken of de verschillen in gevoeligheid voor complement tussen de verschillende borrelia-soorten meetbaar zijn tijdens een infectie in een gastheer.

In **hoofdstuk 3** infecteren we wildtype en muizen met een niet werkend complementsysteem (C3-/-) met verschillende soorten van het *B. burgdorferi* sl complex. Hierdoor kunnen we zien of de efficiëntie van de infectie hoger is in C3-/- muizen en of de mate van complementongevoeligheid van de borrelia-soort effect heeft op de manier waarop de infectie wordt veroorzaakt.
Pathologisch en moleculair (PCR) onderzoek van deze muizen wijst uit dat *B. burgdorferi* en *B. afzelii* een goede infectie kunnen bewerkstelligen, dit was onafhankelijk van de aanwezigheid van complement. *B. bavariensis* was niet detecteerbaar met pathologisch onderzoek, maar wel met PCR. Wederom was dit onafhankelijk van de aanwezigheid van complement. *B. garinii* en *B. valaisiana* waren niet in staat een infectie te geven in de muizen, zowel in de C3-/- muizen als in wildtype muizen. Vermoedelijk speelt de mate van gevoeligheid voor complement wel een grote rol, maar is de infectie van *B. burgdorferi* zo afhankelijk van een hele reeks van factoren en eiwitten, waaronder CRASP 1 t/m 5, dat het moeilijk is het effect van één eiwit in een *in vivo* experiment te onderzoeken. Wel werd ook duidelijk in dit experiment dat PCR gevoeliger is dan pathologie in de detectie van *B. burgdorferi* in de weefsels van de geïnfecteerde gastheer.

Het aantonen van *B. burgdorferi* in de gastheer is belangrijk om een actieve infectie te kunnen bevestigen. Bij het diagnosticeren van infecties van micro-organismen die niet of slecht te kweken zijn is serologie vaak de eerste keus. Bij serologie toont men de antilichamen aan die gemaakt worden door het lichaam als reactie op de infectie. Antilichamen worden gemaakt tegen stukjes eiwit (antigenen) van de bacterie. Antilichamen kunnen levenslang aantoonbaar blijven, maar soms ook weer dalen over de tijd, als het antigeen niet meer aanwezig is.

Bij serologische diagnostiek kan gebruikt worden gemaakt van verschillende technieken: de enzyme immunoassay (EIA) of de immunoblot. Volgens de richtlijnen is de methode van voorkeur een screening van het bloed met EIA en, indien deze positief is, dit te bevestigen met een immunoblot. Het laatste decennium is er veel onderzoek gedaan naar geschikte antigenen voor deze diagnostiek. Daarbij lijkt in het bijzonder het C6-peptide veelbelovend: de antistoffen tegen dit antigeen kunnen al twee weken na een infectie duidelijk aantoonbaar zijn. Daarnaast is het tussen de verschillende soorten *B. burgdorferi* nagenoeg identiek, waardoor het niet noodzakelijk is een mix van alle soorten te gebruiken. Een ander bijzonder voordeel is dat de hoeveelheid antilichaam tegen C6-peptide ook weer snel kunnen dalen na behandeling. Dit is voornamelijk in patiënten die maar kort een infectie hebben gehad, maar minder uitgesproken in patiënten die een langer bestaande infectie hadden.

Wanneer de infectie met *B. burgdorferi* tot uiting komt in de hersenen (neuroborreliose) wordt er niet alleen gekeken naar de antilichamen in het bloed, maar ook naar antilichamen in de hersenvloeistof (liquor). Als er relatief meer antilichamen in de liquor zitten dan in het bloed dan maakt dat een infectie van het zenuwstelsel zeer waarschijnlijk. Dit heeft invloed op de keuze van behandeling. Naast deze antilichaamindex wordt er ook gekeken naar tekenen van ontsteking (inflammatie) aan de hersenen in het algemeen, zoals het aantal witte bloedcellen in de liquor. Na behandeling nemen de tekenen van algemene ontsteking snel af, maar de antilichaamindex kan enkele jaren positief blijven, ook in mensen die helemaal geen klachten meer hebben.

In **hoofdstuk 4** wordt er gekeken naar de toepasbaarheid van een screeningstest op liquor voor C6-peptide om de diagnose neuroborreliose te bevestigen. Het blijkt dat de C6-peptide (IgG/IgM) EIA zeer geschikt is om te screenen op neuroborreliose (gevoeligheid 97%), maar dat deze test niet goed gebuikt kan worden om de diagnose te bevestigen. De specificiteit bij mensen die antilichamen tegen *B. burgdorferi* in het bloed hebben is 83%, dat betekent dat sommige mensen een positieve testuitslag krijgen, terwijl ze geen

neuroborreliose hebben. De hoeveelheid antilichamen verschilde echter tussen de groep met een neuroborreliose en de overige patiënten. De relatieve Lyme index waarden waren gemiddeld veel hoger in de neuroborreliose groep dan in de Lyme patiënten groep, echter op individueel niveau heeft dit weinig praktische waarde.

De sterke beperking van de serologie is het gebrek aan de mogelijkheid een actieve infectie te bevestigen in een patiënt die al serologisch positief is door een eerdere, behandelde of door het eigen afweersysteem opgeruimde, infectie. Een test die een actieve infectie met *B. burgdorferi* kan bevestigen in deze patiëntengroep, is wenselijk.

In **hoofdstuk 5** kijken we naar chemokine CXCL13 in liquor als biomarker voor een actieve infectie met *B. burgdorferi*. In patiënten met neuroborreliose is deze biomarker in alle patiënten licht tot zeer sterk verhoogd. Als op basis van een vergelijking met andere inflammatoire ziekten een afkapwaarde wordt gekozen van 250 pg/ml liquor dan was bij 88% van de patiënten deze biomarker verhoogd. In de meeste patiënten met neuroborreliose is deze biomarker zelfs zeer sterk verhoogd. In de controles zonder inflammatoire aandoening was deze biomarker bij niemand verhoogd. Bij patiënten met inflammatoire aandoeningen was de waarde van CXCL13 zeer wisselend, doch in de overgrote meerderheid van de patiënten lag de waarde onder 250 pg/ml. Een opvallende bevinding was dat bij veel HIV-positieve patiënten CXCL-13 verhoogd was.

Daarnaast bleek bij patiënten met neuroborreliose, dat na het starten van behandeling dat de waarde van CXCL13 in de liquor zeer snel daalt, waardoor deze biomarker een rol zou kunnen krijgen in de follow-up bij behandeling. De marker lijkt daarom een veelbelovende rol te kunnen gaan spelen in de diagnostiek naar neuroborreliose. Het is echter van belang deze marker niet als enige te gebruiken, maar dit te combineren met de klinische uiting, de serologie en eventueel PCR, gezien de aanwezige aspecificiteit bij andere inflammatoire aandoeningen.

Serologie kan levenslang positief blijven, ook als er geen infectie meer is. Een positieve serologische test betekent dus niet per definitie dat de klachten ook worden veroorzaakt door een infectie met *B. burgdorferi*. In **hoofdstuk 6** wordt een patiëntenpopulatie van de vroege artritiskliniek (EAC) gescreend op antislichamen en de aanwezigheid van een infectie met *B. burgdorferi*. In deze populatie blijkt dat ongeveer 2,0-4,5% van de EAC-populatie antistoffen heeft tegen *B. burgdorferi.* Na uitgebreid onderzoek, waaronder het uitsluiten van

andere aandoeningen, bleek dat slechts 0,5 tot maximaal 1,2 % van de totale populatie ook werkelijk de een infectie met *B. burgdorferi* heeft. Dat betekent dat wanneer een patiënt met artritis wordt getest op de ziekte van Lyme, zonder te letten op specifieke kenmerken van Lyme, bij een positieve testuitslag de kans slechts 12-28% is dat deze patiënt daadwerkelijk Lyme heeft. Als men preselecteert op kenmerken van Lyme en alleen die patiënten test die mogelijk een infectie met *B. burgdorferi* hebben op basis van klinische parameters, bijvoorbeeld onder andere "betrokkenheid van de grote gewrichten" dan kan deze kans worden verhoogd naar 42-85%. Deze studie benadrukt het belang van het inschatten van de a priori kans dat de patiënt een infectie met *B. burgdorferi* heeft voordat de dokter besluit *B. burgdorferi* antilichamen te testen.

In **hoofdstuk 7** wordt een HIV-positieve patient met neuroborreliose beschreven. Deze patiënte had een zeer progressieve infectie van de hersenen, met als uiting een forse afwijking aan het looppatroon. Haar infectie met HIV was op dat moment goed onder controle. Op de MRI-scans van de hersenen waren duidelijk afwijkingen waar te nemen. Deze uiting is al eerder beschreven bij een neuroborreliose, maar in deze patiënte met HIV verliep de ziekte sneller dan te verwachten was. Dit leidde tot de hypothese dat een infectie met *B. burgdorferi* mogelijk heftiger verloopt in een patiënt met HIV dan in een verder gezonde patiënt. Een dergelijk verschijnsel is eerder beschreven bij HIV-patiënten, namelijk bij een infectie met *T. pallidum*, oftewel syfilis, een andere spirocheetsoort. Dit afwijkende beloop zou te maken kunnen hebben met de eerder genoemde CXCL13-receptor. Een HIV-infectie heeft onder meer een effect op B-cellen (antilichaam producerende cellen) van het immuunsysteem, waarbij de CXCL13 receptor door de infectie met HIV op deze cellen minder tot uiting komt. Hierdoor verloopt de reactie op antigenen, waar de CXCL13-receptor voor nodig is, minder efficiënt. Dit zou een verklaring kunnen zijn voor de verminderde afweer tegen *B. burgdorferi*, bij een HIV-infectie. Deze hypothese moet echter nog nader worden onderzocht.

De diagnostiek naar een infectie met *B. burgdorferi* heeft te kampen met een aantal problemen. De serologie is niet altijd eenduidig te interpreteren. Er zijn meerdere serologische testen en ook biomarkers die kunnen helpen bij het stellen van de diagnose. Het is van belang alle beschikbare klinische gegevens en de combinatie van testen en hun beperkingen te interpreteren op een individuele basis. Hierbij is samenwerking van de clinicus met een arts-microbioloog wenselijk om de juiste diagnose te kunnen stellen in het belang van de patiënt.

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Curriculum Vitae

Nathalie Daniëlle van Burgel, dochter van Huibert van Burgel en Jannigje Gerritsen, werd in september 1979 geboren in het Beatrix ziekenhuis te Gorinchem. De middelbare school deed ze op het Lyceum Oude Hoven waar ze in 1997 het VWO afgemaakt heeft met acht vakken.

In 1997 is ze begonnen met de studie Biomedische wetenschappen aan de Rijksuniversiteit Leiden een stage daarvan is verricht aan de Universidad Autónoma de Madrid. Het doctoraalexamen Biomedische wetenschappen heeft ze behaald in 2003.
In 1998 is ze begonnen met de studie Geneeskunde aan de Universiteit Leiden waarvan het doctoraal is behaald in 2003 en ze haar artsenbul heeft gekregen in 2004.

Oktober 2004 is ze gestart als AIOS KO voor de specialisatie van arts-microbioloog in het Leids Universitair Medisch Centrum. Deze promotie is voor een deel verricht op de Yale university of medicine in New Haven en de Goethe universiteit in Frankfurt. In haar opleidingstraject heeft ze ook deelgenomen aan enkele besturen: de Nederlandse Vereniging voor AIOS Medische Microbiologie (NVAMM), vertegenwoordiger NVAMM in de Nederlandse Vereniging voor Medische Microbiologie (NVMM), voorzitter van de Trainee Association van de European Society of Clinical Microbiology and Infectious Diseases (ESCMID) en vertegenwoordiger AIOS bij de sectie Medical Microbiology van de Union Européenne des Médecins Spécialistes (UEMS).

Sinds oktober 2011 is ze geregistreerd als arts-microbioloog en werkzaam in het Hagaziekenhuis in Den Haag. Vanuit haar interesse voor Lyme neemt ze nu nog deel aan het consensusberaad Lyme van het RIVM en in de adviescommissie Lyme van de Gezondheidsraad. Recent heeft ze ook plaatsgenomen in het bestuur van de sectie infectieziektenserologie van de Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek (SKML).

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