Lyme Neuroborreliosis

Diagnosis and Treatment

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Cover illustration: Dark field micrograph of *Borrelia burgdorferi* spirochetes at 400x magnification. Image from CDC Public Health Image Library.

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Ineko

Facts are meaningless.

You could use facts to prove anything that's even remotely true.

Homer J. Simpson

ABSTRACT

Lyme neuroborreliosis, the infection of the nervous system by the tick-borne bacterium *Borrelia burgdorferi*, is common in the temperate parts of the Northern hemisphere. Manifestations of the disease include facial palsy, radicular pain, sensory disturbances, and occasionally CNS symptoms such as confusion and paraparesis. The diagnosis of Lyme neuroborreliosis is based on medical history, clinical examination and cerebrospinal fluid (CSF) analysis. Recommended antibiotic treatment is oral doxycycline or intravenous ceftriaxone. The overall aims of this thesis were to improve the diagnosis and treatment of Lyme neuroborreliosis.

In **paper I**, 102 patients with peripheral facial palsy were studied. Onset of symptoms in July to October, additional neurological symptoms and CSF pleocytosis were factors that discriminate patients with peripheral facial palsy caused by Lyme neuroborreliosis from patients with Bell's palsy.

In **paper II**, it was shown that CSF levels of the chemokine CXCL13 are highly elevated in Lyme neuroborreliosis and that levels decline after treatment, but high and overlapping CXCL13 levels were also seen in patients with asymptomatic HIV-infection and the decrease in CXCL13 is correlated to the decrease in CSF cell count. The additional diagnostic value of CXCL13 analysis is therefore limited.

In **paper III**, new reference ranges for CSF cell counts when analyzed with automatic cell counters were determined, based on CSF sampling of 80 healthy volunteers. The differentiation of mononuclear cells into lymphocytes and monocytes was shown to be of limited value in the discrimination between Lyme neuroborreliosis and viral CNS infections.

In **paper IV**, it was shown that treatment with oral doxycycline resulted in a similar decrease in CSF mononuclear cell counts in patients with Lyme neuroborreliosis with CNS symptoms compared with patients with peripheral nervous systems symptoms, and that all patients with CNS symptoms improved on treatment with no need for retreatment. Oral doxycycline can therefore be considered an effective treatment for Lyme neuroborreliosis, irrespective of the severity of symptoms

Keywords: Lyme disease, Lyme borreliosis, Lyme neuroborreliosis, Borrelia burgdorferi, facial palsy, doxycycline, chemokine CXCL13, cell count.

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SAMMANFATTNING PÅ SVENSKA

Borrelia burgdorferi är en bakterie som sprids via fästingar. Infektion med borrelia kan ge upphov till flera olika sjukdomssymptom av vilka den långsamt tillväxande hudrodnaden erytema migrans är den vanligaste. Näst efter hudsymptom är symptom från nervsystemet det vanligaste tecknet på borreliainfektion. Sjukdomen kallas då neuroborrelios och drabbar cirka 500-1000 personer per år i Sverige. De vanligaste symptomen vid neuroborrelios är ansiktsförlamning, strålande smärta som kan vara svår, och känselpåverkan. Mindre vanligt är andra förlamningar och symptom från hjärnan som förvirring och demenslika symptom. Diagnosen neuroborrelios ställs på sjukhistoria, undersökningsfynd och provtagning av ryggvätska. I ryggvätska påvisas förhöjda nivåer av vita blodkroppar och antikroppar mot borrelia. Neuroborrelios behandlas i Sverige med antibiotika av typen doxycyklin i tablettform; utomlands är intravenös behandling med ceftriaxon vanligare. Syftet med denna avhandling var att förbättra diagnostik och behandling av neuroborrelios.

Symptomen vid neuroborrelios liknar de vid ett antal andra sjukdomar. Ansiktsförlamning, som är det vanligaste symptomet vid neuroborrelios, kan ha flera andra orsaker. I delarbete I visas att det går att skilja neuroborrelios från Bells pares, som är den vanligaste orsaken till ansiktsförlamning generellt, genom att patienter med neuroborrelios insjuknar under sommar och tidig höst, har neurologiska symptom från andra delar av kroppen och har förändrad ryggvätska.

Även med analys av ryggvätska är det inte alltid möjligt att helt säkert ställa diagnosen neuroborrelios. De senaste åren har signalmolekylen CXCL13 i ryggvätska framförts som en markör som skiljer neuroborrelios från andra sjukdomar. I delarbete II visas att analys av CXCL13 kan vara av värde i enstaka fall men generellt tillför begränsad information. I delarbete III utvärderas en ny automatiserad metod för analys av vita blodkroppar i ryggvätska som alltmer ersatt tidigare manuella metoder. Nya gränser för normalvärden, som skiljer friska från sjuka, presenteras, baserat på undersökning av 80 friska frivilliga individer.

I delarbete IV studeras behandling av svår neuroborrelios med symptom från hjärna eller ryggmärg som medvetandepåverkan eller förlamning av underkroppen. Studien visar att dessa patienter, precis som patienter med mindre allvarlig neuroborrelios, kan behandlas med doxycyklin i tablettform, vilket stödjer svenska behandlingsriktlinjer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Bremell D, Hagberg L. **Clinical characteristics and cerebrospinal fluid parameters in patients with peripheral facial palsy caused by Lyme neuroborreliosis compared with facial palsy of unknown origin (Bell's palsy)**. *BMC Infectious Diseases. 2011;11:215.*

II. Bremell D, Mattsson N, Edsbagge M, Blennow K, Andreasson U, Wikkelsö C, Zetterberg H, Hagberg L. **Cerebrospinal fluid CXCL13 in Lyme neuroborreliosis and asymptomatic HIV infection**. *BMC Neurology. 2013;13:2*.

III. Bremell D, Mattsson N, Wallin F, Henriksson J, Wall M, Blennow K, Zetterberg H, Hagberg L. **Automated cerebrospinal fluid cell count - New reference ranges and evaluation of its clinical use in central nervous system infections**. *Clinical Biochemistry. 2014; 47(1-2):25–30*.

IV. Bremell D, Dotevall L. **Oral doxycycline for Lyme neuroborreliosis with symptoms of encephalitis, myelitis, vasculitis or intracranial hypertension**. *European Journal of Neurology. Epub ahead of print.*

CONTENT

ABBREVIATIONS

AAN	American Academy of Neurology
ACA	Acrodematitis chronica atrophicans
AI	Antibody index
Bb	*Borrelia burgdorferi*
BP	Bell's palsy
BLC	B lymphocyte chemoattractant = CXCL13
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CXCL13	Chemokine [C-X-C motif] ligand 13 = BLC
EFNS	European Federation of Neurological Societies
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema migrans
EUCALB	European Union Concerted Action on Lyme Borreliosis
IDSA	Infectious Diseases Society of America
IV	Intravenous
LB	Lyme borreliosis
LD	Lyme disease
LLMD	Lyme-literate medical doctors
LNB	Lyme neuroborreliosis

LP	Lumbar puncture
NB	Neuroborreliosis
Osp	Outer surface proteins
PCR	Polymerase chain reaction
PFP	Peripheral facial palsy
PLDS	Post-Lyme disease syndrome
PNS	Peripheral nervous system
VlsE	Variable major protein-like sequence, expressed

1 INTRODUCTION

1.1 Lyme borreliosis

Lyme borreliosis (LB), or Lyme disease (LD), is an infectious disease caused by the spirochete *Borrelia burgdorferi* (*Bb*) sensu lato. Hard ticks of the *Ixodes* genus act as vectors transmitting the spirochetes to humans. LB most commonly affects the skin, nervous system, joints and heart [1]. When LB affects the nervous system it is called Lyme neuroborreliosis (LNB) [2]. LNB and especially the diagnosis and treatment of LNB are the subjects of this thesis. As will be described, there are differences between European and American LB. The studies that form the basis of this thesis were all done on patients with European LB, the thesis will therefore mainly focus on European LB and more specifically, European LNB.

1.2 Historical notes

The first known report on the neurological manifestations of LB was published in 1922 and described a 58-year-old French farmer who presented with severe radiating pain and lymphocytic pleocytosis in CSF. The farmer recalled a tick-bite in the pain-affected area three weeks previously. He improved on treatment with neoarsphenamine, the then drug-of-choice for syphilis [3]. In 1941 the German neurologist Alfred Bannwarth published a report on 14 patients in which he described the now well-recognized triad of facial nerve palsy, radicular pain and lymphocytic meningitis, but he did not connect the neurological symptoms with preceding tick-bites [4]. The terms Bannwarth's syndrome or Garin-Bujadoux-Bannwarth syndrome are now used interchangeably to describe the painful meningoradiculoneuritis of LNB [5]. Even before the reports by Garin and Bujadoux and Bannwarth, articles had been published on other clinical manifestations of LB: the skin manifestation of acrodermatitis chronica atrophicans (ACA) was described by Buchwald in 1883, erythema migrans by Afzelius in 1909 and lymphocytoma by Burckhadt in 1911 [6-8]. Over the years, an increasing number of reports on patients now believed to have been suffering from LB followed. Penicillin became widely available after World War II and the first description of its use and efficacy in treating LB was published by Hellerström in 1950 [9]. In spite of the mounting evidence that a tick-borne organism, susceptible to penicillin and other antibiotics, was

responsible for the diverse clinical manifestations now known as LB, it was not until 1982 that the first report describing the spirochete now known as *Bb* was published [10]. In 1977, Steere and co-workers had published a report on multiple cases of arthritis, especially in children, in the area around the town of Old Lyme, Connecticut, naming the condition Lyme arthritis. A majority of the patients reported systemic and neurological symptoms in addition to arthritis. A tick-borne infectious agent was suspected [11]. Finally, in 1981 Willy Burgdorfer and co-workers succeeded in demonstrating spirochetes in *Ixodes* ticks and that serum from patients with Lyme arthritis contained antibodies reactive to this spirochete [10,11]. The spirochetal etiology of LB was then definitely established in 1983 by Steere and co-workers when the spirochete could be recovered from blood and CSF of patients with LD [12]. Soon afterward, it was shown that a similar penicillin-susceptible spirochete was the cause of the lymphocytic meningoradiculitis seen in Europe [13,14]. In 1984, the newly discovered organism was given the name *Borrelia burgdorferi* [15].

1.3 The vector *Ixodes ricinus*

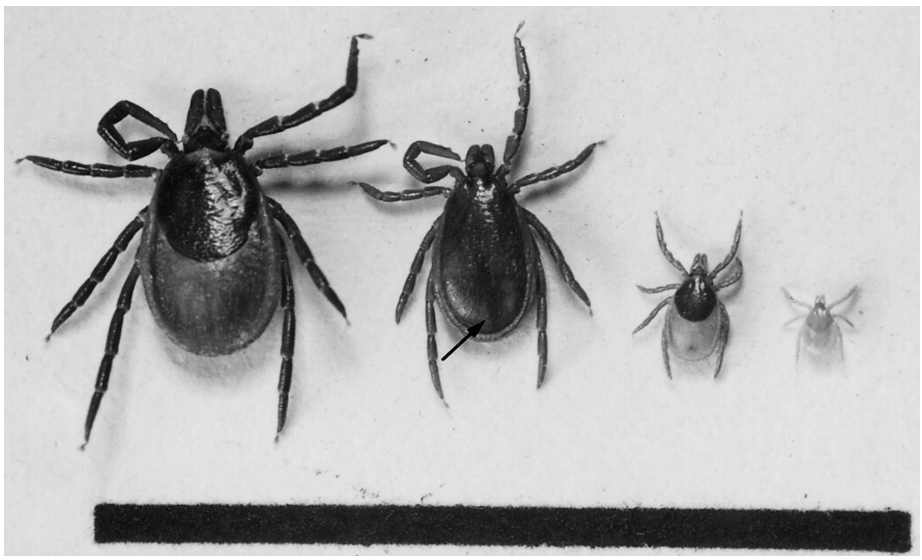

Figure 1. Four stages of unfed Ixodes ricinus. Left to right: female, male, nymph and larva (bar 1 cm). (Reprinted from Parola P, Raoult D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin Infect Dis. 2001 Mar 15;32(6):897–928. By permission of Oxford University Press.)

Bb is transmitted to humans by ticks of the *Ixodes* genus. Different *Ixodes* species act as vectors in various parts of the world: In North America *Ixodes scapularis* and *Ixodes pacificus*, in Asia *Ixodes persulcatus* and in Europe *Ixodes ricinus*. These ticks have similar life cycles and ecological requirements [16]. As this thesis mainly covers European LNB, for which *I. ricinus* is the main vector, this species will be described in more detail. *I. ricinus* occurs in Europe from the Atlantic coast to the Urals and from the Mediterranean to Scandinavia. Only at high altitudes, in very dry areas and in the northernmost parts of Europe is it usually absent [17]. *Ixodes* ticks are active at air temperatures above 4°C [18]. The life cycle of *I. ricinus* consists of three stages: larva, nymph and adult, feeding only once during each stage (figure 1).

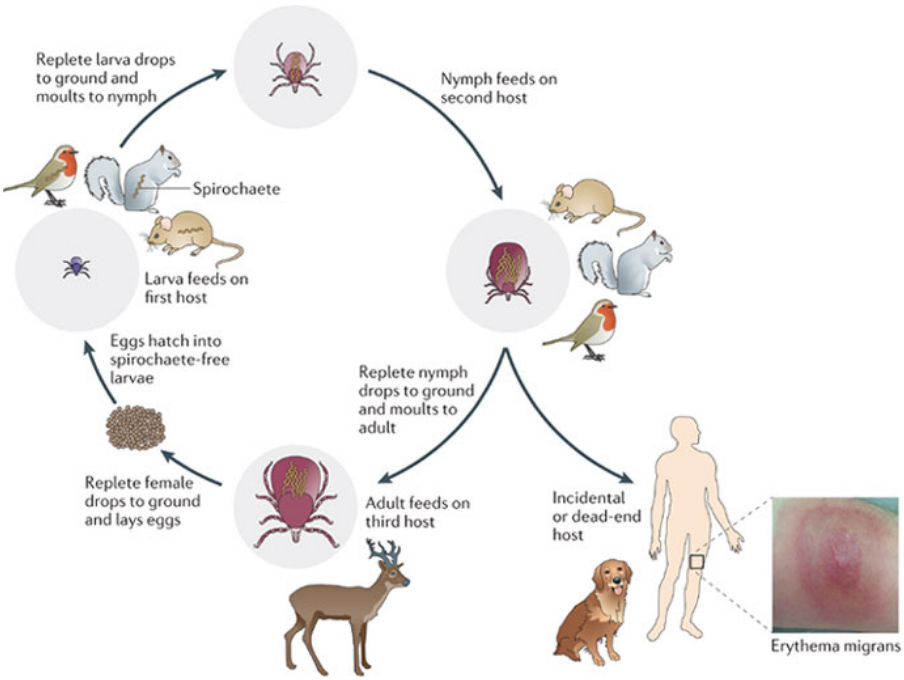

Figure 2. Infectious cycle of I. ricinus and B. burgdorferi. (Reprinted by permission from McMillan Publishers Ltd: [Nature Reviews Microbiology] [doi: 10.1038/nrmicro2714] copyright [2012])

The unfed tick ambushes the host as it passes through the vegetation, grabs hold with its front legs and after having wandered around on its host for several hours, attaches itself with its mouthparts. Undisturbed, the feeding of the tick takes between two and fifteen days and is dependent on

the tick development stage and the type of host [19]. Larvae and nymphs most often feed on small rodents and birds, while adult ticks typically feed on larger mammals such as hares, roe deer and cattle (figure 2). Having finished the blood meal, the tick drops to the ground where it either molts to the next development stage or, in the case of the mated adult female, lay eggs; or if environmental conditions are unfavorable, enters diapause, a stage of reduced metabolism and delayed development. The entire life cycle of *I. ricinus* usually takes two to three years but could be longer or shorter depending on environmental conditions [19]. Between feedings, the ticks seek out areas where the microclimate relative humidity does not drop below 80%. Typically this is near the ground in areas with a good cover of vegetation [16].

I. ricinus ticks acquire the *Bb* infection from a reservoir host as larva or nymph. The infection then persists in the tick through the development to the next stadium. Transovarial infection is considered rare, meaning that unfed larvae are seldom infected with *Bb* [16]. The proportion of ticks infected with *Bb* varies geographically and is usually higher in adult ticks than in nymphs. A meta-analysis on the prevalence of *Bb* in European *I. ricinus* ticks found an overall mean prevalence of 14% [20]. A recent Swedish-Finnish study on ticks removed from humans (in contrast to being collected by flagging lower vegetation), found an infection rate of 26% [21].

1.4 *Borrelia burgdorferi* sensu lato

The genus *Borrelia* belongs to the family *Spirochetacae* in the order *Spirochetales*. Initially, the *Borrelia* spriochetes found in the United States and Europe were thought to be identical. However, further analysis revealed that the various isolates showed strain heterogeneity. The name *Borrelia burgdoferi* was therefore amended to *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.), "sensu lato" meaning "in the broad sense", to cover several genospecies, while the specific genospecies discovered by Burgdorfer and coworkers was named *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), "sensu stricto" meaning "in the narrow sense". *B. burgdorferi* s.l. is now the name of the complex of *Borrelia* genospecies considered causative agents of LD [22-24]. To date, the *B. burgdorferi* s.l. complex consists of 18 separate genospecies. Of these however, only three, *B. burgdorferi* sensu stricto, *B. garini* and *B afzelii*, are considered significant human pathogens [25]. Reports of individual human cases infected with other genospecies have been published: *B. bissettii*, *B.*

lusitaniae, *B spielmanii* and *B. valaisiana*, but there is yet no evidence that these genospecies account for more than a small minority of LD cases [26-29].

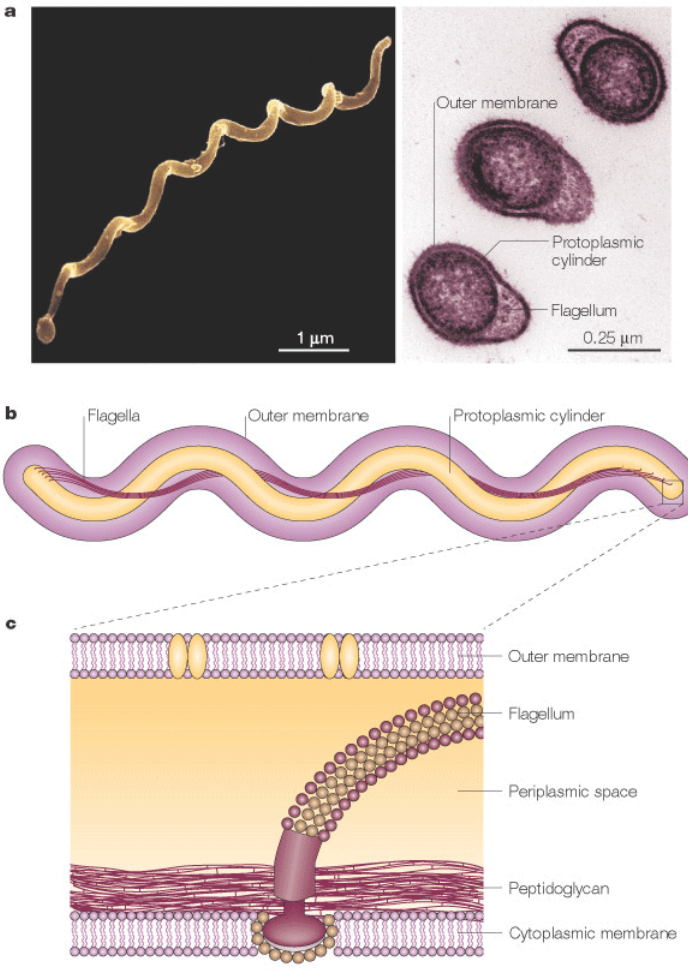

Figure 1. Morphology and structure of B. burgdorferi. a) Scanning (left) and transmission (right) electron micrographs. b-c) Diagram of the spirochete with detail of the flagella attachment points. (Reprinted by permission from McMillan Publishers Ltd: [Nature Reviews Microbiology] [doi: 10.1038/nrmicro1068] copyright [2005])

Bb is a thin, elongated, wave-shaped bacterium measuring 20-30 µm in length and 0.2-0.3 µm in width. Structurally, from the inside out *Bb* consists of the cytoplasm, the inner cell membrane and the peptidoglycan, which together make up the protoplasmic cylinder. Outside the

protoplasmic cylinder is the periplasmic space containing 7-11 flagella parallel with the long axis of the bacterium and attached to the cytoplasmic membrane near the termini of the spirochete (figure 3) [30]. The flagella propel the bacterium forward by generating posteriorly propagating planar waves [31]. This way of motion enables Bb to migrate even through highly viscous environments like human skin [32]. Purified flagellum protein was for the first decades after the discovery of *Bb*, the most commonly used antigen for serological tests [33]. The outermost part of the bacteria is the trilaminar outer membrane surrounded by a mucoid surface layer [30]. *Bb* thus have the double-membrane of gram-negative bacteria but in contrast to most other gram-negative bacteria, the *Bb* outer membrane lacks lipopolysaccharides (LPS) [34]. Major components of the *Bb* outer membrane are instead the outer surface proteins (Osp) A-F and variable major-like protein sequence expressed (VlsE), that are selectively expressed depending on the surrounding environment [35]. Recombinant versions of some of these proteins are now used as antigens in newer diagnostic assays for *Bb* antibodies [36].

Bb is a slow-growing bacterium with a generation time during log-phase growth *in vitro* of 7-20 hours, though it may be as short as 4 hours in blood-fed ticks [37-39]. Optimal growth temperatures vary between *B. garinii* (37°C), *B. afzelii* (35°C) and *B. burgdorferi* sensu stricto (33°C), which might explain part of the differences in organotropism and symptomatology between the genospecies [40].

In nature, the most important reservoirs for *Bb* are rodents such as mice and voles, and some bird species. Larger mammals, such as roe deer, cattle and sheep are incompetent hosts for *Bb* but might play a role in the spread of *Bb* by enabling the transmission of infection between ticks co-feeding on the same animal [41,42].

The geographical distribution of the various genospecies of *B. burgdorferi* s.l. has profound implications. In North America, only *B. burgdoferi* s.s. has been found of the genospecies known to cause disease in humans, while in Europe all three significant human pathogenic genospecies are present. As the different genospecies show different organotropism and give rise to different, if overlapping, clinical pictures, the relative prevalence of each genospecies affect the overall picture of LD [43,44]. Also within Europe, there might be regional differences. A metaanalysis on *Bb* infection in European *I. ricinus* ticks found that, of the infected ticks, 38% were infected with *B. afzelii*, 33% with *B. garinii* and 18% with *B. burgdoferi* sensu stricto, while a Swedish-Finnish study on ticks

removed from humans reported higher numbers for *B. afzelii* and lower numbers for *B. burgdoferi* sensu stricto [20,21].

1.5 Epidemiology

It is usually stated that LB is the most common tick-borne infection in the Northern hemisphere [45,46]. However, except for small, well-defined, high-incidence areas of tick-borne encephalitis (TBE) in central Europe and the Baltic states, other tick-borne infections are rare in Europe and North America, why the above statement is of limited value [47]. Few countries in Europe have made LB a mandatory notifiable disease, meaning that the data used for epidemiological assessment have to be collected in other ways such as through laboratory reports, physician surveys, voluntary reports and hospital diagnostic records. The imprecision of the underlying data makes comparison between countries difficult. It is however, generally accepted that the highest incidence of LB is seen in central Europe with a reported incidence in Slovenia of 206 cases per 100 000 inhabitants. Northern and southern Europe report lower incidence numbers with areas close to the Mediterranean seeing very few cases [48]. In one of the most extensive studies of the epidemiology of LB, carried out in southern Sweden, Berglund and co-workers reported an overall incidence of 69 cases per 100 000 inhabitants. The study found marked regional differences between the studied counties, all considered endemic for LB, with incidence numbers ranging from 26 to 160 cases per 100 000 inhabitants [49]. A Swedish study from 2006, using a different methodology and focusing solely on EM, reported an incidence of 464 cases of EM per 100 000 inhabitants, which is one of the highest incidence numbers ever reported. Part of the difference between the results of these two Swedish studies could be explained by differences in study design but the authors also speculate that there has been a real increase in LB incidence [50].

Data on the relative frequency of the clinical symptoms of LB are even scarcer. A study covering 15 European countries reported that skin manifestations (not specified as EM, ACA or lymphocytoma) were the most common, affecting 59% of patients, followed by neurological manifestations (34%), joint manifestation (15%) and cardiac manifestations (2%). There were considerable differences between countries regarding the relative frequency of each of the clinical manifestations [51]. Two German studies have reported frequencies of LNB as percentages of all LB cases of 3% and 18% [52,53]. The Swedish

epidemiological study described previously reported EM in 77% of patients, LNB in 16%, arthritis in 7%, ACA in 3%, lymphocytoma in 3% and carditis < 1% of patients [49].

The age distribution of patients with LB is bimodal with one peak at 5-10 years of age and the second peak at 60-70 years of age [49,52,53]. The proportion of LB patients presenting with neurological symptoms (LNB) is higher for children than for adults. In adults, it has been shown that tick bites in the head and neck area more frequently give rise to neurological symptoms. As children more often than adults get their tick bites in this area, it has been speculated that this explains their higher propensity for neurological manifestations [49,52].

The individual risk of acquiring LB for a human is dependent on so many factors that trying to present an estimate for the overall risk is almost pointless. Stjernberg and Berglund found a 4% risk of being tick-bitten per 10 hours spent outdoors and a 0.5% risk of developing clinical LB per tick-bite [54]. In another study, Fryland and co-workers found that 6% of the persons bitten by *Bb*-infected tick showed seroconversion but that the majority of these persons had a subclinical infection [55].

1.6 Pathogenesis of Lyme neuroborreliosis

How *Bb* finds its way from the biting tick to the affected human organ and how it there causes the damages that give rise to the clinical manifestations are questions that are still only partly answered. It starts with the unfed tick in which *Bb* resides in the mid-gut. On encountering blood when the tick starts feeding, *Bb* starts dividing and then penetrates the gut wall and translocates to the tick salivary glands. From the salivary glands it enters the tick-bitten host with injected saliva. Most studies show that it takes *Bb* at least 36-48h from the start of the blood meal to complete the migration from the mid-gut to the salivary glands, why infection is presumably less likely if the tick is removed within one or two days [16,38]. Having entered the human host, the most common manifestation of LB, erythema migrans, is caused by the centrifugal migration from the bite of *Bb* spirochetes [56].

How *Bb* then finds its way to other organs and especially the nervous system is poorly understood. There are mainly two ways for *Bb* to reach distant structures in the human body, either via the blood or by migrating

along structures such as peripheral nerves. The finding that *Bb* could be cultured from the blood of a relatively large proportion of American patients with EM suggests a haematogenous route of dissemination [57]. On the other hand, it has been noted since long before the spirochetal etiology of LB was known, that neurological symptoms are more common in the area of the body of the tick-bite, which would suggest that *Bb* migrates along peripheral nerves directly to the nerve roots [58,59]. Part of the answer could be that haematogenous spread is the preferred route for *B. burgdorferi* s.s., the only genospecies known to cause LB in North America, while *B. garinii*, that causes the majority of LNB cases in Europe, to a larger extent migrates along peripheral nerves [59,60].

The anatomical location and the pathophysiological mechanisms behind the neural dysfunctions seen in LNB are still a matter of research and speculation. Reports on the histopathological changes in LNB are too few to draw general conclusions but descriptions of perivascular mononuclear infiltrations points to vasculitis as one of the pathophysiological mechanisms [61,62]. It has also been shown, in the rhesus macaque model that is the best-suited animal model for LNB, that *Bb* can induce inflammation and apoptosis in dorsal root ganglia cells [63]. MRI-scans of patients with LNB show pathological changes only in a minority of cases. The described changes range from multiple white-matter lesions to nerve-root and meningeal enhancement [64,65]. In general, reviews, especially of European LNB, describe a contradiction that is often inadequately explained. The most common symptoms of European LNB are facial nerve palsy and radicular pain [66]. Neuroanatomically, the pathological changes causing these symptoms must be of peripheral nervous system (PNS) origin. On the other hand, CSF pleocytosis, which in other infections is regarded as a sign of CNS involvement, is considered a mandatory finding in LNB in all but the very earliest cases [67]. One explanation is that the dorsal root ganglia, that are commonly the location of the *Bb* infection and by definition part of the PNS, are anatomically located in the subarachnoid space. Inflammation of the dorsal root ganglion will therefore affect the composition of the CSF [68]. Direct *Bb* involvement of the brain or spinal cord parenchyma, i.e. a true CNS infection occurs but is considered and the incidence is unknown [2].

1.7 Clinical characteristics of Lyme borreliosis

Because of the geographical distribution and the different organotropism of the *B. burgdorferi* s.l. genospecies, North American and European LB differ with regard to the clinical manifestations and their relative frequency, as has been described above [43,69]. Borrelial arthritis and carditis are more common in North America while LNB and ACA are more common in Europe. EM is seen on both continents but multiple EM lesions are common in North America and rare in Europe [56]. LNB is the focus of this thesis but other manifestations of LB will also be covered briefly in this section as they sometimes occur simultaneously with the neurological symptoms.

1.7.1 Dermatoborrelioses

The dermatological manifestations of LB are EM, ACA and lymphocytoma. EM is by far the most common of these and the most common manifestation overall. Typically, EM is a bluish-red maculopapular rash appearing at the site of the bite 7-30 days after the bite. It expands slowly, sometimes after a while clearing centrally so that the lesion gets a ring-like appearance [45,56]. ACA is characteristic for European LNB and the large majority of cases are caused by *B. afzelii* [69,70]. It most commonly affect elderly people and is located on the extensor surfaces of the distal extremities, most often the feet and lower legs. ACA starts as a bluish-red discoloration with doughy oedema and then progresses over months to years to an atrophic phase where the skin becomes thin and wrinkled and the discoloration changes to violet. In patients with long standing cases of ACA, peripheral neuropathy is common, affecting both sensory and motor nerves [45,56]. The third skin manifestation is lymphocytoma, which is a bluish solitary, painless nodule, typically located on the earlobe or areola. It is more common in children than in adults [56,71].

1.7.2 Neuroborreliosis

The terms Lyme neuroborreliosis (LNB) and neuroborreliosis (NB) are used interchangeably and mean the same thing: neurological symptoms caused by *Bb* infection. The time from tick bite to onset of neurological symptoms is usually 2-6 weeks but could be 1-12 weeks. Estimates of the time from tick bite to onset of neurological manifestations are imperfect as only one third of the patients can recall being bitten by a tick [66,72]. However, most cases of LNB occur during July to October and cases with

onset of neurological symptoms during winter and early spring are rarely reported, which indicates that the incubation period is very seldom more than three months [72]. LNB is preceded by EM in 30-50% of patients but the skin lesion may disappear before the onset of neurological symptoms [66,72]. Of the symptoms associated with LNB, patients may present with a single symptom or multiple symptoms in various combinations. Traditionally, LB has been divided into stages, with LNB being divided into early and late LNB, symptoms lasting more than six months being the criterion for late LNB [67]. Some of the more severe central nervous system (CNS) manifestations are more common in patients with a long standing *Bb* infection but there is a considerable overlap and the usefulness of the division into early and late LNB has been questioned [46,73].

The triad of symptoms and signs of lymphocytic meningitis, cranial neuropathies and painful radiculoneuritis as described by Garin and Bujadoux in 1922 and by Bannwarth in 1941 are still considered hallmark symptoms of LNB and is now referred to as Garin-Bujadoux-Bannwarth syndrome or just Bannwarth's syndrome [3,4]. Isolated meningitis is rarely seen in European adults but headache is commonly described in association with other neurological symptom. In children and in North American patients, isolated meningitis is more common [74]. As will be described in the diagnosis section, CSF lymphocytic pleocytosis is, on the other hand, mandatory for the LNB diagnosis. A painful radiculoneuritis (also described as radicular pain) is the most common symptom in LNB and is seen in 70-85% of patients [66,72]. The pain is described as lancinating and often more severe than anything before experienced by the patient. It is localized to the extremities or the trunk and may be migratory. Over-the-counter analgesics seldom have any effect [74,75]. For reasons unknown, the pain is often described as being worse at night [59]. Cranial neuropathy affects roughly half the patients with LNB. The facial nerve (cranial nerve VII) is the most commonly affected with 40-50% of LNB patients presenting with peripheral facial palsy (PFP), which can be uni- or bilateral [66,72]. In areas endemic for LB, up to 25% of peripheral facial palsies are caused by LNB [76,77]. Other than the facial nerve, involvement of all other cranial nerves has been described in LNB, with cranial nerve VI the secondly most affected [72,74].

Both cranial neuropathies and radiculoneuritis are neuroanatomically PNS symptoms. Other PNS symptoms seen in LNB are sensory disturbances and pareses outside the cranial nerve area. Sensory disturbances are experienced by 30-50% of LNB patients, are generally of the hyper- or

dysesthesia type and are often concurrent with radicular pain [66,72]. Paresis can affect muscles in the limbs or trunk but more commonly affects legs than arms, possibly because of the higher frequency of tick bites on the lower than on the upper part of the body [72]. The percentage of patients presenting with paresis outside the cranial nerve area is estimated to 15-40% and increases with the duration of symptoms [66,72].

CNS involvement in LNB is rare and the incidence is difficult to estimate. Presumably, no more than 10% of LNB patients have CNS symptoms. The likelihood of developing CNS symptoms is thought to increase with increasing disease duration [72,74]. Patients with CNS LNB can present with a large array of symptoms. If the infection causes encephalitis, encephalomyelitis or myelitis, the patients may show confusion, cognitive deficits, apraxia, ataxia, Parkinsonism, paraparesis and neurogenic bladder. LNB is known to cause cerebral vasculitis leading to transient ischemic attacks and stroke [78]. LNB is also known to cause intracranial hypertension, a diagnosis that is sometimes and somewhat incorrectly, referred to as pseudotumor cerebri [79].

Children with LNB may present with a slightly different clinical picture from that seen in adults. Facial nerve palsy is even more common in children, affecting 60-70% in one Swedish study [80,81]. Meningism, which is rare in adults with European LNB is relatively common in children, as are unspecific symptoms such as fever, fatigue and loss of appetite [80].

1.7.3 Other and rare manifestations of Lyme borreliosis

It was the clustering of cases of Lyme arthritis that eventually led to the discovery of *Bb* as the causative agent of LB [10,11]. Today, and especially in Europe, Lyme arthritis is relatively rare. It is a mono- or oligoarthritis affecting large joints, commonly the knee. The patients have recurrent attacks of pain and joint swelling with symptom-free intervals in-between [45]. Lyme carditis is even rarer with only sporadic cases seen in Europe [51]. Carditis due to *Bb* infection is usually manifested by atrioventricular block as a result of conduction disturbances [71]. Eye involvement in LB has been described but is considered rare. Almost any part of the eye can be affected but diagnosis is difficult and since there are several other eye disorders with similar clinical picture, LB is often a diagnosis of exclusion [45].

1.7.4 The debated entity of "chronic Lyme disease"

The term chronic Lyme disease can have different meanings depending on the opinion of the person concerned. Originally, chronic Lyme disease (or chronic Lyme borreliosis or chronic Lyme neuroborreliosis, the terms are used interchangeably), denoted Lyme borreliosis with a duration of symptoms of more than six months, i.e. what is now more commonly called late LB [66,67]. However, highly vocal patient-activist organizations and a small number of practitioners, on both sides of the Atlantic, use the term chronic LD to describe an ongoing infection with *Bb* that cannot be diagnosed with traditional methods and that is unresponsive to standard short-term antibiotic treatments [82,83]. Symptoms that are often assigned to chronic LD include pain, fatigue and neurocognitive symptoms [1]. Proponents of the chronic LD concept are of the opinion that very long-term (months or years) antibiotic treatment is needed to suppress the symptoms but the underlying infection is considered inherently incurable [82]. The requested long-term antibiotic treatments are often provided by doctors described by themselves, and by their patients, as Lyme-literate medical doctors (LLMD) [84]. The claims of the chronic LD advocacy groups have been subjected to extremely rigorous reviews by professional associations and by government bodies and have been found to be lacking scientific evidence [83,85]. It has also been shown, that the few health care practitioners that promote the concept of chronic LD and treat patients with long-term antibiotic therapy, pose a threat to public health, both because patients suffering from other diseases might be wrongly diagnosed with chronic LD, and because the long term antibiotic therapy prescribed can cause disease and even death [84,86,87].

In contrast to the claims by the chronic LD advocacy groups, persistent symptoms after treatment for LNB, with no ongoing infection, affect a minority of patients. This is commonly called post-Lyme disease syndrome and is covered in section 1.10 [46].

1.8 Diagnosis of Lyme neuroborreliosis

Europe and North America differ in the area of LNB diagnosis as well, with American diagnostic guidelines being less strict and with less focus on CSF analysis. This section will cover the diagnosis of European LNB. European guidelines were first published in 1996 by the European Union Concerted Action on Lyme Borreliosis (EUCALB), an EU-funded

initiative [88]. The EU no longer funds EUCALB but the most recently updated guidelines were produced by clinicians on the EUCALB Advisory Board [5]. The European Federation of Neurological Societies (EFNS) has also produced diagnostic guidelines for LNB that are very similar to the ones produced by the EUCALB clinicians [67].

The diagnosis of LNB is based on a combination of clinical and laboratory findings. For the definite diagnosis of LNB, current diagnostic guidelines require: a) neurological symptoms consistent with LNB and other causes excluded; b) CSF pleocytosis; c) intrathecal *Bb*-specific antibody production [5,67]. The diagnosis of LNB is a step-wise process comprising careful taking of medical history, clinical examination and analysis of laboratory tests. In addition to the laboratory tests included in the diagnostic criteria, there are additional tests that are sometimes of help and that also will be covered here. Non-standard diagnostic methods such as lymphocyte transformation test (LTT) and CD57+/CD3- lymphocyte subpopulation typing will not be covered in this thesis as they have not been shown to be reliable markers of LNB and are not recommended in any national or international guidelines [5,67,89].

Medical history and clinical symptoms

The first part of the diagnostic process is to assess the overall risk that the patient could be infected with *Bb*. This risk depend on geography, season and the recreational habits of the patient [2]. A city-dweller in Stockholm who never leaves town presenting with PFP in March is not likely to have LNB, whereas an avid hunter from rural Blekinge presenting with PFP in September has a relatively high likelihood of having LNB. Other important clues include whether the patient can recall a tick-bite in the months preceding the onset of neurological symptoms and if he or she has noticed a skin lesion that could be EM.

The clinical symptoms could be clear-cut or more unspecific, consistent with LNB but also with other diseases. If the clinical symptoms of the Garin-Bujadoux-Bannwarth syndrome, i.e. both cranial neuropathy and painful radiculoneuritis, are present, the clinical diagnosis is straightforward. If the patient on the other hand presents with an isolated PFP, the cause could be a wide range of infectious and non-infectious diseases including Ramsay-Hunt syndrome, stroke, malignancy and Bell's palsy [90]. Similarly, radicular pain could be caused by sciatica, paresis of the foot by peroneal mononeuropathy and sensory disturbances by diabetes polyneuropathy or multiple sclerosis [1,91]. LNB with CNS symptoms also have a wide range of potential differential diagnoses

including malignancies, dementia and Parkinson's disease [92,93]. In cases where the clinical symptoms overlap those of other diseases, simple and reliable laboratory diagnostic methods are essential.

CSF cell count and albumin

As described above, the demonstration of CSF pleocytosis and intrathecal *Bb* specific antibody production are the standard laboratory criteria for the diagnosis of LNB [5,67]. CSF pleocytosis was described already in the first published account of LNB [3]. The cut-off value for pleocytosis in LNB as in other infectious and inflammatory nervous system diseases is commonly a CSF cell count > 5 cells/μL, though sometimes the limit is set at > 4 cells/μL. The origin of these cut-off values is to a large extent unclear but there are studies from before 1970 stating that the 95th percentile for a healthy population is around 5 cells/μL [94,95]. Pleocytosis is almost invariably observed in LNB but there are case reports describing patients with very short duration of symptoms without pleocytosis (Bremell, unpublished data) and one study indicating that pleocytosis might be less common in LNB caused by *B. afzelii* [96]. However, this latter finding has yet to be repeated. The pleocytosis is typically lymphocytic or mononuclear in contrast to the granulocytic pleocytosis seen in bacterial meningitis [5,97]. The two largest studies on LNB presented mean and median CSF cell counts of respectively 90 cells/μL (range 2-1100) and 160 cells/μL (range 4-1000) [66,72]. The composition of mononuclear cells in CSF from patients with LNB has rarely been investigated but in a small study, Cepok and co-workers presented data showing the majority of cells to be CD4+ and CD8+ T-cells and with a significant number of B-cells and plasma cells. The proportion of monocytes was low but increased after treatment was initiated [98]. The possibility to use the relative levels of different leukocyte subsets to differentiate LNB from other infectious diseases has been sparsely investigated. Bacterial meningitis rarely poses a differential diagnostic problem as both the clinical course and symptomology are essentially different and the CSF pleocytosis is massive and granulocytic [97]. Viral infections however, often present with moderate mononuclear pleocytosis [99]. In a pediatric population, the levels of granulocytes have been shown to be higher in patients with enteroviral meningitis than in patients with LNB and in a small study on adult patients, the proportion of monocytes was shown to be significantly higher in patients with viral CNS infections than in patients with LNB [98,100]. CSF cell count decreases rapidly after initiation of treatment but slightly elevated CSF cell count can persist for weeks or even months [72,98]. Decrease in CSF cell count has been used as a surrogate marker of treatment effect, but

there are no studies on the specific relation between decrease in CSF cell count and reduction of symptoms [101,102].

LNB results in blood-CSF-barrier damage and leakage of protein from blood to the CSF. Earlier studies reported data for the total protein concentration of the CSF; reported values are mean 1.4 g/L (range 0.2-10.7) and median 1.1 g/L (range 0.2-12) in the two largest studies on LNB [66,72]. Protein levels decrease after treatment but more slowly than cell counts [72,103]. Today it has been increasingly common to report values for CSF albumin instead of total protein. There are no large studies on the CSF albumin levels in LNB and no simple algorithm for the transformation between protein and albumin. Still, it is generally accepted that moderately elevated levels of CSF albumin are typical in LNB [74].

Serological diagnosis

The second mandatory laboratory diagnostic criterion for LNB is the demonstration of intrathecally produced *Bb*-specific antibodies. First-generation serology tests used whole-cell lysates of *Bb* as antigens for the capture of *Bb* antibodies. These tests lacked specificity because of cross-reactivity as they contained antigens shared by many bacteria [39]. Second generation tests use purified *Bb* protein as antigen, most commonly the flagella protein flagellin. These tests have lower risk of cross-reactivity but cross-reactivity still exists, especially to syphilis, Epstein-Barr virus and to rheumatoid factor [104]. Third generation tests use recombinant or synthetic proteins as antigens. For these tests the risk of cross-reactivity is lower still and specificities above 93% have been reported when third-generation assays have been tested against panels of potentially cross-reactive sera [105]. The large majority of studies on the discriminatory performance of *Bb* serology tests have been done on serum and relatively little has been published on the risk of cross-reactivity in CSF samples. Nevertheless, given that the risk of cross-reactivity is very low with third-generation tests and that there are no studies showing the risk to be higher in CSF than in serum samples, it is reasonable to assume that the risk of false positive results because of cross-reactivity in CSF samples is low. However, the risk of false positive results in CSF samples because of diffusion of *Bb* antibodies from blood or because of a previous infection still remains, as will be described.

There are several commercial *Bb* antibody test kits in use that differ in method and choice of antigen and also somewhat in diagnostic performance, but a detailed description of their respective weaknesses and strengths are beyond the scope of this thesis and the overall conclusions

on serological diagnosis of LNB are valid for all tests in use today. Serological diagnosis is used for other manifestations of LB, such as Lyme arthritis and ACA, both of which require the demonstration of *Bb* serum antibodies [5]. The diagnostic sensitivity of *Bb* serum antibodies for the diagnosis of LNB is 65-85% [106,107]. The diagnostic specificity of isolated analysis of serum antibodies is limited since the seroprevalence of *Bb* antibodies in the general population can be above 20% in endemic areas [92,108]. The use of *Bb* serum antibodies analysis should be limited to the exclusion of LNB in patients with long-standing disease, as the negative predictive value in patients with duration of symptoms of more than six weeks approaches 100% [109]. Isolated analysis of *Bb* antibodies in CSF also has drawbacks because the risk of diffusion of serum antibodies across the blood-CSF-barrier makes the results difficult to interpret [110]. The method of choice for the demonstration of intrathecally produced *Bb*-specific antibodies is parallel analysis of CSF and serum followed by calculations of an antibody index (AI) that take into account blood-CSF-barrier dysfunction. The AI has a sensitivity of 70-80% in the first weeks after onset of neurological symptoms, reaching 100% after six weeks [111,112]. The specificity of a positive AI is very high but not 100% as a positive AI has been shown to persist for years after treatment of LNB and a positive test thus can reflect a previous infection [113].

Culture and PCR

Bb can be cultured from skin, blood and CSF in specially composed Barbour-Stoenner-Kelly (BSK) medium. Cultures are incubated at 30-34°C for up to 12 weeks because of the slow growth rate of *Bb in vitro* [39]. When *Bb* is cultured from skin specimens from patients with EM or ACA the yield can reach 60% or even higher, but when cultured from CSF or blood, the yield is much lower, 10-17%, making culture unsuitable for routine diagnosis of LNB [39]. In Sweden culture of *Bb* is not routinely performed at any of the microbial laboratories, its use limited to research purposes [89]. PCR technology for the detection of *Bb* is more commonly used than culture, both in Sweden and internationally [89]. PCR-methods for the detection of *Bb* use various target sequences and different oligonucleotide primers and are not standardized, meaning that results obtained by different laboratories may vary considerably [5]. Nevertheless, PCR is useful for the detection of *Bb* spirochetes in skin specimens and in synovial fluid, the sensitivity being above 60% and even higher in some studies [39]. The sensitivity of PCR for detecting *Bb* spirochetes in CSF is unfortunately lower, 15-30%, with the higher numbers seen shortly after the onset of neurological symptoms and

decreasing with the duration of the disease [5,39,114]. In the diagnosis of LNB, PCR is not recommended for routine use but could be helpful in some cases, especially in very early LNB [67,89].

CXCL13

Chemokines are small proteins that direct circulating leukocytes to sites of inflammation or injury [115]. Chemokine [C-X-C motif] ligand 13 (CXCL13), previously known as B lymphocyte chemoattractant (BLC), is produced by stromal cells, monocytes and possibly other cell types [116,117]. CXCL13 directs B-cells to lymphoid follicles and is involved in the formation of ectopic germinal centers within the CNS in inflammatory CNS diseases [116,118]. In 2005, using protein expression profiling, Rupprecht and co-workers identified an upregulation of CXCL13 in CSF of patients with LNB but not in CSF from patients with various other inflammatory and non-inflammatory neurological diseases [119]. Since then, an increasing number of studies on the diagnostic potential of CSF CXCL13 have been published. It has also been shown that analysis of serum levels of CXCL13 are of little use in the diagnosis of LNB [120,121]. In assessing the diagnostic potential of CXCL13, a number of studies have shown levels to be significantly higher in LNB than in several other nervous system diseases such as bacterial- and viral meningitis and encephalitis, Guillain-Barré syndrome, multiple sclerosis and Bell's palsy [121-123]. It has been shown that CXCL13 rises early in the course of disease and that levels decrease rapidly after initiation of treatment [121]. The other diseases in which significantly elevated levels of CXCL13 have been observed are diseases with a low incidence in Lyme borreliosis-endemic areas such as neurosyphilis, cryptococcal meningitis and human African trypanosomiasis (also known as sleeping sickness) [123-125]. As of yet, according to EFNS guidelines, routine analysis of CXCL13 is not recommended for patients with suspected LNB but can be of help in diagnosing seronegative patients during early disease and for control of treatment [67].

1.9 Treatment of Lyme neuroborreliosis

It has been recognized since long before the spirochetal etiology of LB was known that antibiotic treatment affects the clinical course [3,9]. Shortly after the discovery of *Bb*, it was shown that the spirochete was sensitive *in vitro* to a range of antibiotics including penicillin and doxycycline [126]. A large number of further studies have shown that *Bb* is sensitive *in vitro* to, among other antibiotics, penicillin G, amoxicillin,

piperacillin, cefotaxime, ceftriaxone, doxycycline, erythromycin and azithromycin [127]. There are some studies showing that the various *B.burgdorferi* s.l. genospecies differ in their *in vitro* antibiotic susceptibility with *B. garinii* being more susceptible to commonly used antibiotics than *B. afzelii* and *B.burgdorferi* s.s., but other studies have failed to show any such differences and it is not known whether this has any clinical implications [128,129]. To further complicate the picture there are considerable discrepancies between *in vivo* and *in vitro* activities of various antimicrobial agents [130]. *In vitro*-derived minimum inhibitory concentrations (MIC) of penicillin G and amoxicillin indicate only moderate activity against *Bb* but both agents are well proven to be clinically effective in the large majority of LB patients. Conversely, the activity of macrolide antibiotics is likely overestimated from *in vitro* studies. The slow-growing nature of *Bb*, the special, highly enriched culture medium required and the chemical instability of β-lactam antibiotics probably all contribute to the relative unreliability of *in vitro* susceptibility data [130].

When treating LNB, in addition to the effect of the antibiotic on *Bb*, the ability of the antibiotic to cross the blood-CSF/brain-barrier also has to be taken into account. The most commonly recommended antibiotics for LNB, intravenous (IV) penicillin G, IV ceftriaxone, IV cefotaxime and oral doxycycline, have all been shown to achieve CSF concentrations above MIC of *Bb*. The MIC values for *Bb* are generally lower for the β-lactam antibiotics but doxycycline show better blood-CSF/brain-barrier-penetration [131-133]. Several studies have compared the efficacy of IV penicillin G, IV ceftriaxone and IV cefotaxime for LNB and found no significant differences [134-136]. There are also studies showing IV penicillin G and oral doxycycline to be equally effective [136,137]. In practice, penicillin G and cefotaxime are now rarely used for the treatment of LNB since they both need to be given as IV injections three times a day. Ceftriaxone, on the other hand, is given as a once-daily IV injection, which enables ambulatory treatment and which makes ceftriaxone the IV treatment option of choice.

In clinical practice today, both in Europe and North America, the choice of treatment for LNB is between IV ceftriaxone and oral doxycycline. Even if ambulatory treatment is possible with ceftriaxone, IV injections still have to be given by trained nurses, carries the risk of infections and are more expensive compared to oral treatment [138]. Oral doxycycline carries none of the disadvantages inherent to IV treatment but its use is contraindicated in late pregnancy and in children under the age of eight as

there is a risk of staining of developing teeth, even if more recent data suggest that this risk is very low [139]. As for the common side effects of the two treatments, doxycycline carries a higher risk of phototoxicity and gastrointestinal irritation, whereas ceftriaxone more often causes hypersensitivity reactions and *Clostridium difficile* infections [138]. There are a limited number of studies directly comparing the efficacy of ceftriaxone and doxycycline for the treatment of LNB. Nonetheless, the published studies are fairly large and one (Ljøstad et al. 2008) is a double-blind, randomized trial. All studies show no difference in outcome between ceftriaxone and doxycycline [101,102].

There are numerous national guidelines for the treatment of LNB. The Swedish national guidelines published by the Medical Products Agency (Läkemedelsverket) in 2009 recommend doxycycline for all manifestations of LNB with ceftriaxone an alternative for patients in whom doxycycline is contraindicated [140]. In contrast, European guidelines by EFNS and American guidelines by the American Academy of Neurology (AAN) and Infectious Diseases Society of America (IDSA) recommend different treatment regimes for LNB with only PNS symptoms compared to LNB with CNS symptoms. For LNB with PNS symptoms, ceftriaxone and doxycycline are considered equally effective but for LNB with CNS symptoms, ceftriaxone is the sole recommended treatment. The preference of ceftriaxone over doxycycline for LNB with CNS symptoms is not based on clinical studies but reflect the consensus opinion of the guideline authors [46,67,81]. CNS involvement is defined in the EFNS guidelines as the clinical manifestations of encephalitis, myelitis and cerebral vasculitis [67].

Data on the optimal duration of treatment for LNB is scarcer and very few studies have compared different treatment durations with the same antibiotic. One American study on ceftriaxone showed no advantage of 28 days of treatment versus 14 days of treatment [141]. A Finnish study showed no added benefit of adding 100 days of treatment with amoxicillin to 21 days of treatment with ceftriaxone [142]. Even though data is limited, the recommendations in the various guidelines are relatively coherent. Swedish guidelines recommend 10-14 days of treatment, depending on the daily dose of doxycycline [140]. American guidelines from AAS and IDSA recommend 14 days of treatment [46,81]. EFNS guidelines differ in that they recommend 14 days of treatment for early LNB but 21 days of treatment for late LNB [67].

1.10 Prognosis of Lyme neuroborreliosis

Patients with LNB usually experience a dramatic effect on symptoms within a few days of starting antibiotic treatment. In fact, a prompt treatment effect is so typical that the absence of effect after seven to ten days of treatment warrants a re-evaluation of the diagnosis. Pain is usually the first symptom to disappear, while pareses, sensory disturbances and CNS symptoms commonly take longer to be affected [72]. CSF cell count start decreasing rapidly and a significant decline is usually seen if CSF sampling is repeated two weeks after starting treatment [101]. A similar picture is seen for CSF albumin or protein [72]. After the initial rapid decline in CSF cell count and reduction of symptoms, a complete clinical recovery and normalization of CSF commonly takes months. In one study, the median CSF cell count was still slightly elevated 12 weeks after initiation of treatment [72].

The long-term prognosis of LNB has been evaluated in several studies. The studies vary in inclusion criteria, time of follow-up, type of symptoms reported and the use or not of a control group, making comparisons between studies difficult. The proportion of patients with remaining symptoms three to five years after treatment is in most studies reported to be 25-50%. [143-146]. The most common remaining symptoms are facial palsy, fatigue, paresthesias and pain [143,145,147]. Severe and debilitating remaining symptoms are rarer, the proportion of patients with reduced working capacity after three to five years is about five percent [72,143]. Various factors have been shown to influence outcome after treatment for LNB. Long duration of symptoms pre-treatment, a high pre-treatment CSF cell count and CNS symptoms are factors indicating a less favorable outcome [65,143,144,148,149]. Children with LNB have a better prognosis than adults with a higher proportion recovering completely [80,143].

If subjective complaints such as fatigue, sleeping disorders, cognitive impairment and myalgia persist for more than six months after treatment for LNB the condition is often termed post-Lyme disease syndrome (PLDS) [82]. PLDS is reported to be more common in North America and most studies on PLDS have been performed on North American patients in whom PLDS is reported following any manifestation of Lyme disease, not only LNB but also non-neurological manifestations such as EM [82,150]. The subjective symptoms that constitute the majority of the remaining symptoms reported in follow-up studies of European LNB are however, fairly similar to the symptoms of PLDS and it is therefore

sensible to use the term PLDS to describe remaining symptoms in European LNB patients as well. As described in section 1.7.4., there is no evidence for ongoing *Bb* infection in patients with PLDS and several randomized controlled trials have failed to show benefits of prolonged antibiotic treatment that outweigh the risk of the treatment [82,151,152]. In contrast, symptomatic treatment for patients with PLDS, for example tricyclic antidepressants or gabapentine for neuropathic pain, often leads to improvement [1,153].

2 AIMS

The overall aims of this thesis were to improve the diagnosis and treatment of Lyme neuroborreliosis. The specific aims were:

- To identify clinical and laboratory characteristics that differentiate peripheral facial palsy caused by Lyme neuroborreliosis from Bell's palsy.

- To investigate the potential use of CSF CXCL13 in the diagnosis of Lyme neuroborreliosis and as a marker of treatment effect.

- To investigate the use of automatic cell counters for the analysis of CSF cells, including the establishment of new reference ranges and assessment of the method's potential in discriminating Lyme neuroborreliosis from other nervous system infections.

- To investigate the use of oral doxycycline for the treatment of Lyme neuroborreliosis with CNS symptoms.

3 PATIENTS AND METHODS

3.1 Case definitions

The following definitions and/or classifications were used for the patients studied in this thesis.

Lyme neuroborreliosis

Clinical symptoms consistent with LNB and other possible diagnoses excluded were the basis for the diagnosis of LNB in papers I-IV. In addition the following criteria were used.

In paper I, patients were classified as definite LNB or possible LNB. Patients with a preceding EM within three months, or positive *Bb* antibodies (IgG and/or IgM) in CSF and either positive AI or ≥ 2 oligoclonal bands on isoelectric focusing of CSF and serum were classified as definite LNB. Patients with positive *Bb* antibodies (IgG and/or IgM) in CSF and/or serum but negative *Bb* antibody index, < 2 oligoclonal bands on isoelectric focusing of CSF and serum, and no preceding EM within three months, were classified as possible LNB.

In paper II, diagnostic criteria for LNB were CSF mononuclear cell count > 5 cells/μL and positive *Bb* antibodies in CSF, plus one of the following: positive AI or CSF cytological examination consistent with LNB with activated plasma cells.

In paper III, patients having been given the ICD-10 diagnosis of LNB by the clinician at the Department of Infectious Diseases were considered having LNB.

In paper IV, diagnostic criteria criteria for LNB were CSF mononuclear cell count > 5 cells/μL, plus one of the following: preceding EM within three months or the detection of *B. burgdorferi*-specific antibodies in CSF.

Bell's palsy

In paper I, patients with PFP were classified as Bell's palsy if they had no preceding EM within three months and no *Bb* antibodies (IgG and/or IgM) in CSF or serum. Clinical signs of herpes zoster were an exclusion criterion.

3.2 Patients and controls

Patients

During the period studied in this thesis, 1990-2012, 366 patients were diagnosed with LNB at the Department of Infectious Diseases, Sahlgrenska University Hospital, according to electronic hospital records. Of these, 203 were included in the studies of the thesis. Some patients were included in more than one of the studies according to figure 4. It should be noted that the 17 patients classified as possible LNB in paper I are not included in the numbers above or in figure 1 as the majority of these patients were deemed unlikely to be infected with *Bb*.

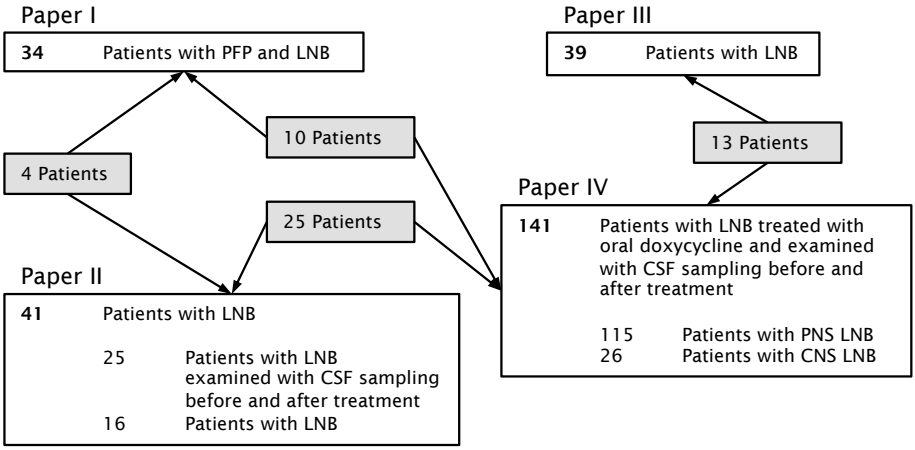

Figure 2. LNB-patients included in the four studies of the thesis. Patients included in more than one study are indicated in the in grey boxes.

Controls

An overview of the controls included in papers I-III is presented in table 1. In paper I, 51 patients with Bell's palsy according to the diagnostic criteria stated above were included as controls. In paper II, 27 patients with asymptomatic HIV-infection and 39 individuals in whom neurologic disease had been ruled out, were included as controls. Inclusion criteria for the HIV-patients were: a) asymptomatic HIV-infection, b) no antiretroviral treatment, c) no neurological symptoms, d) a negative syphilis test. In paper III, 80 neurologically healthy volunteers undergoing

knee or hip surgery were included in the reference range section of the study. In the differential diagnosis section, 39 patients with HIV-infection, 60 patients with viral CNS-infection, 20 patients with bacterial meningitis, 9 patients with other infectious diseases and 14 patients with non-infectious diseases were included as controls. Diagnoses were determined according to standard clinical criteria at the Department of Infectious Diseases, Sahlgrenska University Hospital.

Table 1. Patients and controls included in the studies of the thesis. Some LNB patients are included in more than one study as described in figure 4.

	Paper I	Paper II	Paper III	Paper IV
LNB	34	41	39	141
Possible LNB	17			
Bell's palsy	51			
HIV		27	33	
Healthy controls		39	80	
Viral CNS infection			60	
Other infectious diseases			29	
Non-infectious diseases			14	
Total	102	107	255	141

3.3 Methods

3.3.1 CSF cell count

Two different methods were used for CSF cell counts during the study period. Until December 31, 2009, all CSF cell counts were performed manually (papers I, II, IV). After that date CSF cell counts were performed on an automatic cell counter (papers III, IV). Manual counts were performed on a Fuchs-Rosenthal hemocytometer after 1:2 dilution with methylene blue. Cells were counted in 32 1 mm^2 areas and CSF cells were specified as erythrocytes, polymorphonuclear leukocytes (granulocytes or "poly") or mononuclear leukocytes ("mono"). Mononuclear leukocytes > 5 cells/µL were considered pathologic. From January 2010 CSF cell counts were performed on the automatic cell counter Siemens Advia 2120i (figure 5). The automatic cell counter separates CSF cells into erythrocytes, monocytes, lymphocytes and

granulocytes on the basis of size, absorbance and light scattering characteristics. Analyses were done according to the manufacturer's instructions (Siemens AG, Erlangen, Germany). In paper III the evaluation of the automatic method that was performed before its introduction into clinical routine is described in detail. The reference ranges for cell counts performed on automatic cell counters are still a matter of debate. In paper III a cut-off value for lymphocytes of > 4 cells/μL is suggested.

Figure 3. The automatic cell counter Siemens Advia 2120i.

In paper IV, for coherence, > 5 cells/μL was used as the cut-off for CSF lymphocytes for the patients analyzed after January 1 2010. The automatic cell counter has the drawback of performing less well on bloodstained samples. Therefore, CSF samples with erythrocytes > 250 cells/μL on the automatic cell count were counted manually.

3.3.2 CXCL13

CSF levels of CXCL13 were analyzed in paper III. CXCL13 was measured by ELISA (Human CXCL13/BLC/BCA-1 Quantikine ELISA kit, R&D Systems, Minneapolis, USA), according to instructions from the

manufacturer. Based on measurements of duplicates of the standard samples (concentrations 7.8-500 pg/mL), the average intra-assay CVs were ≤ 10%. Samples with CXCL13 concentrations > 500 pg/mL were diluted stepwise, 1:10, 1:100, until the assay produced a valid result.

3.3.3 *Bb* serology

CSF and serum serology

Two different tests were used to analyze *Bb*-specific antibodies in CSF and serum during the study period. Until June 26, 2006 (papers I, II, IV), samples were analyzed for IgG and IgM with the Dako Lyme Borreliosis ELISA kit using purified native *B. burgdorferi* flagellum as antigen (Dako Cytomation A/S, Glostrup, Denmark). From June 26, 2006 (papers I-IV), samples were analyzed with the Liaison chemoluminescence immuno-assay (CLIA), using recombinant VlsE as antigen for IgG and recombinant OspC as antigen for IgM (Diasorin, Saluggia, Italy). Cut-off values for positive samples were according to the manufacturers' instructions.

Antibody index

The CSF/serum *Bb*-antibody index was used in papers I and II. The *Bb*-antibody index was calculated as the ratio of the CSF/serum quotient of specific antibodies to the corresponding CSF/serum quotient of total immunoglobulins as described by Reiber and Lange. Antibody index values > 1.4 were considered positive and indicative of specific intrathecal antibody synthesis [154].

3.4 Methodological considerations

Different diagnostic criteria for LNB were used in the studies of this thesis as described in section 3.1.1. There were several reasons for this. In paper I, one of the aims was to study differences in CSF parameters between patients with LNB and Bell's palsy. Therefore it was not possible to use CSF mononuclear pleocytosis as a diagnostic criterion for LNB. This might have led to the misclassification of some patients with LNB in whom CSF *Bb*-antibody synthesis was not yet detectible. In papers II and IV, demonstration of CSF pleocytosis was required and in addition the detection of CSF *Bb*-antibodies and additional findings (paper II) or detection of CSF *Bb*-antibodies or preceding EM (paper IV). Thus, even if the diagnostic criteria used in papers II and IV are somewhat less strict than the criteria of EUCALB as described by Stanek et al., the risk that

patients in papers II and IV with other diseases were wrongly classified as having LNB was likely to be small [5]. In paper III the ICD-10 diagnosis registered in the electronic hospital records formed the basis of disease classification for the patients included in the differential diagnosis section of the study. Since all patients presented with mononuclear pleocytosis and were seen by doctors at the Department of Infectious Diseases familiar with LNB and other nervous system infections, the number of patients who might have been misclassified was likely to be very low. In summary, the use of different diagnostic criteria for LNB in this thesis is somewhat unwieldy but unlikely to affect the results or conclusions in a significant way.

The two different tests for the analysis of *Bb*-antibodies used during the study period have been shown to differ in their diagnostic performance, the Liaison kit being somewhat more sensitive than the Dako kit without sacrificing specificity [105,106]. There is a small risk that this might have affected the results of paper I as the detection of *Bb*-specific antibodies was the main determinant for classification into one of the study groups.

3.5 Statistics

The following statistical software was used in this thesis: PASW statistics 18.0 (paper I), GraphPad Prism 5.0 (papers II-IV), MedCalc 12.4 (paper III). The Pearson's χ^2-test was used for the analysis of unpaired categorical data (paper I). The Mann-Whitney *U*-test was used for comparison of continuous variables between two groups (papers I, III, IV). For comparison of continuous variables between three groups, the Kruskal-Wallis test followed by Dunn's post test was used (paper II). For analyses of paired continuous data, the Wilcoxon matched pairs test was used (papers II, IV). Analysis of covariance (ANCOVA) was used for comparison of slope coefficients (paper IV).

3.6 Ethics

Ethical approvals were obtained from the Regional Ethical Review board at the University of Gothenburg: Dnr 358-95 (papers I-IV), Dnr 163-12 (paper III), Dnr 123-10 (papers III, IV), Dnr 588-01 (papers II, III). Informed consent was obtained from the patients in the reference range section of paper III.

4 RESULTS

4.1 Paper I

The study covered the anamnestic, clinical and laboratory diagnosis of patients with PFP. One hundred and two patients with PFP were included in the study. According to the case definitions in section 3.1 they were divided into three groups: definite LNB, possible LNB and BP. There were 34 patients with definite LNB, 17 with possible LNB and 51 with BP. Patients classified as possible LNB did not differ significantly from the patients with BP in any of the studied clinical or laboratory parameters. Comparisons were therefore mainly done between the patients with definite LNB and BP. Patients with definite LNB fell ill in the period May to December with a peak in August-September while the onset of symptoms for the BP patients was more evenly distributed over the year. There was a significant difference in age between the groups definite LNB and BP, patients with definite LNB being older (median age 46 years (7-75) versus 36 years (15-70). The proportion of patients that had noticed a tick bite preceding the onset of symptoms was generally low and there was no significant difference between the groups. Regarding clinical symptoms, both patients with definite LNB and BP experienced pain and sensory disturbances from the side of the face affected by the facial palsy with no significant difference between the groups. On the other hand, neurologic symptoms outside the affected side of the face, including the side of the face unaffected by paresis, were significantly more common in the definite LNB group. These symptoms included radicular pain, sensory disturbances and other pareses. Patients with definite LNB had significantly higher levels of mononuclear cells and albumin in CSF than patients with BP.

4.2 Paper II

The study dealt with the diagnostic potential and performance of CSF CXCL13 and consisted of two sections. In the longitudinal section 25 patients with LNB who had undergone CSF sampling before and after treatment were analyzed with regard to the CSF levels of mononuclear cells and CXCL13. In the cross-sectional section 16 patients with LNB, 27 patients with asymptomatic HIV-1 infection and 39 individuals with no neurologic disease designated “healthy controls”, were analyzed, with regard to the CSF levels of mononuclear cells and CXCL13.

In the longitudinal section of the study, CSF levels of both CXCL13 and mononuclear cells decreased significantly after treatment. The decrease in CXCL13 was about one order of magnitude higher than the decrease in mononuclear cells but the decrease in the two parameters correlated significantly.

In the cross-sectional section of the study, CSF CXCL13 levels differed significantly between patients with LNB, HIV and healthy controls. All but one of the healthy controls had immeasurably low levels. For the LNB and HIV patients, even though the difference on group level was significant, there was a considerable overlap and the HIV patient with the highest CXCL13 concentration had a higher value than seven of the sixteen LNB patients.

The correlation between CSF levels of CXCL13 and mononuclear cell counts were analyzed. There was a significant correlation for HIV patients as well as for LNB patients, both in the longitudinal and the cross-sectional part of the study.

For the assessment of the clinical usefulness of CSF CXCL13 ROC curve analysis was used. The combined group of LNB patients from the pre-treatment part of the longitudinal section and the cross-sectional section were analyzed against the combined group of HIV patients and healthy controls. The CXCL13 concentration maximizing sensitivity and specificity in this study was 62 pg/mL, giving a sensitivity of 90% and a specificity of 88%.

4.3 Paper III

The study covered laboratory methods and diagnostic performance of the CSF cell count. The first section of the study described the initial evaluation of the automatic cell counter Siemens Advia 2120i for the analysis of CSF cell counts. For this section, 121 consecutive CSF samples from patients evaluated for various neurological diseases were used. The study showed a very high degree of conformity between the results from the automatic and the manual cell counts for samples with an erythrocyte count < 250 cells/μL. The correlation was weaker for samples with cell counts < 3 cells/μL.

The second section aimed to establish reference ranges for CSF cell counts analyzed with automatic cell counters. CSF from 80 neurologically healthy volunteers undergoing orthopedic surgery was used. The 95th

percentile was 3.0 cells/μL for lymphocytes, 1.0 cell/μL for monocytes and 1.0 cell/μL for granulocytes. Taking into account that the linearity could not be established for samples with cell counts < 3 cells/μL, the resulting reference ranges would be < 4 cells/μL for lymphocytes, < 3 cells/μL for monocytes and < 3 cells/μL for granulocytes.

The third section of the study explored the potential of the more detailed results obtained from the automatic cell counter to be used for differential diagnosis. Consecutive CSF samples from 175 patients with results above the upper reference limit established in section two of the paper were used. The patients were grouped according to discharge diagnosis into six groups: bacterial meningitis, HIV, LNB, viral CNS infection, other infection and non-infectious disease. Comparisons were done between the groups LNB and viral CNS infection. There were no significant differences between the groups for either of the cell types lymphocytes, monocytes or granulocytes.

4.4 Paper IV

The study evaluated the efficacy of oral doxycycline for severe LNB. Patients with LNB, treated with oral doxycycline, who had undergone CSF sampling before and after treatment were included. Patients were grouped according to symptoms into one group with symptoms indicating CNS involvement and one group with symptoms only from the PNS. Twenty-six patients were included in the CNS symptoms group and one-hundred-and-fifteen in the PNS symptoms group. Of the patients with CNS symptoms, more than half had had symptoms for less than two months and three quarters for less than six months. All patients with CNS symptoms showed marked improvement after treatment with no patient relapsing and no patient needing retreatment. Sixty-two percent of the patients with CNS symptoms had sequelae at the end of follow-up but only fifteen percent had severe sequelae substantially affecting the person's daily life. There was no significant correlation between the duration of symptoms pre-treatment and the severity of the sequelae. All patients in both groups showed a decrease in CSF mononuclear cell count at follow-up. On group level, the decrease was significant for both groups. The decrease in CSF albumin level was also significant for both groups. The slopes of the regression lines describing the decrease in CSF mononuclear cell count did not differ between the groups of patients with CNS and PNS symptoms.

5 DISCUSSION

This thesis and the published studies that form its basis highlight the important role of medical history and simple routine CSF examinations for the diagnosis of Lyme neuroborreliosis. Practical tools are presented that can help in the differentiation of peripheral facial palsy caused by LNB from Bell's palsy within hours of admission. Additional analyses such as the chemokine CXCL13 and separate lymphocyte and monocyte CSF cell count by mode of automatic CSF cell counting are shown to be of limited value in the routine diagnosis of LNB. Furthermore, data is presented that supports the use of oral doxycycline also for LNB with severe symptoms, which has been questioned by international experts.

The strengths of the studies include the large clinical material on which they are based, the fact that all patients were seen at the same center and examined by experienced infectious disease doctors, the use of a uniform antibiotic regime throughout the study period and the frequent use of follow-up CSF sampling. Weaknesses of the studies include the retrospective study design, the lack of systematic long-term follow-up routines for part of the study period and the absence of neuropsychological testing in the assessment of sequelae. Furthermore, some of the persons included as healthy controls in the studies were individuals examined for diffuse symptoms and in whom somatic disease was ruled out.

Often, public opinion on Lyme borreliosis, and in particular Lyme neuroborreliosis, seems to be that it is a disease surrounded by controversy, that the disease can present itself in almost any way, that the diagnostic methods are unreliable and that the recommended treatment regimens are ineffective. These miscomprehensions are fueled by uncritical journalists and in particular the possibility of patient-activist groups to organize themselves online. The Norwegian doctor and epidemiologist Preben Aavitsland, on describing the misconceptions about diagnosis and treatment of LB, put it aptly as "chronic Lyme borreliosis is a disease you catch from the Internet, not from the woods" [155]. In contrast to the unscientific claims by certain patient-activist groups and charlatan doctors, there are however, real shortcomings to the diagnostic methods used today. Also, given that some patients suffer from remaining symptoms after treatment, current treatment regimens cannot be considered perfect. These are areas of research to which knowledge is added by the studies on which this thesis is based.

5.1 Diagnosis of Lyme neuroborreliosis

5.1.1 Medical history and clinical examination

Though it is commonly said that the diagnosis of LNB should be based on medical history, clinical examination and an assessment of tick-exposure risk, this statement merits to be repeated [5]. As the definite diagnosis of LNB can only be made by CSF examination, the clinician first has to decide, after having taken a medical history and performed a clinical examination, whether or not to perform a lumbar puncture (LP) for CSF sampling. Although severe complications following LP are rare, post-LP headache affects 10-25% of patients and can render the patient incapacitated for days [156]. An LP should therefore only be performed when needed for the diagnostic process to move forward. For patients considered to have a low risk of LNB and who have had symptoms for more than six weeks, performing an LP may not be necessary, as a negative serum *Bb*-antibody test rules out LNB [109].

The first aspect to consider in the medical history is the possibility of tick-exposure. As the incubation period from tick-bite to onset of neurological symptoms is usually 2-6 weeks, this is the period that should be investigated. If the patient has noticed a biting tick, the exposure is established. As shown in paper I, and in line with previously published data, however, only about one third of patients with LNB can recall a tick-bite [66,72]. Whether or not the patient has noticed a tick-bite, skin lesions possible being erythema migrans should be inquired for. For slowly expanding, sharply demarcated, bluish-red lesions with little local symptoms of pain or itch, there are few differential diagnoses. A preceding EM means the diagnosis of Lyme borreliosis is established, but an EM may not remain at the time of onset of neurological symptoms [66]. For the patient with no known tick-bite or EM to have been exposed to ticks, he or she must have moved through tick-infested areas while the outside temperature was above 5°C [18]. A detailed inquiry into the patient's recreational habits is necessary, having walked only on paved footpaths in city parks means no risk of tick-exposure. Another important piece of information is in what season the neurological symptoms started. As we show in paper I, the peak incidence of LNB is in August and September with fewer cases seen in May to July and October to December, and no cases seen in January to April. This is in line with previous reports in which very few cases are seen between January and April [49,72].

In the patient's description of the symptoms there are certain details to pay attention to. Headache, vertigo and fatigue without additional symptoms are very seldom indicative of LNB [71]. Pain described as lancinating and worse during night-time, is on the other hand, typical of LNB [59]. In paper I, it is shown that patients with peripheral facial palsy regardless of its cause, often experience pain and/or sensory disturbances from the side of the face affected by the paresis, but that patients with peripheral facial palsy caused by LNB in contrast to patients with Bell's palsy, often experience pain and/or sensory disturbances from the unaffected half of the face or from other parts of the body. This knowledge will be of help in deciding whether further investigation is warranted or not in the rather common situation when a patient presents with peripheral facial palsy during tick-feeding season but without having noticed a tick-bite or EM.

The clinical examination focuses on objective symptoms and when possible tries to quantify them. This can be relatively easy, as in the case of pareses, or more difficult, as in the case of symptoms indicative of CNS involvement. In paper IV, the need for strict criteria for the diagnosis of CNS involvement in LNB is emphasized and the perceived symptoms of fatigue and mild memory impairment are contrasted to the objective symptoms of ataxia and paraparesis. Confusion and hallucinosis are rare symptoms of CNS LNB that at least to an extent can be made objective by thorough psychiatric examination. A full-body examination is necessary as it is not uncommon to find an EM unnoticed by the patient. In clinical practice, neurological symptoms together with an EM is sometimes considered sufficient evidence to start treatment of LNB without LP but this is not recommended by European or Swedish guidelines [5,67,89].

In conclusion, in everyday clinical practice, knowledge of key signs of LNB in the medical history and clinical examination is of great help to the clinician trying to establish a provisional diagnosis and deciding whether or not to proceed with further analyses.

5.1.2 Laboratory diagnosis

According to European guidelines, definite diagnosis of LNB requires the demonstration of CSF pleocytosis and *Bb*-specific intrathecal antibody production. Culture and PCR can provide supporting evidence but are not recommended as routine diagnostic tools because of low sensitivity [5,67]. Analysis of the chemokine CXCL13 in CSF is not recommended in the European guidelines from 2010 and 2011 but the more recently

published Swedish diagnostic guidelines state that it can be useful in patients with early LNB in whom the antibody index is still negative [5,67,89]. In papers II and III the properties of the CSF cell count and CXCL13 were studied and their usefulness as diagnostic tools were assessed and put into perspective.

CSF cell count

The CSF cell count is the most important analysis in the diagnosis of LNB. In European guidelines, both from EFNS and EUCALB, as well as in Swedish guidelines, demonstration of CSF pleocytosis is the central criterion in establishing the diagnosis of LNB [5,67,89]. CSF cell counts have been done manually in more or less the same way since the first LP was performed more than 100 years ago [157]. Manual cell counts are labor intensive and require trained laboratory personnel on duty 24 hours a day, which can be challenging for small hospitals. The results are also prone to inter- and intra-operator variability and the coefficient of variation (CV) is, at or above 20%, higher than for most other laboratory tests [158]. The recently introduced automatic cell counters have the advantage of shorter turn-around-times, lower costs and possibly also better precision [159,160]. They also provide more detailed results as cells specified only as mononuclear leukocytes in manual counts are further separated into lymphocytes and monocytes. However, with the new method, several questions arise. The first is what references ranges to use. Reference ranges used for manual counts are not automatically transferrable to automatic counts and their origin is furthermore somewhat obscure. As the pleocytosis in LNB is typically mild to moderate using a too low or too high cut-off value could result in over- or underdiagnosis. Another question that arises is if the additional detail of the results can be of clinical value. These two questions were addressed in paper III. CSF samples from 80 neurologically healthy persons were collected. Using this material, one of the largest set of CSF samples from neurologically healthy persons compiled for decades, new reference ranges were determined for granulocytes, < 3 cells/μL, lymphocytes, < 4 cells/μL, and monocytes, < 3 cells/μL. These new reference ranges are now in use at the Sahlgrenska University Hospital. On the second question, of the potential clinical usefulness of the additional detail provided by the automatic counters by the separation of mononuclear cells into lymphocytes and monocytes, the results of paper III show that this information is likely of limited use in discriminating between LNB and viral CNS infections. Both LNB and viral CNS infections present with mild to moderate mononuclear pleocytosis and moderately elevated CSF albumin and in some cases overlapping symptoms [99,100,161,162]. In two previous

smaller studies, one (Shah et al.) found a significantly higher percentage of granulocytes in enteroviral meningitis than in LNB, and one (Cepok et al.) found significantly higher percentage of monocytes in viral meningitis than in LNB [98,100]. Unfortunately, none of these findings could be verified in the larger patient material of paper III, where 39 patients with LNB were compared with 60 patients with viral CNS infection. There were no significant differences between the groups for neither granulocytes nor lymphocytes nor monocytes.

Even though guidelines require the demonstration of elevated CSF cell count for the diagnosis of LNB, the question whether LNB can present without CNS pleocytosis often arises. LNB without pleocytosis is considered rare and to occur primarily in very early LNB but the frequency with which it occurs is unknown as is the maximum time interval from onset of symptoms to rise in CSF cell count [5,67]. There are indications that pleocytosis might be less common in LNB caused by *B. afzelii*. In a report from 2006, Strle and co-workers present data on 33 patients in whom *Bb* had been isolated from CSF. Pleocytosis was seen in 19/23 patients infected with *B. garinii* but only in 2/10 patients infected with *B. afzelii* [96]. However, these results have yet to be repeated and have not so far influenced LNB diagnostic guidelines.

In addition to its use in the diagnosis of LNB, CSF cell count is also used as a surrogate marker of treatment effect. The customary definition of a surrogate marker is a validated laboratory measurement or physical sign that is used as a substitute for a clinically meaningful endpoint. However, very few laboratory tests used as surrogate markers fulfill the requirement of being properly validated [163]. The ultimate endpoint in the treatment of a bacterial nervous system infection would be the sterilization of CSF but as previously described, CSF culture of *Bb* has too low sensitivity to be clinically useful [39]. The clinically relevant endpoint used in prospective studies is the reduction of symptoms, but in retrospective studies data on clinical symptoms is less reliable [101]. In LNB, there are no studies that have explicitly studied the relationship between reduction of symptoms and decrease in CSF cell count. Nevertheless, the decrease in CSF cell count after initiation of treatment is well recognized and there are no reports of persistent *Bb* infection in patients with normalized CSF cell count [72]. In the large majority of studies on the treatment of LNB, decrease in CSF cell count is used as a primary or secondary endpoint [101,102,137].

CXCL13

Since the discovery in 2005 of highly elevated CSF levels of CXCL13 in LNB, a considerable number of articles have been published on the possible role of CXCL13 both in the diagnosis of LNB and as a marker of treatment effect [119]. As it is not so far recommended in international guidelines, its use in clinical practice is limited and relatively few laboratories yet analyze CXCL13 routinely. In paper II, the differential diagnostic potential of CXCL13 was studied, as was its use as a marker of treatment effect.

Table 2. CXCL13 in CSF in studies published to date (including paper III) with suggested cut-off values. [a]Median of the combined group of LNB-patients from the longitudinal and the cross-sectional part of the study. [b]Mean instead of median. [c]Samples with CXCL13 ≥ 500 pg/mL were not further diluted to obtain a final value; actual medians were likely > 500 pg/mL [120,122,123,166-168].

Study	Population	Number of patients	Median CXCL13 (pg/mL)	Suggested CXCL13 cut-off (pg/mL)
Cerar et al. (2013)	adult	46	113	18.9
Sillanpää et al. (2013)	pediatric	24	3485	103
Bremell et al. (2013)	adult/pediatric	41	2297[a]	61
van Burgel et al. (2011)	adult/pediatric	58	1183	250
Schmidt et al. (2011)	adult	17	15 149[b]	1229
Wutte et al. (2011)	adult/pediatric	22	500[c]	not specified
Tjernberg et al. (2010)	adult/pediatric	124	500[c]	142

The weak link in the diagnosis of LNB is the delay from onset of symptoms to the time when intrathecal antibody production can be detected; it is primarily in this time period that analysis of CXCL13 can provide additional information [122]. For the test to be clinically relevant in these situations, it needs to be both highly sensitive and highly specific. In most published studies, the sensitivity of CXLC13 in detecting definite LNB is above 90-95% [121,122,164]. However, in every study, the authors have defined their own cut-off for CXCL13 to discriminate between LNB and other diagnoses. In table 2, median CXCL13 in patients with LNB as well as suggested cut-offs in published studies are presented. Early studies are not included as they used the CXCL13 to CSF protein ratio instead of the absolute CXCL13 concentration. As can be seen, both

median and cut-off values varies between studies by two orders of magnitude. In paper II, the implications of these differences are clearly illustrated, for example if the cut-off of 1229 pg/mL suggested by Schmidt and co-workers had been used in the patients in paper II, the sensitivity would have been 56% [122]. All studies presented in table 2 used the same commercial ELISA kit for CXCL13 determination (Human CXCL13/BLC/BCA-1 Quantikine ELISA kit, R&D Systems, Minneapolis, USA) why the differences cannot be attributed to the specific method. Intra-assay and especially inter-laboratory differences are however, well-known problems of novel biomarkers [165].

The specificity of CXCL13 in diagnosing LNB in published studies is even higher, often above 96% [120-122]. The compositions of the control groups in the studies vary considerably. Some use patients without CSF pleocytosis in whom LNB has been excluded and some use disparate groups of patients with various infectious and non-infectious CNS diseases [120-122,164]. In paper II, neurologically healthy persons and patients with asymptomatic HIV infection were included as control groups. The rationale behind the inclusion of HIV patients was that elevated serum levels of CXCL13 are reported in HIV, and that moderate mononuclear CSF pleocytosis is fairly common in HIV patients [169,170]. The elevated CSF CXCL13 levels found HIV patients and the considerable overlap in CXCL13 levels between patients with LNB and patients with HIV add to the doubts that CXCL13 is as specific for LNB as it is sometimes claimed. In addition, the problem with determining a universally accepted cut-off obviously affects the specificity of CXCL13 as much as the sensitivity. The overall problem with the use of CXCL13 in diagnosing LNB is common to that of most biomarkers, that in the relevant population, the true diagnostic accuracy is impossible to accurately assess. There is no gold standard for the diagnosis of LNB and in patients with suspected early LNB without intrathecal antibody production for whom analysis of CXCL13 could be of help, there is no way of definitely determining if they have LNB or not.

However, even with all the shortcomings of CXCL13 described here, the analysis could still provide supportive information in the 25-50% of patients with early LNB in whom intrathecal antibody production is not yet detectable and in whom PCR is negative or unavailable [106,112]. As long as each laboratory designates its own cut-off and ensures that an elevated CXCL13 value is not automatically interpreted as a proof of LNB, it is reasonable to analyze CXCL13 in selected cases.

As a marker of treatment effect, the case for CXCL13 is weaker. Senel and co-workers describe a rapid decrease in CXCL13 after treatment initiation, even more rapid than the decrease in CSF cell count, but three weeks after start of treatment the relative decrease in the two parameters was not significantly different [121]. As have been highlighted in the previous section, it is difficult to develop surrogate markers for treatment effect. For CSF cell count, at least, there is substantial clinical experience of its use as a marker of treatment effect in LNB while for CXCL13 the experience is very limited. In paper II, it is shown that both CXCL13 and CSF cell counts decrease significantly after treatment of LNB and that the decrease in the two parameters correlates. Taken together, as a marker of treatment effect, analysis of CXCL13 cannot be recommended as it gives no further information than the CSF cell count.

A third potential role for CXCL13 could be in helping diagnosing the rare cases of LNB that present without pleocytosis, either being very early cases, or as described above, possibly cases caused by *B. afzelii* [96]. There are no published reports on elevated CXCL13 levels in LNB patients without pleocytosis. In unpublished data on one patient presenting with very early LNB without pleocytosis and in whom the diagnosis was made several weeks later after renewed CSF sampling showed CSF pleocytosis, there was no increase in CXCL13 in the initial CSF sample (Bremell, unpublished data).

5.1.3 Diagnostic flow chart

Based on the arguments presented mainly in the section on laboratory diagnosis, an algorithm for the diagnosis of LNB is presented as a flow chart (figure 6). The flow chart shows that with the analysis of CSF cell count, *Bb* antibodies in serum and CSF, and in selected cases, CSF CXCL13, the diagnosis of LNB can be established or excluded fairly accurately in the large majority of cases.

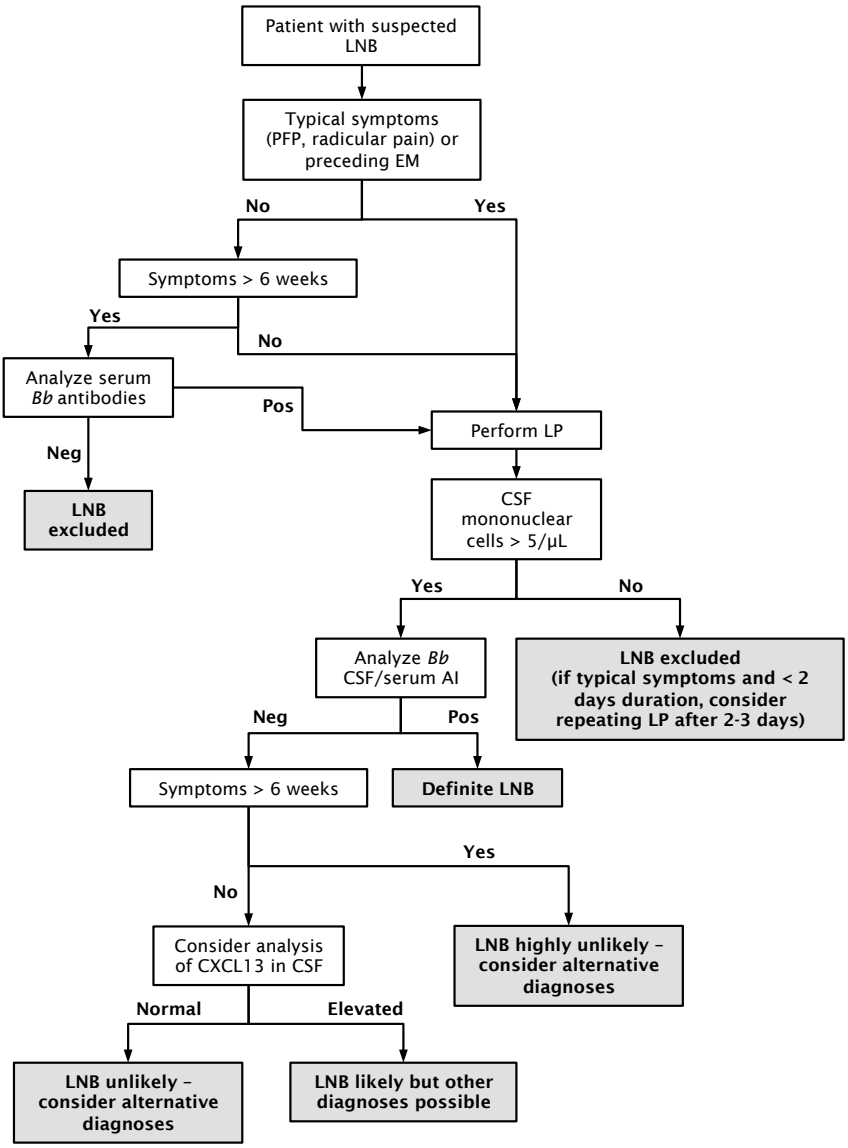

Figure 4. Flow chart for the diagnosis of LNB. Cut-off for CSF mononuclear cells is dependent on the method used; for automatic cell counts the cut-off for lymphocytes is > 4 cells/µL. For CXCL13, discrimination between normal and elevated values is at the discretion of the analyzing laboratory.

5.2 Treatment of Lyme neuroborreliosis

As described in section 1.10, in everyday clinical practice, intravenous ceftriaxone and oral doxycycline are the two treatment options for LNB. By tradition, intravenous treatment has been preferred in America, while European doctors were earlier to adopt oral treatment with doxycycline. In Sweden oral doxycycline has since long been the standard treatment for all manifestations of LNB and is recommended in the current national guidelines [140]. At the Department of Infectious Diseases, Sahlgrenska University Hospital, the dosing regimen for oral doxycycline has, for more than 25 years, been 400 mg daily for 10 days. This dose is based on studies showing that CSF concentrations of doxycycline in patients treated with 400 mg daily were more likely to exceed MIC for *Bb* than were CSF concentrations in patients treated with 200 mg daily [133]. In addition, the long half-life of doxycycline means that it takes several days to reach targeted steady-state levels; this time can be shortened with a higher dose [171]. It should be noted though, that the higher dose have not been shown to be clinically superior and treatment failures are exceedingly rare with both dosing regimens. Internationally, the trend has been towards increased use of oral doxycycline but intravenous ceftriaxone is still the recommended treatment for LNB with symptoms indicating CNS infection [46,67,81].

In paper IV, data on the treatment of LNB with CNS symptoms with oral doxycycline is presented. The number of patients with CNS symptoms is, at 26, the largest material published to date on the treatment of LNB with CNS symptoms. The distinction between LNB with PNS and CNS symptoms is not always clear-cut as been described previously. In paper IV, patients with mild or unspecific cognitive symptoms such as fatigue or perceived memory impairment were not classified as CNS LNB. Even with these stricter criteria though, in the absence of radiologic or electrophysiological findings, it was impossible to completely prove actual parenchymal involvement in the patients classified as LNB encephalitis.

All patients improved on treatment and none were considered treatment failure. Although the proportion of patients with remaining symptoms at the end of follow-up was, at 62%, somewhat higher than is commonly reported, only a minority had severe remaining symptoms [144]. Compared with LNB with only PNS symptoms, it is reasonable to assume that LNB with CNS symptoms has a somewhat less favorable prognosis. Decrease in CSF cell count was used as a marker of treatment effect as

previously described. The decrease was highly significant. When compared to the control group consisting of 115 patients with LNB with PNS symptoms, the slopes of the regression lines did not differ. The findings in paper IV strongly support the Swedish therapeutic tradition of oral doxycycline for all manifestations of LNB. Given the rarity of LNB with CNS symptoms, it is unlikely that a randomized controlled trial comparing oral doxycycline with intravenous ceftriaxone for this indication will be performed and treatment recommendations therefore will have to be based on sufficiently large retrospective studies such as the one in paper IV.

6 CONCLUSIONS

- In an area endemic for *B. burgdorferi*, onset of symptom in late summer - early fall, associated neurological symptoms and CSF mononuclear pleocytosis are predictive factors discriminating Lyme neuroborreliosis from Bell's palsy as the cause of peripheral facial palsy.

- Levels of CSF CXCL13 are highly elevated in LNB and decrease after treatment. The decrease is significantly correlated to the decrease in CSF mononuclear cells. CSF levels of CXCL13 in LNB overlap those in asymptomatic HIV infection. The diagnostic value of CXCL13 in LNB remains to be established.

- For CSF samples analyzed with automatic cell counters, new reference ranges are suggested: < 4 cells/μL for lymphocytes, < 3 cells/μL for monocytes and < 3 cells/μL for granulocytes. The differentiation of mononuclear cells into lymphocytes and monocytes is of limited value in the discrimination between LNB and viral CNS infections.

- Treatment with oral doxycycline results in a similar decrease in CSF mononuclear cell counts in patients with LNB with CNS symptoms compared with patients with LNB with PNS symptoms. All patients with LNB with CNS symptoms show clinical improvement on treatment with oral doxycycline with no need for retreatment. Oral doxycycline can be considered an effective treatment for Lyme neuroborreliosis, irrespective of the severity of symptoms.

7 FUTURE PERSPECTIVES

As has been shown in this thesis, the knowledge of all aspects of Lyme borreliosis and Lyme neuroborreliosis have been growing rapidly since the spirochetal etiology of the disease was first discovered 33 years ago. However, there are still many areas in which knowledge is sketchy, the lack of understanding of how *Bb* makes it way from the site of the tick-bite to the nervous system being an obvious candidate for more research [59]. The disturbing finding that LNB caused by *B. afzelii* may present without CSF pleocytosis also needs to be investigated further, as detection of CSF pleocytosis is the basis for laboratory diagnosis of LNB [96].

In clinical practice, the need for improved diagnostic methods is the most obvious area of improvement. A method of diagnosing LNB without the need for CSF sampling would have been desirable. As there is no other nervous system infection that can be reliably diagnosed solely from blood tests, such a method is unlikely to be available in the near future. As for the possible improvements in the methods currently in use, it is reasonable to assume that the sensitivity of PCR for the detection of *Bb* can be significantly improved, hopefully to the point where the analysis can be implemented in the routine investigation of LNB. PCR methods for the detection of other infectious agents are constantly improving and some of the new methods might be adapted for *Bb*. As for culture, the slow-growing nature of Bb makes cultures unsuitable for routine diagnosis even if the yield could be significantly improved from today's low levels. Analysis of CSF CXCL13 can already be of help in the diagnosis of LNB but more research on larger patient materials is needed to establish generally accepted cut-offs.

In the area of LNB treatment, there are no ongoing trials of new antibiotics or radically different dosing regimes with existing antibiotics registered in clinical trial databases. As the proportion of patients with remaining symptoms after treatment is reported to be 19-70%, there is however, room for improvement [144]. In other nervous system infections, addition of corticosteroids to antibiotic therapy has been shown to improve outcome, for example in bacterial or tuberculous meningitis [97]. It has also been shown that treatment with corticosteroids improve the cure rate in Bell's palsy [172]. This is the basis for an ongoing study at the Department of Infectious Diseases, Sahlgrenska University Hospital, on patients with peripheral facial palsy caused by LNB who receive high-dose corticosteroid therapy in addition to standard therapy

with doxycycline. If the results of this study are positive, the logical next step will be studies on adjunctive corticosteroid therapy for all manifestations of LNB.

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