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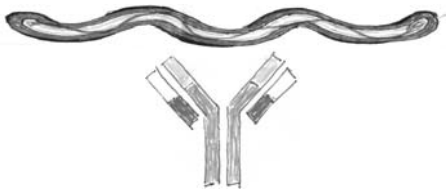
Laboratory Diagnosis of Lyme Borreliosis

Anti-*Borrelia* Antibodies and the Chemokine CXCL13

Ivar Tjernberg



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Cover illustration: Gunnar Tjernberg, displaying a Lyme borreliosis spirochete with flagella and an IgG antibody.

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CONTENTS

ABSTRACT

Lyme borreliosis (LB), the most common tick-borne disease in Europe and North America, is caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex. The spirochetes can invade several different organs, thereby causing many different symptoms and signs. Diagnosis of LB relies on patient history, physical examination, and detection of anti-*Borrelia* antibodies. However, anti-*Borrelia* antibodies are not always detectable, and they commonly persist even after LB is successfully treated or spontaneously healed.

The aim of my work was to study diagnostic aspects on clinical cases of LB and control subjects in an area endemic to LB, with a focus on newly developed anti-*Borrelia* antibody tests. A total of 617 patients with symptoms and/or signs consistent with LB, as well as 255 control subjects, were studied. The diagnostic panel included the following new LB tests: Immunetics Quick ELISA C6 *Borrelia* assay kit (C6), invariable region 6 peptide antibody assays (IR6), Liaison *Borrelia* CLIA (Li) and the chemokine CXCL13. Results were compared with the older Virotech *Borrelia burgdorferi* ELISA (VT) and with a Western blot method, the Virotech *Borrelia* Ecoline IgG/IgM Line Immunoblot (WB EL), when appropriate.

In general, no significant differences were noted between the C6, VT and Li tests regarding serosensitivity in various LB manifestations. However, the seropositivity rate was lower for the C6 test compared with the VT and Li tests 2–3 and 6 months after diagnosis of erythema migrans (EM), indicating normalization of antibody levels. In addition, EM patients reporting a previous LB episode had a C6 seropositivity rate similar to that of patients without a previous LB episode, and seroprevalence in healthy blood donors was lower in the C6 test than the VT and Li tests. Taken together, these results support the recommendation of the serum C6 test as a *Borrelia* serological test due to its ability to reflect ongoing or recent infection.

Although the majority of EM patients at presentation showed concordant serological responses to IR6 peptides representing the three main *Borrelia* species and the C6 peptide, there were also clinical EM cases that were C6-negative and could be detected mainly by a seroresponse to a *B. burgdorferi* sensu stricto-derived IR6 peptide. Thus, an antibody test combining antigens could be of value in the serodiagnosis of LB in Europe.

The serosensitivity of the C6 test in cases of Lyme neuroborreliosis (LNB) was shown to be associated with symptom duration. A serosensitivity rate of 93% was found in LNB patients ≥ 12 years of age with a symptom duration of more

than 30 days. Therefore, a negative C6 test in serum in such a patient argues against an LNB diagnosis.

The presence of chemokine CXCL13 in cerebrospinal fluid was confirmed to be a reliable marker of LNB. CXCL13 differentiated LNB from other conditions and also indicated a high probability of LNB in children with short symptom duration where anti-*Borrelia* antibodies were still lacking in the cerebrospinal fluid.

A two-tiered approach (C6 test in combination with WB EL) showed no significant improvement in specificity over the C6 test alone. However, WB EL may be useful in diagnosing suspected cases of *acrodermatitis chronicum atrophicans* and Lyme arthritis, usually displaying multiple IgG bands.

In conclusion, although the serodiagnosis of LB remains to be settled, this thesis provides some practical tools regarding the use and interpretation of *Borrelia* serology including proposed diagnostic routines.

SAMMANFATTNING PÅ SVENSKA

Borrelios är den vanligaste fästingburna infektionen i Europa och Nordamerika. Infektionen orsakas av spiralformade bakterier med samlingsnamnet *Borrelia burgdorferi* sensu lato. Dessa bakterier kan invadera många olika organ och därmed ge upphov till många olika symptom. Vid lokal hudinfektion, så kallat erythema migrans, baseras diagnostiken på sjukhistoria och typiska fynd vid kroppsundersökning. Vid övriga tillstånd utgör påvisning av borreliaantikroppar ett komplement för att ställa korrekt diagnos. Borreliaantikroppar kan dock inte alltid detekteras, särskilt tidigt i förloppet. Borreliaantikroppar kan dessutom ofta påvisas under lång tid även efter framgångsrikt behandlad eller spontanläkt infektion och det kan också förekomma falskt positiva reaktioner vid andra sjukdomstillstånd.

Syftet med avhandlingsarbetet var att undersöka nyttan av nya borreliatester hos patienter med olika former av borrelios och olika kontrollgrupper i ett område med hög förekomst av borrelios. Totalt studerades 617 patienter med symtom förenliga med borrelios samt 255 kontrollpersoner. Följande borreliatester användes: De antikroppsdetekterande testerna Immunetics Quick ELISA C6 *Borrelia* assay kit (C6), ELISA baserad på "invariable region 6 peptider" (IR6), Liaison *Borrelia* CLIA (Li) och för jämförelse även Virotech *Borrelia burgdorferi* ELISA (VT) samt Virotech *Borrelia* Ecoline IgG/IgM Line Immunoblot (western blot) i tillämpliga fall. Vidare gjordes koncentrationsbestämning av det immuncellsattraherande signalämnet, kemokin CXCL13, hos patienter med misstänkt borreliainfektion i nervsystemet (neuroborrelios).

C6-, VT- och Li-testerna var lika effektiva att upptäcka antikroppar hos patienter med borrelios. Däremot var C6-testet bättre på att påvisa sjunkande nivåer efter en infektion eftersom andelen positiva i C6-testen var lägre jämfört med VT- och Li-testerna i uppföljande prover 2-3 och 6 månader efter erythema migrans. Detta fynd stöds av att de som tidigare haft borrelios hade samma frekvens av positivt test med C6-metoden jämfört med de som inte haft borrelios tidigare. Dessutom var andelen friska blodgivare med borreliaantikroppar lägst med C6-testen jämfört med VT- och Li-testerna. Sammantaget lämpar sig därmed C6-testet som borreliaantikroppstest, framför allt baserat på dess förmåga att bättre påvisa aktuell infektion.

Antikroppssvaret vid erythema migrans mot olika IR6-peptider och C6 visade sig vara övervägande samstämmigt, men det fanns C6-negativa fall där ett antikroppssvar mot en av IR6-peptidtesterna kunde påvisas. Möjligen kan därför en kombination av olika antigen vara av nytta för att höja känsligheten i framtida borreliantikroppstester.

Hos patienter med neuroborrelios visade det sig att andelen med positivt C6-test i blodet ökade ju längre patienterna haft symtom. Nittiotre procent av patienter med symtom i mer än 30 dagar var positiva med C6-testet. Detta gällde dock inte barn under 12 år. Hos en vuxen patient som haft symtom passande med neuroborrelios i mer än 30 dagar talar alltså ett negativt C6-test emot diagnosen.

Kemokinet CXCL13 i spinalvätska visade sig vara en pålitlig markör för neuroborrelios, även hos barn med kort sjukhistoria där befintliga antikroppstester inte hunnit bli positiva.

Någon säkert minskad andel falskt positiva borreliaantikroppsresultat kunde inte påvisas med ett internationellt rekommenderat två-stegsförfarande som inkluderar metoden western blot vid jämförelse med C6-testet ensamt. Däremot kan western blot fylla en diagnostisk funktion vid borrelia-orsakade tillstånd i hud (akrodermatit) och leder (borreliaartrit).

Avslutningsvis är laboratoriediagnostiken av borreliainfektion fortsatt svår, men denna avhandling har visat på vilka sätt borreliaserologi kan användas, bland annat i en föreslagen diagnostik rutin.

LIST OF PAPERS

I. **I Tjernberg,** G Krüger, I Eliasson: C6 peptide ELISA test in the serodiagnosis of Lyme borreliosis in Sweden, Eur J Clin Micorbiol Infect Dis. 2007 Jan. 26(1):37-42.

II. **I Tjernberg,** T Schön, J Ernerudh, AC Wistedt, P Forsberg, I Eliasson: C6-peptide serology as diagnostic tool in neuroborreliosis, APMIS. 2008 May. 116(5):393-9.

III. **I Tjernberg,** H Sillanpää, I Seppälä, I Eliasson, P Forsberg, P Lahdenne: Antibody responses to borrelia IR(6) peptide variants and the C6 peptide in Swedish patients with erythema migrans, Int J Med Microbiol. 2009 Aug. 299(6):439-46.

IV. **I Tjernberg,** AJ Henningsson, I Eliasson, P Forsberg, J Ernerudh: Diagnostic performance of cerebrospinal fluid chemokine CXCL13 and antibodies to the C6-peptide in Lyme neuroborreliosis. J Infect. 2011 Feb. 62(2):149-58.

ABBREVIATIONS

ABA	anti-*Borrelia* antibodies (including AI and ITA)
ACA	*acrodermatitis chronicum atrophicans*
AI	specific anti-*Borrelia* antibody index
AUC	area under curve
A-V	atrioventricular
B.b. s.l.	*Borrelia burgdorferi* sensu lato
B.b. s.s.	*Borrelia burgdorferi* sensu stricto
BL	Borrelial lymphocytoma
C6 Imm.	Immunetics Quick ELISA C6 *Borrelia* assay, manufacturer cut-off
C6 IH	Immunetics Quick ELISA C6 *Borrelia* assay, in-house cut-off
CDC	Centers for Disease Control and Prevention
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
EBV	Epstein-Barr virus
EL	EcoLine Virotech *Borrelia* immunoblot
ELISA	enzyme-linked immunosorbent assay
EM	erythema migrans
ESR	erythrocyte sedimentation rate
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IR6	invariable region 6 peptide
ITA	intrathecal anti-*Borrelia* antibodies
LA	Lyme arthritis
LB	Lyme borreliosis
LC	Lyme carditis
Li	Diasorin Liaison *Borrelia* chemiluminescence assay
LNB	Lyme neuroborreliosis
n.d.	not determined
n.s.	not significant
OD	optical density
Osp	outer surface protein

PCR	polymerase chain reaction
PcV	phenoxylmethyl penicillin
RF	rheumatoid factor
ROC	receiver operating characteristic
VlsE	variable protein-like sequence, expressed
VT	Virotech *Borrelia burgdorferi* ELISA
WB	Western blot

INTRODUCTION

Lyme borreliosis

Lyme borreliosis (LB) is the most common tick-borne disease in both Europe and North America. The disease is caused by spirochetes of the *Borrelia burgdorferi* (*B. burgdorferi*) sensu lato (s.l.) complex. In Europe hard ticks, mainly *Ixodes ricinus*, can transmit the bacteria to humans, in whom infection may present different symptoms depending on which organs are affected. The most common manifestation of LB is erythema migrans (EM), a localised skin condition at the site of the tick bite. In addition, spirochetes may also disseminate to other organs and sites of the body such as the nervous system, joints and heart (Berglund et al., 1995, Stanek and Strle, 2003, Wormser et al., 2006).

Historical notes

The first known article describing a manifestation of LB was published in 1883 by the German physician Alfred Buchwald, who described a case of diffuse idiopathic skin atrophy (Buchwald, 1883). This chronic cutaneous manifestation of LB was later named *acrodermatitis chronica atrophicans*, or ACA (Herxheimer and Hartman, 1902). A few years later a Swedish dermatologist described another LB manifestation, EM (Afzelius, 1910), also a manifestation on the skin, described as an annular, red skin lesion associated with a tick bite. The third type of cutaneous manifestation, lymphocytoma, most often found on the earlobe or the nipple, was described first in 1911 (Burckhardt, 1911) and thereafter in 1943, when a Swedish dermatologist coined the term *lymphadenosis benigna cutis*, encompassing both borrelial and nonborrelial types of benign skin hyperplasia (Bäfverstedt, 1943).

In 1922 the French physicians Garin and Bujadoux reported neurologic symptoms associated with tick bites, and in 1941 Bannwarth described the classical triad of lymphocytic meningitis, cranial nerve palsy and radiculoneuritis (Garin and Bujadoux, 1922, Bannwarth, 1941). Regarding treatment, Thyresson, a Swedish dermatologist, described successful treatment of ACA with penicillin in 1949, and in 1951 more evidence for curative treatment of EM and meningitis was presented (Hellerström, 1951, Hollström, 1951).

Many years later Allen Steere investigated clustered cases of arthritis among children and young adults in Lyme, Connecticut, USA, and described yet another manifestation of LB, namely Lyme arthritis, or LA (Steere et al., 1977). Subsequently, Lyme carditis (LC) was also described as a manifestation of LB among young adults with fluctuating degrees of atrioventricular (A-V) block (Steere et al., 1980).

Although spirochetal etiology had been suggested early, for instance by Carl Lennhoff in 1948 (Lennhoff, 1948), it was not until 1982 that William Burgdorfer finally identified spirochetes in ticks as the causative agent of LB (Burgdorfer et al., 1982). Two years later the spirochetes were named *B. burgdorferi*, after the discoverer (Johnson et al., 1984).

Hard ticks are vectors of Lyme borreliosis

In Europe the principal vector of *Borrelia* spirochetes is *Ixodes ricinus*, and in North America the main vectors are *Ixodes scapularis* and *Ixodes pacificus* (Piesman and Gern, 2004, Masuzawa, 2004).

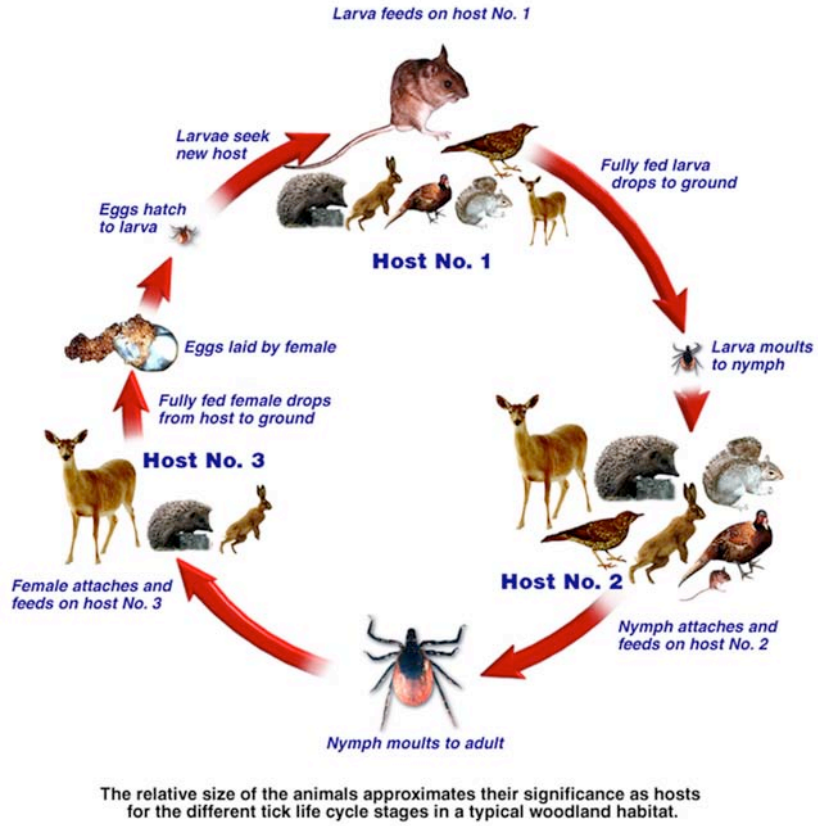
The life cycle of *Ixodes ricinus* involves four stages: egg, larva, nymph and adult. A blood meal is required for the tick to develop into the next stage in the life cycle. Usually, the tick stays attached to the host for several days during a blood meal. As the bites most often are painless, they may go unnoticed for lengthy periods of time. These attributes contribute to the vector potential of the tick (Parola and Raoult, 2001). Many ticks lack eyes, and even when eyes are present, it is doubtful that they enable a detailed perception of the environment. However, ticks have a variety of sensory organs that enable ticks to locate their hosts. One common host-seeking behaviour pattern of *Ixodes ricinus* is to climb up vegetation and wait for passing hosts, holding their front legs out in the manner of insect antenna. Tick larvae often feed on small hosts such as birds, rodents, lizards and hedgehogs, while nymphs and adult ticks more often take their blood meals from bigger mammals such as roe deer and humans (Figure 1). Once replete, the tick detaches from the host and finds a place to digest the blood meal in order to moult to the next feeding sage; alternatively, it may enter diapause, as in winter - a state characterized by reduced metabolism and delayed development (Parola and Raoult, 2001). *Ixodes ricinus* ticks are widely distributed throughout Europe, implying that this tick can survive various environmental conditions. However, *Ixodes ricinus* is sensitive to desiccation, especially when seeking hosts; a humidity of at least 80% is a prerequisite for tick survival (Kahl and Knulle, 1988, Randolph et al., 2000). After the winter, ticks become active when air temperature exceeds 4-6°C (Duffy and Campbell, 1994).

The life cycle of a tick may vary from six months to six years, depending on environmental conditions, but usually takes approximately three years, as is also the case in southern Scandinavia.

Although roe deer are common and important hosts for ticks, they seem incapable of infecting ticks with *B. burgdorferi*, thereby failing to act as reservoir hosts: instead, rodents, smaller mammals and birds act as reservoir hosts (Jaenson and Talleklint, 1992, Talleklint and Jaenson, 1993, Gern et al., 1998, Olsen et al., 1993). After feeding on a reservoir host spirochetes and/or other pathogens may persist in the tick until the next blood meal, when pathogens may be transmitted to the next host. In the case of *B. burgdorferi*, it has been reported that increased duration of tick attachment increases the risk of transmission to the host. In an experimental animal model, spirochetes were transmitted to 1 of 14 rodents within 24 hours and to 13 of 14 rodents after at least 72 hours (Piesman

et al., 1987). Different *Borrelia* genospecies may also affect the time for spirochetes to transfer from the vector to the host (Crippa et al., 2002).

Figure 1. The tick life cycle, printed with permission from Jeremy Gray (EUCALB, 2009).



The spirochete complex *Borrelia burgdorferi* sensu lato

The genus *Borrelia* belongs to the family *Spirochaetaceae* in the order Spirochaetales. This family also includes *Treponema pallidum*, the causing agent of syphilis (Rosa, 1997, Tilly et al., 2008). The term *B. burgdorferi* s.l. includes *Borrelia* genospecies within the same complex. To date at least 15 different *Borrelia* genospecies have been identified within the complex (Margos et al., 2009, Rudenko et al., 2009a). The spirochetes of the complex are Gram-negative, supposedly mainly extracellular with a helical shape, and measure 10-30 μm in length and 0.2-0.5 μm in width (Rosa, 1997, Pal and Fikrig, 2003, Ma et al., 1991). *Borrelia* consists of a protoplasmic cylinder containing cytoplasm and a linear chromosome as well as a number of linear and circular plasmids (Fraser et al., 1997, Casjens et al., 2000, Tilly et al., 2008). The protoplasmic cylinder is surrounded by a periplasmic space with 7 to 11 flagella that are attached

subterminally to the protoplasmic cylinder. The flagella are arranged parallel to the long axis of the cell and they wound around the cylinder. The flagellar apparatus is a motility mechanism of the spirochete that propels the bacteria forward by propagating flat waves. Thanks to the flagella LB spirochetes are highly mobile and able to penetrate through various tissues (Hovind-Hougen, 1984, Rosa, 1997, Sal et al., 2008). The main structural component of the flagella is flagellin, a 41-kDa protein. Native purified flagellum has been used since the 1990s as an antigen in LB serological tests (Hansen et al., 1988, Karlsson, 1990). Finally, a trilaminar outer surface membrane surrounds the periplasmic space (Rosa, 1997). This outer surface membrane contains a number of abundant lipoproteins such as outer surface proteins (Osp) OspA, OspB, OspC, and variable major protein-like sequence, expressed (VlsE) (Rosa, 1997, Eicken et al., 2002). Together, OspE, OspF and the outer surface E/F-like leader peptide paralogues constitute OpsE/F-related proteins (Erps). Osps seem to interact with cellular and interstitial components of the tick and the mammalian tissue (Singh and Girschick, 2004).

In Europe, several *Borrelia* genospecies have been described, including *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), *B. garinii*, *B. afzelii*, *B. spielmanii*, *B. valaisiana*, *B. lusitaniae*, *B. bavariensis*, *B. bissettii* and a *B. miyamotoi*-like *Borrelia* species (Piesman and Gern, 2004, Fraenkel et al., 2002, Richter et al., 2004, Margos et al., 2009, Rudenko et al., 2009b). In Sweden, the prevalence of *Borrelia*-infected ticks ranges from 3% to 23% (Gustafson et al., 1995, Fraenkel et al., 2002, Wilhelmsson et al., 2010). The prevalence of *Borrelia* has also been shown to be greater in adult ticks than in nymphs (Rauter and Hartung, 2005, Wilhelmsson et al., 2010). Although the prevalence of different identified genospecies varies among different investigations and geographical areas in Europe, *B. afzelii* and *B. garinii* seem to be the most commonly identified genospecies. See Table 1 for the results of two Swedish investigations.

Table 1. Prevalence of *Borrelia* species in *Ixodes ricinus* ticks collected in Sweden.

	Fraenkel et al., 2002	**Wilhelmsson et al., 2010**
Borrelia prevalence in ticks	32/301 (11%)	75/399 (19%)
Frequency of *Borrelia* species		
B. afzelii	44%	61%
B. garinii	31%	23%
B. burgdorferi s.s.	13%	1%
B. valaisiana	6%	13%
B. lusitaniae	0%	1%
B. miyamoto-like	6%	1%

Although several *Borrelia* species have been associated with human LB infection, the three main pathogenic species in Europe are still considered *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s (Rauter and Hartung, 2005, Rudenko et

al., 2009b).
The situation in North America is quite different. The predominant genospecies, and the only one demonstrated to infect humans, is *B. burgdorferi* s.s. carried by *Ixodes scapularis* in northeast North America and by *Ixodes pacificus* in western North America. However, the vast majority of human LB cases in North America are acquired through the bites of *Ixodes scapularis* (Piesman and Gern, 2004). The main vectors and *Borrelia* species related to human infection are summarised in Table 2.

Table 2. The principal vectors and the *Borrelia* genospecies of main human relevance in Europe and North America.

	Europe	**North America**
Ixodes Vector	*Ixodes ricinus*	*Ixodes scapularis* *Ixodes pacificus*
Borrelia species	*B. burgdorferi* s.s. *B. afzelii* *B. garinii*	*B. burgdorferi* s.s.

Various studies have suggested an association between clinical manifestations and *Borrelia* species.
Although all relevant *Borrelia* genospecies seem able to cause all clinical manifestations, there is evidence that the different genospecies are more or less associated with various clinical manifestations (van Dam et al., 1993, Ryffel et al., 1999, Eiffert et al., 1998). For instance, *B. burgdorferi* s.s. is often associated with arthritis, particularly in North America, whereas *B. garinii* is associated with neurological symptoms and *B. afzelii* with skin manifestations of LB (Ohlenbusch et al., 1996, Lunemann et al., 2001, Rauter and Hartung, 2005).

Epidemiology of Lyme borreliosis in Europe

LB is the most common tick-borne disease in the Northern Hemisphere (Stanek and Strle, 2003, Wormser et al., 2006). In an epidemiological study of LB in the south of Sweden, the overall incidence of LB was 69 per 100.000 inhabitants per year (Berglund et al., 1995). However, great variations were noted between counties. The highest annual incidence was noted in the southeastern counties of Blekinge and Kalmar, with 133 to 160 cases per 100.000 inhabitants. Interestingly, the annual incidence of EM in Blekinge has increased rapidly since 1995 and was shown to reach as many as 664 per 100.000 inhabitants in 2000 (Bennet et al., 2006b). In addition, strong positive correlations were found between EM incidence and mean temperature during the summer months, as well as between EM incidence and milder temperatures during the winter (Bennet et al., 2006b).
Surveillance strategy of LB varies throughout Europe; therefore, direct comparison between countries is difficult. Even if LB is diagnosed, there is often a lack of reporting, as only a few countries have made LB a compulsorily notifiable disease. Although this makes it difficult to compare incidence rates among European countries, it appears that disease incidence and antibody prevalence are higher in the central and eastern parts of Europe than in the

western parts. A gradient of decreasing incidence from south to north in Scandinavia and from north to south in Italy, Spain and Greece has also been noted. The highest incidences of LB in northern Europe are found on the Åland Islands, in the Baltic States and Sweden and in central Europe in Austria, the Czech Republic, Germany and Slovenia (Wahlberg et al., 1993, Carlsson et al., 1998, Lindgren and Jaenson, 2006). A low annual LB incidence, 0.32 per 100.000 inhabitants, has been reported in the United Kingdom (Smith et al., 2000).

The risk of a human acquiring LB naturally depends on many factors, such as behaviour, tick abundance in a given season and geographical distribution of ticks in the area, as well as the prevalence of *Borrelia* species in the ticks. As previously described, the duration of the tick bite may also affect the risk of transmission of spirochetes from the tick to the host (Randolph, 2001, Piesman et al., 1987, Stjernberg and Berglund, 2005, Robertson et al., 2000b, Crippa et al., 2002). According to one study, in southeastern Sweden the risk of acquiring LB through a single tick bite is 0.5% (Stjernberg and Berglund, 2002).

As the various *Borrelia* genospecies may be more or less associated with the various LB manifestations, and their relative frequency seems to vary among various European geographical regions, one could suspect that the relative frequency of the different clinical LB manifestations also vary. For instance, in Scandinavia and Slovenia *B. afzelii* is more common than *B. garinii* in ticks, while the opposite seems to be the case in northern and central Germany as well as in Austria and Switzerland (Rauter and Hartung, 2005). EM is by far the most commonly reported manifestation all over Europe. In the south of Sweden 77% of LB cases were EM, and in Slovenia EM represents as much as 90% of registered cases (Dandache and Nadelman, 2008, Berglund et al., 1995, Strle and Stanek, 2009). Despite *B. garinii* having been frequently isolated from ticks in Germany, Lyme neuroborreliosis (LNB) only accounts for 3% of all LB cases in eastern Germany (Rauter and Hartung, 2005, Fulop and Poggensee, 2008). However, in another German survey, LNB was reported in 18.4% of 3935 patients (Priem et al., 2003). In Sweden LNB accounts for 16% of LB cases (Berglund et al., 1995). Case reporting systems may be weak and case definitions quite different across studies. Thus, comparisons between different studies and countries should be made with caution. Recently, European case definitions have been published that may aid in future comparative studies (Stanek et al., 2010). Although one German study showed LA to amount to 24.5% of LB cases, other studies in Europe show considerably lower frequencies of 2-7% (see Table 3). The finding that LA is more common than LNB in Germany was repeated in another smaller study of 313 patients (Huppertz et al., 1999). Again, differences in case definitions may affect these findings. Regarding ACA, borrelial lymphocytoma (BL) and LC, their relative frequencies tend to be below 5% (Priem et al., 2003, Berglund et al., 1995, Strle and Stanek, 2009).

Table 3. Relative frequencies of clinical LB manifestations in Sweden, Germany and Slovenia.

	Berglund et al., 1995	**Priem et al., 2003**	**Strle et al., 2009**
	n=1471	n=3935	n=1020
EM	77%	51%	82%
LNB	16%	18%	9%
LA	7%	24%	3%
ACA	3%	2%	5%
BL	3%	5%	1%
LC	<1%	n.d.	n.d.

n = number of patients studied
n.d. = not determined

There also appear to be differences between the relative frequencies of manifestations in adults and children. BL and LNB manifestations are more common in children than in adults. The physical distribution of reported tick bites also differs. Children more often report tick bites on the head and neck region, while adults more often report tick bites on the lower extremities. An association was also found between tick bites on the head and neck region and LNB. Overall, studies show that LB peaks at 5–9 and 60–74 years of age (Berglund et al., 1995, Strle and Stanek, 2009).

Pathogenesis of Lyme borreliosis

After transferral from the tick to the host, motility plays a major role in the spreading of *Borrelia* spirochetes. Using a technique called intravital microscopy in mice, *Borrelia* spirochetes have been shown to move and disseminate from the microvasculature through a multistage process that includes tethering, dragging, stationary adhesion and extravasation (Moriarty et al., 2008, Norman et al., 2008). However, spirochetes also encounter the immune system of the host. At first, many components of the host's innate immune response, such as the complement system and phagocytic cells at the site of the tick bite, meet the spirochetes. The cells of the innate immune system, such as dendritic cells, monocytes/macrophages and granulocytes, may recognise a microbe through molecular patterns that are specific to microbes and not found in mammalian cells. One important example is Toll-like receptors (TLRs) expressed by cells of the innate immune system that may recognise and bind lipoproteins, gram-negative bacterial lipopolysaccharides and other microbial products. This binding further activates the cell through intracellular signalling pathways, leading to gene transcription and the subsequent expression of co-stimulatory molecules, as well as the secretion of cytokines involved in the ensuing adaptive immune response (Abbas et al., 2007). Regarding *Borrelia* spirochetes, TLR2 has been shown in mice to be key to mammalian recognition of lipoprotein antigens. Mice deficient in TLR2 have increased spirochete loads and ankle swelling (Hirschfeld et al., 1999, Wooten et al., 2002, Wang et al., 2004).

The complement system consists of several plasma proteins that are activated by, for example, microbes and promote the destruction of microbes and inflammation. To avoid damage to normal cells, the activation of the complement system is tightly regulated by several factors, for example, factor H. However, microbes may evade the attack by recruiting these host complement regulatory proteins. Many pathogens, including *Borrelia,* have evolved proteins that facilitate the recruitment of factor H to their cell membranes in the defence against the complement system (Abbas et al., 2007, Ekdahl et al., 2007). *B.burgdorferi* s.l. produces several different outer surface proteins collectively termed complement regulator-acquiring surface proteins (CRASPs). These lipoproteins share affinities for the host fluid phase negative regulators of complement factor H and/or factor H-like protein 1 (FHL-1) (Bykowski et al., 2008). Erp proteins are also able to bind complement inhibitory factor H (Singh and Girschick, 2004).

Not only do spirochetes have to defend themselves against components of the innate immune system, but they must also contend with the adaptive immune system. Humoral immunity mediated by secreted antibodies is the principal protective response to extracellular bacteria and it functions to block infection and eliminate the microbes. Protein antigens of a pathogen may be presented on the surface of antigen-presenting cells to immature T lymphocytes. Under the right conditions these T lymphocytes are activated into mature T-helper (Th) and T-cytotoxic lymphocytes. Th lymphocytes may further help pathogen-specific B lymphocytes to mature into plasma cells able to secrete large amounts of pathogen-specific antibodies of IgG-class. These antibodies in turn activate complement and stimulate phagocytosis of bacteria by granulocytes and macrophages. Several of these complicated processes involve the signalling of various cytokines (Abbas et al., 2007).

Chemokines are a large family of structurally homologous cytokines that stimulate leukocyte movement and regulate the migration of leukocytes from the blood to the tissues. Chemokines are produced by leukocytes and by several types of tissue cells, such as endothelial cells, epithelial cells and fibroblasts. Chemokines are important in recruiting cells of the host defence to sites of infection (Abbas et al., 2007). In the case of LB, high levels of the T lymphocyte attractant chemokines CXCL9 and CXCL10 have been established in EM and ACA (Mullegger et al., 2007). In the case of BL and LNB, on the other hand, the B lymphocyte attractant chemokine CXCL13 has been found (Mullegger et al., 2007, Rupprecht et al., 2005). Data suggests that CXCL13 plays a key role in B cell migration to the cerebrospinal fluid (CSF) in LNB patients (Rupprecht et al., 2009). A major purpose of these T and B lymphocyte responses in LB seems to be to promote antibody production, since the opsonisation of spirochetes with antibodies results in more effective killing of spirochetes (Montgomery et al., 2002). In addition, the involvement of cytotoxic effector mechanisms has been suggested (Ekerfelt et al., 2003). The differences in chemokine signatures among various manifestations of LB may be explained by the differences in tissue

tropism among the *B. burgdorferi* s.l strains (Mullegger et al., 2007, Rupprecht et al., 2005, Rupprecht et al., 2009).

Borrelia spirochetes also use strategies such as antigenic variation to avoid destruction by the immune system. The adaptive immune system of infected vertebrates mounts an immune response against the original infecting serotype, but this specific response may be ineffective against newly emerging variants (Barbour and Restrepo, 2000). One mechanism that could contribute to the survival of the spirochete is the recombination at the variable major protein-like sequence (vls) gene locus (Zhang et al., 1997, Anguita et al., 2001). The vls gene cluster consists of a single vlsE (vls expression site) and 15 silent vls cassettes in *B. burgdorferi* s.s. strain B31 (Anguita et al., 2001, Wang et al., 2001). It has been postulated that infection induces sequence changes and thus alters the antigenic properties of the vlsE, leading to immune evasion through antigenic variation. The generation of new antigenic variants is thought to occur through the exchange of DNA cassettes by the process of recombination. This recombination could potentially help spirochetes to escape an antibody-mediated defence against the vlsE protein variants arising during infection (Zhang et al., 1997, Anguita et al., 2001). It has also been shown that although each *B. burgdorferi* s.s. spirochete contains a single OspC gene copy, different strains of *B. burgdorferi* s.s. express different OspC proteins in rodents with diverse sequences (Barbour and Restrepo, 2000). In contrast, OspA shows little heterogeneity within species, supposedly because it is not under the same immune selection pressure as OspC (Nordstrand et al., 2000, Wilske et al., 1995). Yet another survival strategy that is unique to *Borrelia* spirochetes among pathogenic bacteria is the ability to survive very limited iron resources. This has been accomplished by eliminating most of the genes that encode proteins that require iron as a cofactor (Posey and Gherardini, 2000).

In conclusion, *Borrelia* spirochetes have a strong potential for adaptation in the invertebrate as well as in the vertebrate host. During this process it adopts different molecular strategies for survival in these different environments (Singh and Girschick, 2004).

Clinical characteristics of Lyme borreliosis

Human LB caused by *B. burgdorferi* s.l. may present with a variety of clinical signs and symptoms and several variations in the course of the disease. Although *B.burgdorferi* s.l. may cause a clinical infection with symptoms and/or signs, the infection may also be asymptomatic in a considerable proportion of cases, especially in Europe but also in the USA (Fahrer et al., 1991, Gustafson et al., 1990, Strle and Stanek, 2009). The main clinical manifestations of LB are EM, LNB, ACA, LA, BL and LC (Berglund et al., 1995, Stanek and Strle, 2003, Strle and Stanek, 2009). Other clinical manifestations such as eye involvement and case reports of myositis, osteomyelitis, diffuse fasciitis, eosinophilic fasciitis and panniculitis have been interpreted as manifestations of LB. However, these manifestations are very rare, and strict confirmation of *B. burgdorferi* s.l. as the

causative agent in human cases of these manifestations seems even rarer (Stanek and Strle, 2003, Strle and Stanek, 2009). The main six manifestations with variants have also been classified into three stages depending on the dissemination and duration of the infection (see Table 4) (Wilske, 2005). This classification is also reflected in the expanding antibody response to an increasing number of *Borrelia* antigens as the infection progressess (Craft et al., 1986, Bunikis and Barbour, 2002).

Table 4. Staging of manifestations of Lyme borreliosis, adopted from Wilske 2005.

Stage	Localisation	Time after tick bite	Clinical manifestation
I	Localised, early	Days to weeks	EM
II	Disseminated, early	Weeks to months	Multiple EM
			BL
			LC
			LNB
III	Persistent, late	Months to years	LA
			ACA
			Late LNB

Erythema migrans (EM)

EM is defined as an erythematous skin lesion that develops days to weeks after infection at the site where *Borrelia* spirochetes were inoculated into the skin. It typically begins as a red macula or papule and expands over a period of days to weeks, usually to an oval or round lesion, with (annular) or without (non-annular/homogenous) central clearing. For a reliable diagnosis, a single lesion must reach ≥ 5 cm in size. Secondary lesions may also occur and multiple EM is defined as the presence of two or more skin lesions.

EM affects all ages and both sexes. In Europe, characterisation of *Borrelia* spirochetes isolated from skin revealed that EM is most often (67-94%) caused by *B. afzelii*, less frequently by *B. garinii* (5-33%) and rarely by *B. burgdorferi* s.s. (Bennet et al., 2006a, Ornstein et al., 2001, Cerar et al., 2008b, Ciceroni et al., 2001, Carlsson et al., 2003). It seems that EM caused by *B. garinii* develops more rapidly and is more often are non-annular than *B. afzelii*. Interestingly, it has also been shown that women with EM caused by *B. afzelii* develop non-annular erythemas more often than men (Bennet et al., 2006a, Carlsson et al., 2003). The median time from tick bite to onset of EM has been shown to be 17 days for EM caused by *B. afzelii* in Europe, compared to 11 days for EM caused by *B. burgdorferi* s.s. in the USA (Strle et al., 1999). The median time from tick bite to diagnosis of *B. garinii* has been shown to be 14 days (Bennet et al., 2006a). The EM might be accompanied by systemic complaints such as headache, fatigue and arthralgia. In Europe these complaints have been reported in some 31-40% of EM cases (Strle et al., 1996b, Tjernberg et al., 2009). In the USA, however, systemic complaints seem more common and have been reported in up to 80% of cases (Dandache and Nadelman, 2008). Other findings include

briefer duration of EM, greater frequency of multiple EM, abnormal findings on physical examinations and greater frequencies of fever and non-annular EM in American patients compared to European patients. However, local symptoms at the site of the EM, such as mild itching, burning or pain are reported in about half of EM patients both in Europe and in the USA (Strle et al., 1999, Stanek and Strle, 2008).

Borrelial lymphocytoma (BL)

BL is a solitary swelling with a diameter of up to a few centimetres, consisting of a dense lymphocytic infiltration of cutis and subcutis as a result of borrelial infection. Although BL may appear at the site of a tick bite, it sometimes appears at a distance from the causative tick bite. BL may occur together with or be preceded by EM; it may also occur together with other second- or third-stage manifestations of LB. Clinically, there is a tumour-like bluish-red swelling nodule which, in the majority of cases, is accompanied by regional lymphadenopathy. Predilection sites are the earlobes in children and the nipples or *areola mammae* in adults (Asbrink and Hovmark, 1988).

Information regarding genospecies involved in BL is limited, as are studies on clinical characteristics. However, the large majority of isolates from BL tissue have been found to be *B. afzelii*, although in some patients *B. garinii* and *B. burgdorferi* s.s. have been isolated. BL seems very rare in North America (Maraspin et al., 2002, Picken et al., 1997, Busch et al., 1996, Ruzic-Sabljic et al., 2000, Ruzic-Sabljic et al., 2002). There seems to be an even distribution between sexes, however as previously noted, BL is more common among children than adults (Berglund et al., 1995, Strle et al., 1992).

Lyme carditis (LC)

LC is heart involvement related to a *Borrelia* infection that usually presents with the acute onset of varying degrees of intermittent A-V heart block as a result of conduction disturbances, sometimes in association with clinical evidence of myopericarditis (Strle and Stanek, 2009). LC usually occurs within two months after onset of infection, and EM and/or LNB may occur concomitantly or in close proximity and therefore be diagnostically helpful (Wormser et al., 2006, Steere et al., 1980). Information regarding the relative frequency of this manifestation is incomplete. LC has earlier been reported to occur in 0.3-4% of European patients with LB and in 4-10% of corresponding patients in the USA (Strle and Stanek, 2009, Wormser et al., 2006). In the Swedish epidemiological study by Berglund et al. (1995), 7 of 1471 (0.5%) LB patients were diagnosed with LC, and in Slovenia LC may also represent up to 0.5% of LB cases (Strle and Stanek, 2009). If the true frequency of LC has diminished it may be the result of improved recognition and treatment of EM (Wormser et al., 2006). This manifestation seems to affect men more frequently than women with a male-to-female ratio of 3:1. In a study of 105 cases of LC in Europe and North America, transient A-V heart block was the most frequent manifestation of LC. The relative frequencies

of various degrees of A-V heart block were similar in European and North American patients with LC. Third-degree A-V block was noted in 49% of the patients, second-degree in 16% and first-degree in 12% (van der Linde, 1991). Patients with LC reported palpitations, syncope, shortness of breath, chest pain and dizziness (Steere et al., 1980).
There are no direct data on the *Borrelia* species causing LC; in the USA, however, LC should be caused by *B. burgdorferi* s.s. One European heart isolate was identified as *B. burgdorferi* s.s (Strle and Stanek, 2009).

Lyme neuroborreliosis (LNB)

LNB is the involvement of the central and/or peripheral nervous systems in an infection with *B. burgdorferi* s.l. LNB may appear early, during the first weeks or months, or late in the course of LB. LNB typically comprises lymphocytic meningitis and involvement of cranial and peripheral nerves (Kristoferitsch et al., 1983). Usually, the most pronounced clinical symptom is pain as a result of radiculoneuritis. Patients may have severe pain, usually in the thoracic or abdominal region, which is often belt-like and most pronounced during the night. Patients may be deprived of sleep for weeks. Radicular pain is generally more frequent and more pronounced in adults than in children. Patients with borrelial meningitis usually have mild or intermittent headaches, but in some patients headache may be excruciating. In European patients fever, nausea and vomiting seem more to be less common and milder than in North America (Pachner and Steere, 1984, Henningsson et al., 2010, Stanek and Strle, 2003, Strle and Stanek, 2009). In a Swedish study facial palsy, neck pain, fever and fatigue were more common in patients under the age of 40 and patients over 40 years of age reported muscle and joint pain, radiating pain, paresthesias, vertigo and concentration problems more often than patients under 40 years of age. Patients under 40 years of age also had symptoms of shorter duration prior to diagnosis than patients over 40. Tick bites had been noticed by 32% of the patients, and 24% had EM (Henningsson et al., 2010). The causal relationship between an individual tick bite and LNB is rather uncertain except when the bite is followed by EM. However, a median of three weeks has been reported to elapse from the time of the bite to the onset of neurologic symptoms (Strle and Stanek, 2009). Any cranial nerve may be affected in LNB but the facial nerves are by far the most frequently involved with a frequency of approximately 80% (Berglund et al., 1995, Strle and Stanek, 2009).

While LNB in North America is caused by *B. burgdorferi* s.s., LNB in Europe is most often caused by *B. garinii*. In a study of 304 characterised LNB European patients, the causative agent was *B. garinii* in 63% of the cases, *B. afzelii* in 23%, *B. burgdorferi* s.s. in 11% and other species in 4% of the cases (Strle and Stanek, 2009). A comparison of patients with *B. garinii or B. afzelii* isolated from CSF found that patients with *B. garinii* infections have a clinical presentation distinct from that of patients with *B. afzelii*. *B. garinii* causes what, in Europe, is understood as typical LNB with meningeal signs and typical radicular pain, whereas the clinical features associated with *B. afzelii* are much less specific and

more difficult to diagnose. The authors speculate that although *B. afzelii* is able to pass through the blood-brain barrier, it has restricted ability to initiate substantial inflammation in the central nervous system (CNS) (Strle et al., 2006).

Long-standing, chronic, borrelial infection of the CNS, although very rare, includes long-lasting (at least six months) manifestations such as chronic meningitis, encephalomyelitis and radiculomyelitis (Ackermann et al., 1988, Hansen and Lebech, 1992). These two studies included detection of inflammatory signs in the CSF, such as pleocytosis together with the detection of specific anti-*Borrelia* antibodies in the CSF. However, a firm confirmation of chronic ongoing infection using culture or PCR of *Borrelia* spirochetes from CSF was not performed.

Lyme arthritis (LA)

LA, the main joint manifestation in the course of LB, is an inflammatory arthritis associated with *B. burgdorferi* s.l. infection. LA affects both children and adults and is predominantly a monoarticular or oligoarticular form of arthritis. It typically involves the knee. LA is often intermittent if untreated, with episodes of joint inflammation spontaneously resolving after a few weeks to a few months. Persistent swelling of the same joint for more than 12 months would be an unusual presenting manifestation of LA (Wormser et al., 2006, Steere et al., 1987). In the late 1980s LA was reported to occur in 60% of patients with untreated LB (Steere et al., 1987). In more recent publications, however, LA frequencies equal to or less than 10% have been reported. This change may be explained by improved recognition and earlier treatment of patients with early LB (Wormser et al., 2006). In Europe, relative frequencies of LA seem to vary from 2-7% (Berglund et al., 1995, Strle and Stanek, 2009). However, these figures contrast with a report from the Centers for Disease Control and Prevention (CDC), which reports an LA frequency of 30% in the USA based on 32095 patient records (Centers for Disease Control and Prevention, 2007). Similarly, a German survey reported LA in 24.5% of 3935 patients with LB (Priem et al., 2003). Possible explanations for these varying figures are confusion between arthritis and arthralgia by the health care provider and differences in serodiagnostic interpretations (Strle and Stanek, 2009, Wormser et al., 2006). Indeed, arthralgia is also relatively frequently reported in patients with LB, in patients with EM before therapy and even in some patients after antibiotic treatment. In an early study from North America, arthralgia was reported in as many as 48% of 314 patients with EM (Steere et al., 1983). In Europe the combination of EM and arthralgia does not seem quite as frequent, but in Slovenia it has been reported in 27% of 85 patients with *B. afzelii* culture confirmed EM (Strle et al., 1999). LA can be preceded or accompanied by other manifestations of LB, such as EM, LNB or ACA (Berglund et al., 1995). The period from tick bite or EM to the onset of LA ranges from 10 days to 16 months (median 3 months), according to a European report; therefore, since the latent period is highly variable, there is no seasonal peak in the occurrence of LA (Herzer, 1991, Strle and Stanek, 2009). In Europe, unlike the USA, there have

been reports stating an association between the extremity affected by the tick bite and/or EM and the extremity in which LA begins (Herzer, 1991, Kryger et al., 1990).
LA is often preceded by intermittent migratory joint pain. The joint involvement is usually asymmetric, the onset of arthritis is acute and accompanied by effusion and the skin over the affected joint is warm but of normal colour (Steere, 1989, Strle and Stanek, 2009). Joints are usually painful, but patients with large knee effusions may have disproportionately mild pains. Joint inflammation usually lasts a few days to weeks, sometimes several months. The course of LA is highly variable, usually recurring, and it may continue for several years. In the beginning, the attacks of arthritis are more frequent and short, but later they may be more prolonged. Every year about 10-20% of patients have complete resolution of the attacks even without antibiotic treatment. About 10% of untreated patients develop chronic arthritis with a duration of a year or longer; in some of them, erosions may develop (Wormser et al., 2006, Steere et al., 1987). In about 10% of patients with LA in the USA, joint inflammation persists for months or even several years after the apparent eradication of the spirochete, *B. burgdorferi* s.s., from the joint through antibiotic treatment. An autoimmune mechanism for this antibiotic treatment-resistant arthritis has been proposed for susceptible patients based on sequence homology between an epitope of OspA of the *Borrelia* spirochete and the human lymphocyte function associated antigen-1. However, this needs to be confirmed (Steere et al., 2001, Steere and Angelis, 2006).

Regarding the European aetiology of LA, reports are somewhat inconsistent. According to two separate studies, one Dutch, one French, *B. burgdorferi* s.s. is the main pathogen (van der Heijden et al., 1999, Jaulhac et al., 2000). However, a German study of 13 LA patients with positive PCR in synovial fluid reported *B. burgdorferi* s.s. in 27%, *B. afzelii* in 33% and *B. garinii* in 40% of the cases (Vasiliu et al., 1998).

Acrodermatitis chronica atrophicans (ACA)

ACA is a chronic skin manifestation of LB seen almost exclusively in Europe. ACA is predominant in females and mainly observed in patients over 40 years of age, but it may also occur in young persons. The onset is usually gradual and insidious and characterised by the appearance of a bluish-red discolouration and doughy, swollen skin. This starts at one extremity, most commonly an acral site such as the extensor parts of the hand or foot or the olecranon area. ACA primarily involves one or more extremities. Some patients remember having other signs of LB, such as EM, neurologic involvement or arthritis, before the onset or diagnosis of ACA, but most patients do not. Thus, ACA can be the first and only sign of LB. The lesion enlarges very slowly over a period of months to years, and the region is usually oedematous. Peripheral nerves and joints in the areas of the affected skin may also be involved (Asbrink and Hovmark, 1988, Asbrink et al., 1986, Strle and Stanek, 2009).

According to the results of polymerase chain reaction (PCR) and isolation of *Borrelia* spirochetes from the skin, the large majority of ACA cases are caused by *B. afzelii*, however, in some patients *B. garinii* and *B. burgdorferi* s.s. have been isolated from the skin lesion (Busch et al., 1996, Ohlenbusch et al., 1996, Ruzic-Sabljic et al., 2000). Therefore, reports on this skin condition from North America are rare and predominantly limited to descriptions of its manifestation in immigrants from Europe (DiCaudo et al., 1994).

Swedish treatment recommendations for Lyme borreliosis

Treatment recommendations for LB may vary for different manifestations, but recently published Swedish recommendations generally follow the European LB treatment recommendations published by the European Union Concerted Action on Lyme Borreliosis (EUCALB) (Wormser et al., 2006, Swedish Medical Products Agency, 2009, EUCALB, 2009). A simplified summary of the Swedish treatment recommendations for adults is shown in Table 5.

Table 5. Adult antibiotic treatment recommendations for Lyme borreliosis in Sweden adopted from the Swedish Medical Products Agency, 2009.

Manifestation	Adults	Alternative drug
EM	PcV 1g x 3 for 10 days	Doxyc. / Azitromycin
EM+fever / Multiple EM	Doxyc. 100 mg x 2 for 10 days	
BL	Doxyc. 100 mg x 2 for 14 days	PcV
LNB	Doxyc. 200 mg x 1 for 14 days	Ceftriaxone
LC	Doxyc. 100 mg x 2 for 14 days	Ceftriaxone
ACA	Doxyc. 100 mg x 2 for 21 days	PcV
LA	Doxyc. 100 mg x 2 for 14 days	Ceftriaxone

PcV = phenoxylmethylpenicillin
Doxyc. = Doxycycline

In cases of phenoxymethylpenicillin (pcV) allergy, either doxycycline or azitromycin may be used in adults with EM. Doxycycline is not recommended during the last two trimesters of pregnancy, and azitromycin is not recommended during the first trimester. In these cases pcV or ceftriaxone may be used. Parental ceftriaxone may be used in patients with LNB, but oral doxycycline appears to be as effective and easier to administer (Dotevall and Hagberg, 1999, Borg et al., 2005). Treatment of children is different than treatment of adults when it comes to the choice of drug. Doxycycline is not recommended for children under eight years of age due to the risk of discolouration of the teeth (Grossman et al., 1971). In cases of LNB in children under eight years of age, parenteral ceftriaxone may be used instead. In children with EM and fever, multiple EM or EM on the head/neck region, doxycycline is recommended except for children younger than eight years of age, for whom amoxicillin is recommended. When amoxicillin cannot be used due to pcV allergy, azitromycin may be used instead (Swedish Medical Products Agency, 2009).

Prognosis of Lyme borreliosis

As for EM, prognosis is generally good. In one five-year follow-up study EM disappeared with antibiotic treatment in 689 of 706 patients (98%) (Bennet et al., 2003). In Europe it has been reported that most EM heals spontaneously even without antibiotic treatment within 10 weeks, but it may last up to a year (Asbrink and Olsson, 1985). In the USA, LA was reported to occur in as much as 60% of patients with untreated EM (Steere et al., 1987).

BL also heals effectively after antibiotic treatment within a few weeks, but other manifestations of LB may develop in the course of untreated long-lasting BL (Asbrink and Hovmark, 1988, Strle et al., 1996a, Strle et al., 1992).

Although LC seems to be rare and therefore hard to study, the prognosis appears to be good, with normal cardiac findings on examination and up to seven years of follow-up examinations (Midttun et al., 1997, Steere et al., 1980). Complete heart block would be the only cause of a lethal outcome in patients with LB: fortunately, this is an extremely rare occurrence (Cary et al., 1990, Stanek and Strle, 2003, Steere, 1989). Patients with various A-V blocks are hospitalised and kept under surveillance with permanent electrocardiography (ECG). In the case of complete heart block, insertion of a temporary pacemaker may be life-saving. In both antibiotically treated and untreated patients, complete heart block usually disappears within a week, whereas symptoms of heart involvement and ECG abnormalities usually vanish within 3-6 weeks (Steere et al., 1980, Strle and Stanek, 2009, van der Linde, 1991, Wormser et al., 2006).

The prognosis of LNB is more complicated. In a study of 177 children being evaluated for LNB, clinical recovery was reported to be good after a six-month follow-up, with no patients displaying recurrent or progressive neurologic symptoms. However, persistent facial nerve palsy caused dysfunctional and cosmetic problems in 11% of patients and persistent symptoms were reported in 21% of cases at the six-month follow-up. The major complaints among patients were headache and fatigue; in a non-borrelial matched control group, surprisingly, headache and fatigue were actually reported more frequently. No prognostic factors could be identified (Skogman et al., 2008). In other mixed studies of children and adults, persistent complaints were reported in 19-50% of patients, with follow-up times varying from six months to eight years after antibiotic treatment for LNB. The most common complaints were persistent facial palsy, paraesthesia, headache, arthralgia and various cognitive complaints (Berglund et al., 2002, Hammers-Berggren et al., 1993, Karkkonen et al., 2001, Tjernberg et al., 2010, Vrethem et al., 2002, Ljostad and Mygland, 2010). Data suggest that early recognition and treatment of LNB would be of great help in avoiding sequelae (Berglund et al., 2002, Dotevall et al., 1999, Ljostad and Mygland, 2010). The cause of such persistent complaints remains unclear. Different clinical outcomes after LNB may be differences in the immune response. When an immune response leads to activation of Th lymphocytes, these cells may differentiate into subsets of effector cells that produce distinct

sets of cytokines and therefore perform distinct effector functions. Th lymphocytes may differentiate into either Th-1 or Th-2 cells. Interferon-γ (INF-γ) is the signature cytokine of Th-1 cells, and interleukin-4 (IL-4) is a defining cytokine of Th-2 cells. Each subset amplifies itself and cross-regulates the reciprocal subset. Therefore, once an immune response develops along one pathway, it becomes increasingly polarised in that direction, and the most extreme polarisation is seen in chronic infections (Abbas et al., 2007). In the case of persistent LNB, as well as the other late manifestations of ACA and LA, data suggest that these patients have a persistent INF-γ (Th-1) response, while LNB and EM patients who do not develop persistent or late LB also up-regulate an IL-4 response after the initial INF-γ response (Widhe et al., 2004, Gross et al., 1998, Oksi et al., 1996). There have also been speculations regarding secondary autoimmune reactions in LNB (Martin et al., 1988, Pohl-Koppe et al., 1999). Finally, astroglial and neuronal proteins have been detected in the CSF of LNB patients pre-treatment, suggesting CNS parenchyma involvement. High concentrations of such markers have been associated with post-infectious objective neurological sequelae (Dotevall et al., 1999). In summary, persistent symptoms and complaints after LNB could either be explained by differences in the host immune response, autoimmune mechanisms or tissue damage or possibly a combination of these factors. Chronic persistent infection with viable *Borrelia* spirochetes as a cause of these complaints following treatment is probably very rare today (Marques, 2008).

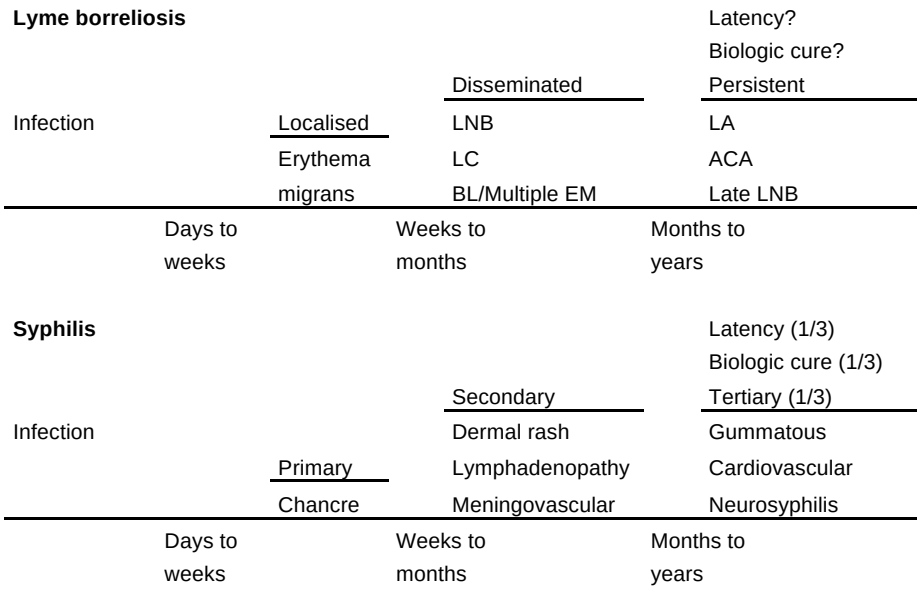
In the early 1980s various penicillin regimens cured 35-55% of patients with LA. However, since the late 1980s intravenous ceftriaxone, oral doxycycline or amoxicillin have been used and seem effective in approximately 90% of patients with LA. The remaining 10% of patients have been termed antibiotic-refractory LA (Dattwyler et al., 1988, Dattwyler et al., 2005, Steere et al., 1994, Steere and Angelis, 2006). Antibiotic-refractory LA may result from persistent infection or from postinfectious immune phenomena. However, persistent infection is likely not an explanation for antibiotic-refractory LA in most patients as detection of *Borrelia* spirochetes or DNA in synovial fluid or tissue is very rare. As previously described, an autoimmune process could possibly explain antibiotic-refractory LA. These patients have sustained or even higher levels of proinflammatory cytokines in synovial fluid and synovial tissue post-antibiotic treatment and Steere et al. hypothesise that in most antibiotic-refractory LA patients synovial inflammation persists after the near or total eradication of *Borrelia* spirochetes (Steere and Angelis, 2006).

There are only few studies regarding the prognosis of ACA. However, prognosis generally seems good, although it may depend on the length of antibiotic treatment and retreatment because of incomplete regression of skin changes, neuropathy or arthralgia (Aberer et al., 1996, Hulshof et al., 1997, Asbrink et al., 1986).

Comparative thoughts on Lyme borreliosis and syphilis

Neither *Borrelia* nor *Treponema pallidum* subspecies *pallidum* (*T. pallidum*), the causative agent of syphilis, produces toxins, yet both spirochetes are capable of invading virtually any mammalian tissue and cause infection and disease manifestations for months to years (Figure 2). Both syphilis and LB exhibit local, disseminated and persistent manifestations in which tissue pathology appears to be due primarily to host reactions (Radolf and Samuels, 2010).

Figure 2. Comparison of Lyme borreliosis with syphilis in humans, adopted from Radolf and Samuels (2010).



However, there are also important differences between the two spirochetes. The mode of transmission for *T. pallidum* is primarily sexual contact or transfer over placenta, while *B. burgdorferi* s.l. is transmitted via tick bites (Singh and Romanowski, 1999). Although transplacental transmission of *B. burgdorferi* s.l. has been found in animal models, clinical, serological and epidemiological studies have failed to confirm a causal association between *Borrelia* infection and a pregnancy-adverse outcome (Mylonas, 2010). Another striking difference is the genomic structure. As previously described, the genome of *Borrelia* contains a linear chromosome as well as a number of linear and circular plasmids, while *T. pallidum* contains a single circular chromosome with no extrachromosomal elements, thereby making its total genome approximately 25% smaller than that of *B. burgdorferi* s.s. (Porcella and Schwan, 2001). The large majority, over 90%, of genes on the *B. burgdorferi* s.s plasmids actually have no convincing similarity to genes outside *Borrelia*. This suggests that they perform specialized functions, perhaps contributing to the ability of this pathogen to survive and maintain its complex life cycle, with hosts alternating between

warm-blooded animals and cold-blooded ticks (Casjens et al., 2000, Porcella and Schwan, 2001). There are also distinct clinical differences. Whereas EM consists of a diffuse, expanding rash, the chancre of syphilis is a well-demarcated, ulcerative lesion. In addition, the cardiovascular manifestation of LB usually includes various degrees of A-V heart block, whereas long-term syphilitic infection can result in the weakening of the lamina media of the aorta and other elastic arteries, leading to aortitis and aneurysm formation. Finally, although vague bone and joint pain has been reported in patients with secondary syphilis, osteitis and arthritis are rarely described, in contrast to LA (Radolf and Samuels, 2010, Singh and Romanowski, 1999).

Laboratory tests and diagnosis of Lyme borreliosis

For a diagnosis of LB to be considered, the patient must have been exposed to the risk of a tick bite; however, a documented history of a tick bite is not essential, because many tick bites go unnoticed (Stanek et al., 2010, Stiernstedt et al., 1988). When diagnosing LB it is important to acknowledge some basic facts that are often neglected or not properly recognised. One such fact is that LB is a disease (Strle and Stanek, 2009, Stanek and Strle, 2003). *Disease* is defined as any deviation from or interruption of the normal structure or function of any body part, organ or system that is manifested by a characteristic set of symptoms and signs and whose aetiology, pathology and prognosis may be known or unknown (The Free Dictionary, 2010). Therefore, there is no disease without signs and/or symptoms, and consequently there is no diagnosis of LB in the absence of clinical manifestations. The mere proof of an infection with *Borrelia* spirochetes is not sufficient, because the infection may not always result in illness. Asymptomatic infection has been reported to occur both in the USA and perhaps at an even higher rate, in Europe (Steere et al., 2003, Fahrer et al., 1991, Gustafson et al., 1990, Ekerfelt et al., 2001). In addition, demonstration of antibodies to *B. burgdorferi* s.l. does not discriminate between active infection and an immunologic imprint of previous symptomatic or asymptomatic infection. Because signs and symptoms form the basis for recognition of the disease, good knowledge of clinical features is important in diagnosing LB (Strle and Stanek, 2009). Although LB is very similar in Europe and in North America, there are important clinical differences (Stanek and Strle, 2003, Stanek et al., 2010). Published case definitions may aid in establishing the basis for diagnosing LB (see Table 6) (Stanek et al., 2010, Wormser et al., 2006).

Due to the nonspecific nature of many of the LB manifestations, laboratory support is essential for diagnosis except for EM, which in most cases is considered a clinical diagnosis (Stanek et al., 2010, Wilske, 2005, Nadelman and Wormser, 1998, Wormser et al., 2006).

Table 6. European Lyme borreliosis case definitions, based on Stanek et al. (2010).

Term	Clinical case definition	Laboratory evidence: Essential	Laboratory/clinical evidence: Supporting
EM	Expanding red or bluish-red patch (≥5 cm in diameter)*, with or without central clearing. Advancing edge typically distinct, often intensely coloured, not markedly elevated.	None	Detection of *B. burgdorferi* s.l. by culture and/or PCR from skin biopsy
BL	Painless bluish-red nodule or plaque, usually on earlobe, ear helix, nipple or scrotum; more frequent in children (especially on ear) than in adults.	Seroconversion or positive serology**; histology in unclear cases	Histology. Detection of *B. burgdorferi* s.l. by culture and/or PCR from skin biopsy. Recent or concomitant EM
LC	Acute onset of atrioventricular (I-III) conduction disturbances, rhythm disturbances, sometimes myocarditis or pancarditis. Alternative explanations must be excluded.	Specific serum antibodies**	Detection of *B. burgdorferi* s.l. by culture and/or PCR from endomyocardial biopsy. Recent or concomitant erythema migrans and/or neurologic disorders.
LNB	In adults mainly meningoradiculitis, meningitis, with or without facial palsy**;** rarely encephalitis, myelitis; very rarely cerebral vasculitis. In children mainly meningitis and facial palsy.	Pleocytosis and demonstration of intrathecal specific antibody synthesis***	Detection of *B. burgdorferi* s.l. by culture and/or PCR from CSF. Intrathecal synthesis of total IgM and/or IgG and/or IgA. Specific serum antibodies. Recent or concomitant EM
LA	Recurrent attacks or persisting objective joint swelling in one or a few large joints. Alternative explanations must be excluded.	Specific serum IgG antibodies, usually in high concentrations**	Synovial fluid analysis. Detection of *B. burgdorferi* s.l. by PCR and/or culture from synovial fluid and/or tissue.
ACA	Long-standing red or bluish-red lesions, usually on the extensor surfaces of extremities. Initial doughy swelling. Lesions eventually become atrophic. Possible skin induration and fibroid nodules over bony prominences.	High level of specific serum IgG antibodies**	Histology. Detection of *B. burgdorferi* s.l. by culture and/or PCR from skin biopsy

* If less than 5 cm in diameter, a history of tick bite, a delay in appearance (after the tick bite) of at least two days and an expanding rash at the site of the tick bite are required.

** Specific antibody levels in serum may increase in response to progression of infection or decrease due to abrogation of the infection process. Samples collected a minimum of three months apart may be required in order to detect a change in IgG levels; as a rule, initial and follow-up samples must be tested in parallel in order to avoid changes due to inter-assay variation.

*** In early cases intrathecally produced specific antibodies may still be absent.

Indirect laboratory methods

A number of laboratory methods have been used in the diagnosis of LB. Indirect methods such as those that detect anti-*Borrelia* antibodies are the most common and widespread. These methods include immunofluorescent-antibody assay (IFA), enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) or immunoblot.
The first method used was IFA, in which serum antibodies may react and bind to cultured organisms fixed onto glass slides. After the addition of fluorescein isothiocyanate-labeled anti-human immunoglobulin (IgG or IgM), the presence of antibodies is detected through fluorescence microscopy. Limitations of this assay include the need for fluorescence microscopy and well-trained personnel and the subjectivity involved in reading and interpreting fluorescence microscopy. The method may still be in use, but it has been superseded by ELISA and Western blot in most countries (Aguero-Rosenfeld et al., 2005, EUCALB, 2009).

ELISA is the most frequently used format for testing for antibodies to *B. burgdorferi* s.l. Although there are various types of ELISA, the principals of the method may be summarised in a few steps. Diluted patient serum (or CSF) is incubated in microwells that are coated with an antigen. Antigen-specific antibodies bind to the antigen, and unbound antibodies are removed by washing. Next, secondary enzyme-linked anti-human antibodies are added. After removing unbound secondary antibodies, the substrate for the enzyme is added, and a visible signal is produced that is measurable and associated with the concentration of patient antibodies to the investigated antigen. Regarding LB, first-generation ELISA tests were based on sonicate antigens. Second-generation tests were improved, for example, by purification of antigens in order to reduce cross-reactivity. Third-generation tests may use recombinant antigens or synthetic peptides. ELISA is superior to IFA, as it is more suitable for large-scale testing and eliminates the need for subjective interpretation (Aguero-Rosenfeld et al., 2005, EUCALB, 2009, Wilske, 2005). However, an important limitation of ELISA in terms of the detection of *B. burgdorferi* s.l. antibodies is the lack of standardization. Variations exist between assays in terms of antigenic composition and the detection of specific immunoglobulin classes. Such variations may occur among different commercial kits as well as between lots of the same kit (Aguero-Rosenfeld et al., 2005, Ekerfelt et al., 2004).

Finally, in a common LB WB, antigenic proteins are transferred from *Borrelia* to a nitrocellulose membrane, which is cut into strips. These antigen-carrying strips are incubated with patient serum, and antigen-specific antibodies may bind to their respective antigens. These bound antibodies may be visualised in a way similar to ELISA. These strips may form various banding patterns depending on which antigen-specific antibodies are present. This may be important, depending on the clinical symptoms and signs of the particular patient (Wilske, 2005). However, WB is limited by the lack of standardisation of the antigen source, visual scoring and subjective interpretation of band intensity, which may lead to

false positive readings; it is further limited by the cost and the variability of antibody responses in patients with the same clinical manifestation of LB (Aguero-Rosenfeld et al., 2005). WB interpretation criteria vary between North America and Europe and may also vary among different areas within Europe (Dressler et al., 1993, Engstrom et al., 1995, Robertson et al., 2000a, Wilske, 2005), perhaps due to geographical differences in the occurrence of different *Borrelia* genospecies.

Performance of ELISA tests for LB

The interpretation of serological LB tests is difficult. There are many commercial serological assays for LB, but in general, the sensitivity (frequency of positive tests in a population with the disease) of antibody detection methods is low in the early stage of LB, stage I, ranging from some 20% to 50% (see Table 4 for LB stages). In stage II, the sensitivity increases to 70-90% and in stage III the antibody sensitivity is nearly 100% and IgG antibodies should be found (Wilske, 2005). The specificity (frequency of negative test results in a population that does not have the disease) of the serological assays also varies for a number of reasons. First of all, various conditions and factors are known to increase the risk of cross-reactions in LB serology, thereby giving rise to false positive test results. Such conditions include Epstein-Barr virus infection, syphilis and rheumatoid factor (RF) (EUCALB, 2009, Magnarelli, 1995, Steere, 1989). Apart from cross-reactions, once a patient's serology is positive it is common for the antibodies to persist, even after treatment and resolution of the infection (Hammers-Berggren et al., 1994b, Hammers-Berggren et al., 1994a). This may be reflected by the presence of anti-*Borrelia* antibodies in a healthy population (seroprevalence), such as blood donors. The seroprevalence varies among blood donor populations within Europe, from a few percent up to nearly 20% in the Åland Islands (Carlsson et al., 1998, Chmielewski and Tylewska-Wierzbanowska, 2002, Hristea et al., 2001, Tjernberg et al., 2007). These positive test results may be genuine *Borrelia* antibodies formed as a result of a previous episode of a symptomatic or asymptomatic LB infection, or they may be the result of cross-reactive factors producing a false positive LB serology. Regarding LB serology, the meaning of *specificity* should also be clarified. Laboratory specificity mainly deals with determining whether positive reactions actually stand for genuine anti-*Borrelia* antibodies. The clinician, on the other hand, not only deals with that question but, in the case of true anti-*Borrelia* antibodies, also determines whether these antibodies are actually related to the present symptoms and complaints of the patient or remain from a previous, non-ongoing, encounter with *Borrelia* spirochetes.

Two-step testing

Since 1995 in the USA and later on in Europe, a two-step (two-tier) approach has been recommended. The first step is usually a sensitive ELISA followed by a confirmatory WB in equivocal or positive cases (Johnson et al., 1996, Wilske, 2005, Centers for Disease Control and Prevention, 1995). The approach was

originally an attempt to improve test accuracy in the USA by increasing the specificity of *B. burgdorferi* s.s. antibody testing while slightly decreasing the sensitivity (Aguero-Rosenfeld et al., 2005). However, the development of highly sensitive and specific ELISAs based on recombinant antigens has raised the question of whether the two-step approach can be modified or even abandoned (Aguero-Rosenfeld et al., 2005, Wilske, 2005, Bacon et al., 2003, Stanek et al., 2010).

C6 peptide and VlsE-based LB serology

In the late 1990s the surface antigen VlsE was characterised in *B. burgdorferi* strain B31. The gene that codes for the VlsE surface lipoprotein has been found, together with 15 silent vls cassettes on a 28-kb linear plasmid of *B. burgdorferi* B31. Of these 16 vls cassettes in total, only VlsE is expressed, hence its name. The vls cassettes contain six highly conserved regions (invariable regions, IR1-6) that are interspersed with six variable regions (Zhang et al., 1997). The invariable region 6 (IR6) of VlsE has been shown to be immunodominant and conserved between the *B. garinii* IP90 strain and the *B. burgdorferi* strains B31 and 297. The synthetic peptide, C6, is based on the IR6 of *B. garinii*, and it was first evaluated and introduced as an ELISA LB serological test in the USA (Liang et al., 1999b, Liang et al., 1999a) and later commercialised by Immunetics®. The vls loci of *B. garinii* IP90 and *B. afzelii* ACAI have been further cloned and characterised, showing 11 silent vls cassettes and 14 silent vls cassettes, respectively. In both organisms, the silent cassettes resemble the *B. burgdorferi* B31 vls sequences in overall amino acid composition and in the presence of six variable regions interspersed among six relatively invariant regions. Although the VlsE expression sites of the *B. garinii* and *B. afzelii* isolates have not been isolated, transcripts of VlsE have been detected (Wang et al., 2003). Further studies of the VlsE protein among the three genospecies have shown that the IR6 regions of some vls cassettes in *B. afzelii* and *B. garinii* strains can vary up to five amino acids compared to the IR6 region of the VlsE protein of *B. burgdorferi* B31. Although antibody response has been shown to be strong to the C6 peptide in both mice and humans infected by the three main genospecies present in Europe, these variations in IR6 regions could have diagnostic relevance (Liang et al., 2000, Gottner et al., 2004). Recombinant VlsE-based antibody tests and the synthetic peptide C6 have been evaluated and established in LB serology over the last few years (Liang et al., 1999b, Philipp et al., 2001, Panelius et al., 2003, Nyman et al., 2006, Smismans et al., 2006, Sillanpaa et al., 2007).

CSF/Serum antibody index

In suspected cases of LNB, paired CSF and serum samples make it possible to determine the presence of intrathecally produced anti-*Borrelia* antibodies by calculating a specific anti-*Borrelia* antibody index (AI) (Wilske, 2005). Kaiser et al. compared different ELISA techniques and calculation methods and found that they were almost equivalent in demonstrating intrathecal synthesis of *Borrelia*-

specific antibodies (Kaiser and Lucking, 1993). In one commonly used commercial ELISA assay for AI, microwells are pre-coated with anti-human IgM or IgG antibodies. Diluted serum is then added to a pre-coated IgM and IgG well. The corresponding diluted CSF from the same patient is also added to an IgM and IgG well. The same total quantity of IgM and IgG antibodies is bound to the wells. After removal of excess proteins by washing, flagellum conjugate is added to the wells. Only human IgM and IgG anti-flagellum antibodies will bind to the conjugate, and unbound flagella will be washed away. By finally adding a chromogenic substrate to the wells, the quantity of bound flagella conjugate can be visualised. The colour intensity in the well corresponds to the concentration of either IgM or IgG anti-flagellum antibodies. By this method the proportion of specific anti-flagellum antibodies in serum and CSF can be compared in the same patient. If the proportion of specific anti-flagellum antibodies is higher in CSF than in serum, the AI is considered positive (IDEIA™ Lyme neuroborreliosis kit, K602811, Oxoid Limited, UK).

However, AI may be negative for the first few weeks, and in addition, a positive AI may persist for several months and even years despite appropriate treatment (Blanc et al., 2007, Brouqui et al., 2004, Hammers-Berggren et al., 1993, Hansen and Lebech, 1991, Stanek and Strle, 2003, Strle et al., 2006, Ljostad et al., 2007).

Current recommendation of LB serology

Blood serology is indicated in all cases of clinically suspected LB except for EM, which in most cases is considered a clinical diagnosis (Stanek et al., 2010, Strle and Stanek, 2009, Wilske, 2005, Wormser et al., 2006). The current European recommendation regarding suspected LNB is to obtain paired samples of CSF and serum for further analysis, including specific antibody calculations and determination of the presence of CSF pleocytosis (elevated numbers of leucocytes such as lymphocytes and plasma cells in the CSF) (Mygland et al., 2010, Wilske, 2005, Stanek et al., 2010, Swedish Medical Products Agency, 2009).

Direct detection methods

The two main methods of direct antigen detection in LB are cultures of *B. burgdorferi* s.l. and PCR for detection of *B. burgdorferi* s.l.-specific DNA (Aguero-Rosenfeld et al., 2005, Wilske, 2005).

B. burgdorferi s.l. may be cultured in modified Kelly's medium from skin biopsies of EM, BL and ACA, from CSF in LNB, from synovial fluid in LA and generally in blood or blood components such as plasma (Aguero-Rosenfeld et al., 2005, Wilske, 2005). Although isolation of the infectious agent through culture is a major method used to diagnose infectious diseases, culture of *B. burgdorferi* s.l. is not commonly used in the routine diagnosis of LB because it is time consuming (generation time of *B. burgdorferi* s.l. is about 7-20 hours), and cultures are incubated for up to 12 weeks before being considered negative. The

sensitivity is also reported to be low, especially in body fluids (Arnez et al., 2001, Cerar et al., 2008a, Karlsson et al., 1990, Wilske, 2005). Culture from skin lesions such as EM and ACA seem to have the highest sensitivity, with a typical isolation rate of at least 40% in untreated EM (Aguero-Rosenfeld et al., 2005). In summary, culture may be of help in individual seronegative cases with clinical suspicion of LB but is usually not employed in the routine clinical diagnosis of LB.

The first PCR assay for detection of a chromosomally encoded *B. burgdorferi* s.l. gene was reported in 1989 (Rosa and Schwan, 1989). Various PCR protocols have subsequently been developed for detection of *B. burgdorferi* s.l. DNA in clinical specimens. A variety of clinical specimens have been tested, such as skin biopsies and various body fluids depending on the clinical manifestations of the patients and using a number of different gene targets for amplification, including the plasmid-borne gene OspA; or chromosomal genes, such as the genes for the flagellar protein or p66; or from regions o the 16S rRNA gene. Generally, the sensitivity of PCR performed on body fluids is low, similar to that of culture. The low yield could be a reflection of the lack of or transient spirochetemia as well as low-level spirochetemia or the presence of PCR-inhibitors in the extracted DNA. The highest sensitivity has been found in skin biopsies from EM and ACA. Specificity, on the other hand, has been reported as 93-100%. As for culture, sensitivity using PCR is generally low in LB involving systems other than skin, with the exception of LA (Aguero-Rosenfeld et al., 2005, Wilske, 2005). A promising approach to increase the sensitivity of the detection is to base the PCR assay on complementary DNA (cDNA) instead of DNA, by extracting RNA from the sample, followed by cDNA-synthesis using reverse transcription. Since ribosomal RNA molecules are more abundant than the ribosomal RNA genes in the bacterial cell, the PCR assay will be more sensitive if using primers targeting the ribosomal RNA genes (Ornstein and Barbour, 2006, Wilhelmsson et al., 2010). During the last decade the real-time PCR technology has been improved and increasingly used in clinical diagnostics. It offers a number of advantages compared to ordinary PCR; the possibility to perform a more accurate quantification, a higher sensitivity than conventional PCR and improved possibilities to perform high throughput analysis. For detection and even for quantification of *Borrelia* there are a number of different assays available (Babady et al., 2008, Ivacic et al., 2007, Wilhelmsson et al., 2010). However, currently, PCR is not commonly used in the routine clinic, but it may be a useful tool in special cases.

In conclusion, the sensitivity of culture or PCR performed on skin biopsies taken from EM or ACA has been reported as approximately 50-70%. Information regarding BL is limited; according to one report sensitivity of culture was 24%. In LA, culture is rarely successful, but the sensitivity of PCR is approximately 50-70% using synovial fluid and perhaps even higher using synovial tissue. The reported sensitivity of culture or PCR in CSF from patients with LNB is low, 10-30%, although it is higher when disease duration is less than two weeks (Eiffert

et al., 1998, Karlsson et al., 1990, van Dam et al., 1993, Vasiliu et al., 1998, von Stedingk et al., 1995, Jaulhac et al., 1996, Wilske, 2005, Maraspin et al., 2002).

Other laboratory methods and laboratory tests

Commonly used routine clinical chemical laboratory blood tests such as C-reactive protein (CRP), total white blood cell count, platelet count and levels of immunoglobulins, may show normal or slightly elevated levels in LB, but these tests generally do not add any useful information in the clinical diagnosis of LB. In two studies of patients with EM, slightly elevated levels of C-reactive protein and erythrocyte sedimentation rates (ESR) were noted in 21-53%, slightly elevated levels of IgM in 33% and elevated white blood cell levels and platelet counts in 6-8% (Bennet et al., 2006a, Steere et al., 1983). In one study of LNB, the serum level of CRP was increased in only 15% of the patients (Pietruczuk et al., 2005). Also, in six well-characterised patients with LA, most routine laboratory blood tests were normal. In two patients, elevated levels of either total IgM or IgA and ESR were noted (Berglund et al., 1996). In CSF of patients with LNB, however, pleocytosis is commonly but not always found (Oschmann et al., 1998, Blanc et al., 2007, Strle et al., 2006). A substantial number of patients also have elevated CSF protein and oligoclonal IgG bands in CSF (Oschmann et al., 1998).

Other diagnostic tests for LNB have been described, such as the lymphocyte transformation test and detection of antibodies that bind in circulating immune complexes. However, results from studies of these tests are contradictory. Therefore, there is not enough evidence to recommend these methods as routine diagnostic tools. In addition, tests for *B. burgdorferi* s.l. antigens and DNA in urine from patients with LB have been suggested, but sensitivity is low and highly variable, and specificity and reproducibility are poor (Mygland et al., 2010, Aguero-Rosenfeld et al., 2005).

Finally, in 2005, using protein expression profiling, Rupprecht el al. showed that the chemokine CXCL13 was upregulated in the CSF of LNB patients but not in patients with non-inflammatory or various other inflammatory neurologic diseases (Rupprecht et al., 2005). Although promising, few studies have been published on the use of CXCL13 as a diagnostic test in LNB, and further confirmation is needed (Ljostad and Mygland, 2008, Senel et al., 2010, Mygland et al., 2010).

INITIATION OF THE STUDY

After graduating as a medical doctor from Uppsala University in the spring of year 2000, I moved to Linköping and worked for half a year in the Infectious Diseases Department. There, I was first presented with the complicated matter of diagnosing patients with LB, an issue that would stay in my mind. A couple of years later, while finishing my internship in Kalmar, I had the opportunity to choose a department for an elective period of three months. I contacted the head of Clinical Microbiology, Dr. Ingvar Eliasson, who warmly welcomed me. Ingvar introduced me with great enthusiasm to the fascinating world of microbiology in general and *Borrelia* serology in particular. One day, he also introduced me to one of his friends in the field of borreliosis, Dr. Johan Berglund.

Johan had just returned from the USA, and he informed us of a new, very promising *Borrelia* serology - the C6 test. I learned that Johan had written a well-received thesis on the epidemiology and clinical manifestations of Lyme borreliosis in the south of Sweden in 1996. Together, we discussed the issue of evaluating this new serology in Europe, and thus began my first project.

Less than two years later we contacted Professor Pia Forsberg in Linköping, whom I already knew from my time at the Department of Infectious Diseases in Linköping. After a couple of meetings, I decided to move forward with my research of laboratory diagnosis of LB. I was accepted as a PhD student at Linköping University, with Pia as my main supervisor and Ingvar Eliasson and Professor Jan Ernerudh (specialist in clinical immunology and neurology) as co-supervisors. In the final year of this project, Ingvar Rydén (specialist in clinical chemistry) joined the team as a local co-supervisor in Kalmar.

AIMS OF THE STUDY

To study clinical cases of LB and control subjects in an area endemic to LB with a focus on immunological and biochemical responses, mainly antibody responses to newly discovered borrelial antigens and their implications for the clinical diagnosis of various manifestations of LB.

Aims of the investigations, presented in each paper:

I. To describe the prevalence and follow dynamics of C6 peptide antibodies in comparison with antibody levels detected by the Virotech *Borrelia burgdorferi* ELISA and the Liaison *Borrelia* chemiluminescence immunoassay using serum samples from patients with LB manifestations, healthy blood donors and patients with conditions known for cross-reactions in anti-*Borrelia* antibody assays.

II. To study clinical cases of suspected LNB with respect to the presence and absence of AI and pleocytosis and to investigate the usefulness of the C6 peptide antibody response in serum as a diagnostic tool in LNB.

III. To evaluate and compare the kinetics of serum antibody responses to the VlsE protein IR6 peptide variants and the C6 peptide in clinical cases of EM.

IV. To evaluate the usefulness of the chemokine CXCL13 and C6 peptide antibodies separately and in combination in paired serum/CSF samples in the laboratory diagnosis of LNB.

PATIENTS AND METHODS

Definitions used in this thesis

The following definitions or classification were used for patients and controls investigated in this thesis:

Erythema migrans (papers I, III)

Cases of EM were clinically diagnosed based on the patient's history and physical examination, i.e., a rash typical of EM with a diameter of ≥ 5 cm.

Borrelia lymphocytoma (paper I)

Cases of BL were clinically diagnosed based on the patient's history and physical examination, i.e., a bluish-red nodule resembling a lymphocytoma, found most commonly in the earlobes of children and occasionally in the nipples of adults.

Lyme neuroborreliosis (papers I, II, IV)

In paper I, a case of LNB was defined as a positive AI in paired serum/CSF samples using the IDEIA™ Lyme neuroborreliosis kit, K6028, at that time manufactured by DakoCytomation, Cambridgeshire, UK (from September 2006 rebranded and sold by Oxoid Limited, UK). AI-positive patients could further be categorised based on the presence or absence of CSF pleocytosis. Patients with a positive AI and pleocytosis could be classified as definite LNB; patients with a positive AI but without pleocytosis or patients sampled for LNB with pleocytosis but a negative AI as possible LNB; and finally, patients negative in AI and without pleocytosis as non-LNB, Table 7 (Mygland et al., 2010). In paper IV, intrathecal anti-*Borrelia* antibody (ITA) production, based on the CSF sample only, was determined for patients from Jönköping County using the same test antigen; flagellum (Lyme Borreliosis ELISA kit 2nd Generation, Dako Cytomation, A/S, Glostrup, Denmark). AI and ITA are collectively termed CSF anti-*Borrelia* antibodies (CSF ABA) in this thesis.

Table 7. Classification of Lyme neuroborreliosis.

	Definite LNB	Possible LNB 1	Possible LNB 2	Non-LNB
CSF Anti-*Borrelia* antibodies[a]	+	+	–	–
CSF Pleocytosis	+	–	+	–

CSF = Cerebrospinal fluid

Pleocytosis = Total white blood cell count ≥ $6*10^6$/L CSF

[a] A positive specific anti-*Borrelia* antibody index or intrathecal anti-*Borrelia* antibodies

Acrodermatitis chronicum atrophicans (paper I)

Prospective cases of ACA were clinically diagnosed based on the patient's history and physical examination. Retrospective cases of ACA were clinically identified by dermatologists together with a typical IgG reactivity in an independent WB (Virotech Western Blot *Borrelia burgdorferi* EcoBlot IgG/IgM, Genzyme Virotech, Rüsselheim, Germany).

Lyme arthritis (paper I)

Three retrospective cases of LA were defined as clinical cases of arthritis with a positive PCR result from synovial fluid using 16S rRNA partial sequencing (Marconi and Garon, 1992).

Possible Lyme borreliosis (paper I)

Paper I included patients with symptoms or signs that were consistent with LB or for whom a LB serological test would have been requested in the routine clinic regardless of the study. The clinical suspicion of LB in these cases was weak, and a final clinical diagnosis could not be determined. However, these patients were still included, as they are part of the clinical practice. These patients were classified as possible LB.

Blood donors (papers I, III)

In this thesis, blood donors were healthy individuals between the age of 18 and 70 who were asked to give an extra serum sample when donating blood. Only volunteers accepted for blood donation with an approved declaration of health were asked and included in paper I. No information regarding previously known or suspected LB episodes was collected.

Epstein-Barr virus-positive (paper I)

Patients with serological evidence of EBV-infection were retrospectively included as controls in paper I. Patients were positive for both EBV IgM and

IgG-antibodies but negative for EBV nuclear antigen (EBNA) antibodies, a pattern highly consistent with primary, ongoing EBV infection (Linde, 1996).

Rheumatoid factor-positive (paper I)

Patients positive for RF in serum were retrospectively included as controls in paper I. Patients with various levels of RF positivity were included, with a titre ranging from 1:80 to 1:>1280.

Patients positive in syphilis serology (paper I)

Patients who tested positive in syphilis serology were included in paper I. Sera positive in both the *Treponema pallidum* particle agglutination (TPPA) test and the rapid plasma reagin test (RPR) were considered consistent with active syphilis infection, while sera positive only in the TPPA test were interpreted as indications of healed syphilis infection.

Patients and controls (papers I–IV)

In total, 872 individuals of all age groups were included in this thesis. Several patients were included in more than one paper. Patients and controls in papers I–IV are described in Table 8. Further description of the patients with symptoms and signs consistent with various manifestations of LB is provided in Table 9.

Except for six cases of ACA collected between 2001 and 2003 and three cases of LA collected between 1999 and 2003, all individuals were sampled between 2003 and 2005. The 872 cases consisted of 617 patients with symptoms and/or signs consistent with various forms of LB. The remaining individuals were controls: either blood donors (n=200) or patients with diseases or conditions known to cross-react in LB serological tests (n=55).

The majority (n=757) of the 872 individuals were recruited from Kalmar County in the southeast of Sweden; the remaining 115 patients, investigated for suspected LNB, were recruited from the neighbouring county of Jönköping. LB is endemic to both counties (Berglund et al., 1995).

Erythema migrans (papers I, III)

In the prospective study of LB in Kalmar County in 2003 (paper I), a total of 158 patients with EM were included (Table 9). Various different clinical symptoms and signs, as well as the duration of the EM and previous episodes of LB were registered for each patient. All EM patients were treated with antibiotics, either with pcV (n=145) or with doxycycline (n=13). Each patient then gave serum samples on three occasions: at the onset of the study, at 2-3 months and at 6 months after onset. Of these 158 complete sets of serum samples, 148 were available for further studies (Table 9) (paper III).

Table 8. Numbers of individuals included and studied in the thesis.

Description	Paper I (n)	Paper II (n)	Paper III (n)	Paper IV (n)
Patients				
Prospective patients	244		158	
Retrospective patients	29*	248*		363
Insufficient samples	44		10	102
Remaining patients	229	248	148	261
Controls				
Blood donors	200		200	
EBV-positive	22			
RF-positive	23			
Syphilis-positive	10			
Total individuals studied (including remaining patients)	484	248	348	261

* 19 patients from paper I were also included in paper II but not in paper IV.
If patients are included in more than one paper, they share the same background cell colour.

Table 9. Description of Lyme borreliosis patients included in this thesis.

Description	Paper I (n)	Paper II (n)	Paper III (n)	Paper IV (n)
EM	158		148	
BL	2			
Definite LNB	21	93		124
Possible LNB	5	62		45
Non-LNB	n.d.	93		92
LA	3			
ACA	9			
Possible LB	31			
Total	229	248	148	261

Definite LNB = Positive for anti-*Borrelia* antibodies in cerebrospinal fluid (CSF) together with pleocytosis (total white blood cell count ≥ $6*10^6$/L CSF)
Possible LNB = Either positive for anti-*Borrelia* antibodies in CSF or pleocytosis in patients sampled for LNB.
Non-LNB = Negative for CSF anti-*Borrelia* antibodies and no pleocytosis of the CSF
n.d. = Not determined
Possible LB = Patients with symptoms or clinical signs consistent with LB, but where a clinical diagnosis could not be determined.

Lyme neuroborreliosis (papers I, II, IV)

Patients positive for CSF ABA were considered consistent with LNB. In total, 221 such individuals were included in the thesis together with 149 patients with symptoms consistent with LNB but without CSF ABA.

In paper I, six AI-positive patients were prospectively included together with 20 retrospectively collected patients identified in 2004 in Kalmar County. Paired

serum/CSF samples from 19 of these 20 retrospectively collected AI-positive cases were also included in paper II.

In paper II, these 19 retrospective AI-positive patients from paper I were included together with an additional 105 AI-positive individuals, in total 124 AI-positive patients. These 124 AI-positive patients were also matched with 124 AI-negative cases, all identified between 2003 and 2005 in Kalmar County for whom a CSF cell count had been performed. In all 248 patients included in paper II, a lumbar puncture had been performed in order to confirm or rule out LNB. These 248 cases were also included in paper IV together with 115 patients from Jönköping County sampled for suspected LNB during 2003–2005 (Tables 8 and 9).

Other Lyme borreliosis manifestations (paper I)

Serum samples from three prospective and six retrospective patients with ACA were studied together with serum samples from three patients with LA. In addition, 31 patients with possible LB were included (Table 9).

Blood donors (paper I, III)

In April of 2003, i.e., before the active tick season in Kalmar County 200 healthy adult blood donors were prospectively included. The group consisted of 100 female and 100 male blood donors. These blood donors were included in order to determine the seroprevalence in LB serological tests in the healthy adult population in an area highly endemic for LB (Table 8).

Cross-reactive conditions (paper I)

To determine the frequency of cross-reactions, serum samples from patients with conditions known to cross-react in LB serological tests were retrospectively included. Serum samples from 22 patients, 11 female and 11 male, with EBV-infection were included. Also, 23 RF-positive patients were included. Of these 23 patients, 12 were female and 11 male. Samples were chosen based on titres of RF. Four patients each with titres 1:80 to 1:>1280 were included, except for three patients with a titre of 1:320. Ten patients with serological evidence of syphilis infection were finally included. These 10 patients, sex unknown, included five patients positive for both TPPA and RPR (i.e., active syphilis), while the other five patients were positive only for TPPA test (i.e., healed syphilis) (Table 8).

Excluded patients or insufficient samples

In paper I, 44 patients did not complete the study and were therefore excluded (Table 8). These 44 patients could be divided into 23 with EM, 7 with facial nerve palsy, 3 with sensory radiculititis and 2 with arthritis. The remaining nine patients could not be classified. Seventeen of the 44 patients were under the age of 15 years.

For paper III, a complete set of three serum samples per EM patient was available for 148 of the 158 patients from paper I (Table 9), thus in 10 cases, 7 female and 3 males, serum sets were incomplete, and these 10 patients were therefore excluded from paper III (Table 8).

A total of 248 patients were included in paper II. These 248 patients from Kalmar County were included together with an additional 115 patients from Jönköping County. Lumbar puncture had been performed in all 363 patients in order to rule LNB in or out (Table 8). However, in 102 of these cases serum or CSF samples were either missing or of insufficient volume to be analysed in paper IV and could consequently not be studied (Table 8). These 102 patients included 50 females and 52 males with a median age of 13 years (range 2–84 years). These 102 patients could also be divided into definite, possible or non-LNB based on their original serum/CSF results: 57 definite LNB patients (CSF ABA positive with pleocytosis), 25 patients with possible LNB (either CSF ABA positive or with pleocytosis) and, finally, 20 non-LNB patients (CSF ABA negative without pleocytosis).

Laboratory methods

In general, ELISA was employed in the majority of the methods used in this thesis (see Introduction for a description of the ELISA). In the case of commercial kits, methods were carried out according to the manufacturer's instructions. If not otherwise specified, results were also interpreted according to the manufacturer's instructions. Personnel at the Departments for Clinical Microbiology and Chemistry, Kalmar County Hospital, carried out the majority of analyses in this thesis in order to reproduce the performance found in everyday practice. Below are descriptions of the various methods used.

Immunetics® Quick ELISA C6 Borrelia assay kit (C6 test)

From 2003 until the execution of analyses in paper IV in 2009, three slightly different C6 kits were provided from the manufacturer. For papers I and III, Immunetics® Quick ELISA C6 *Borrelia* assay kit Cat. No. DK-E392-096, CF-E392-800 was used. For samples analysed between 26 July and 27 December, 2004, a variant of the same kit was provided, both Cat. No. DK-E392-096. Both the first and the second kits were used in paper II. Finally, in paper IV, a third variant of the C6 kit was utilised on CSF, Immunetics® C6 Lyme ELISA kit, Cat. No. DK-E601-096, CF-E601-801.

Regarding the first and second kits, the principles were basically the same. Each kit was based on a synthetic peptide antigen (C6 peptide) in microwell ELISA format. The antigen was derived from the VlsE protein, which has been shown to elicit an immune response consisting primarily of IgG antibodies. Serum samples were added to microwells together with a conjugated antigen as a secondary measure to detect antibodies specific to the C6 peptide (i.e., a double antigen

assay, personal communication Immunetics®). After removal of unbound antibodies by washing, bound antibodies and conjugate were detected by adding a chromogenic peroxidase substrate. A blue product was formed that was quenched by diluted sulphuric acid, which caused the colour to change to yellow. The optical absorbances of the wells were then measured at 450 nm using an ELISA microplate reader. Positive and negative control samples were included in the kit and were analysed on each microplate. For the first kit, OD results < 0.125 were considered negative, OD > 0.15 were considered positive and OD values in between were considered indeterminate. In paper III, a modified, in-house (C6 IH), cut off value (≥ 0.0689) of the first version of the test was tried, based on the mean OD value plus three standard deviations (SD) of 171 blood donors without IgG bands in an independent WB. For papers I and III, calculations of positive results were based on either the manufacturer cut-off (C6 Imm.) or the in-house cut-off (C6 IH).

In the second kit, OD values ≥ 0.12 were considered positive and OD < 0.12 negative, with no indeterminate values. In the first version, serum samples were diluted 1:2, whereas undiluted samples were used in the second version.
As the first two versions of the kit were double antigen assays, they were not selective for immunoglobulin classes. Finally, in the third version of the test kit, the patient sample was diluted 1:20 and added to microwells. After removal of unbound antibodies, bound antibodies were detected using a horseradish peroxidase-conjugated goat anti-human IgG/IgM conjugate. After washing a colour change could be visualised by adding a substrate and stopping the reaction, similar to the other versions of the kit. For each kit, the cut-off value was determined by adding 0.3 to the absorbance of an included calibrator. A Lyme index value could then be calculated by dividing using the obtained patient absorbance value by the cut-off value. Lyme index values ≤ 0.90 were considered negative and values ≥ 1.10 positive. Values in between were considered indeterminate.

Virotech (VT) Borrelia burgdorferi ELISA IgG/IgM test kit (REV 031127-05)

This assay was used for paper I. In contrast to the C6 assays, this method detects *Borrelia* IgM and IgG antibodies separately. The manufacturer did not describe the antigen or antigens used in this test kit. The antibody sought in the human serum sample formed an immune complex with the antigen coating the microplate. Unbound antibodies were washed away and enzyme conjugate (either IgM or IgG conjugate) was added. After removal of unbound conjugate, substrate was added, which, in the presence of the enzyme, resulted in a blue dye. Finally, adding a stopping solution changed the colour to yellow, and OD values could be measured at 450 nm. By dividing the obtained patient OD value with the OD value of an included cut-off control sample, the Virotech unit was calculated for the patient sample. For both the IgM and the IgG assay, Virotech unit values < 0.90 were considered negative, values > 1.10 positive and values in between as indeterminate.

Diasorin Liaison (Li) Borrelia IgM (310890), IgG (310880)

This assay was used for paper I. The Liaison *Borrelia* test for IgM and IgG antibodies is a chemiluminescence immunoassay. Recombinant OspC is the antigen used in the IgM assay and recombinant VlsE is used in the IgG assay; both are derived from *B. burgdorferi,* according to the kit insert. These recombinant antigens were attached to magnetic particles. Antibodies in calibrators, controls and patients samples bound to the antigens, and unbound substances were removed by washing. In the second step, monoclonal isoluminol conjugate mouse anti-human IgM or IgG antibodies were added for detection. After removal of unbound antibodies, start reagents for the chemiluminescence reaction were added, and the light signal produced was measured in relative light units using a photo multiplicator, indicating the concentration of IgM or IgG antibodies in the sample. The analyses were performed by an instrument that automatically calculated an index for IgM and an absorbance unit value per mL (AU/mL) for IgG. An IgM index < 0.90 was considered negative, and an index ≥ 1.10 was considered positive. Values in between were considered indeterminate. IgG values < 10 AU/mL were considered negative and values ≥ 15 AU/mL positive, while values in between were indeterminate.

IR6 peptide antibody analyses

These assays were used for paper III. Three synthetic IR6 peptides were produced at the Core Facility of Protein Chemistry, Haartman Institute, University of Helsinki (Table 10). The amino acid sequence of the peptide 1 (P1) corresponded to the IR6 region of the VlsE gene sequence of *B. burgdorferi* strain B31 and peptide 2 (P2) corresponded to the IR6 region of the VlsE gene of the *B. garinii* strain IP90 (Liang et al., 1999a, Liang et al., 1999b, Zhang et al., 1997). The amino acid sequence of peptide 4 (P4) corresponded to the IR6 region of vls recombination cassette 4 of *B. afzelii* ACAI (Wang et al., 2003). All peptides were biotinylated at their amino terminus.

Table 10. IR6 peptide sequences used in IgG ELISA tests.

Code	Strain of origin	Amino acid sequence	MW	Length
P1	*B. burgdorferi* sensu stricto B31 VlsE gene	*[H]MKKDDQIAAAIALRGMAKDGKFAVK[OH]	2903	25 aa
P2	*B. garinii* IP90 VlsE gene	*[H]MKKDDQIAAAMVLRGMAKDGQFALKD[OH]	2852	26 aa
P4	*B. afzelii* ACAI vls recombination cassette 4	*[H]MKKSDKIAAAIVLRGVAKDGKFAVA[OH]	2588	25 aa

MW = Molecular weight

* = Site of biotin label

aa = amino acids

The IR6 peptide IgG ELISAs were carried out according to Sillanpää et al. (2007). Ninety-six-well microtitre plates were coated with recombinant streptavidin in phosphate-buffered saline (PBS) and incubated at 4°C overnight. The plates were post-coated with 5mg/ml human serum albumin in PBS for 2 h at 37°C with agitation. Plates were washed five times with washing solution (0.9% NaCl, 0.025% Tween 20; 300ml per well) using a plate washer. Biotinylated peptides were added at an amount of 20 ng per well, and plates were incubated with agitation for 2h at room temperature (23°C). Plates were washed as described earlier. Background control wells without peptide antigens were used for each serum sample. The samples were diluted 1:100 in buffer consisting of 0.5% (v/v) human serum albumin with 0.05% (v/v) Tween 20, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride (PMSF) and 0.06% (v/v) NaN3 in PBS, and plates were incubated with agitation for 2h at room temperature (23°C). Plates were washed as described earlier. The secondary antibody was alkaline phosphatase-conjugated rabbit anti-human IgG at a 1:5000 dilution in 0.5% (v/v) bovine serum albumin with 0.04% Tween 20 and 0.06% NaN3 in PBS. Plates were incubated with agitation for 2 h at room temperature (23°C) and then washed as described earlier. 4-Nitrophe-nylphosphate was used as the substrate. The OD at 405 nm was measured with an iEMS Reader MF 15 min after addition of substrate. For each sample the OD value of the blank control well was subtracted from the OD value of the patient sample well (Sillanpaa et al., 2007). Samples from 171 blood donors (paper I and III) without IgG bands in an independent WB were used to define the cut-off value for each antigen (mean OD plus 3 SD). The following OD cut-off values were used in paper III: P1 0.04856, P2 0.05126, P4 0.08155.

DakoCytomation IDEIA Lyme neuroborreliosis test kit K6028

This assay was used for papers II and IV. The LNB kit is designed to detect the specific proportion of IgM and IgG anti-flagellum antibodies in CSF and serum making it possible to determine whether the specific proportion is higher in CSF than in serum, indicating LNB. Briefly, diluted paired patient samples with CSF

and serum were added to microwells coated with anti-human IgM or IgG. The dilution of samples guaranteed that the well was saturated with either IgM or IgG antibodies. Therefore, the same total amount of antibodies was captured in the well for both CSF and serum. After washing, biotinylated native *B. afzelii* strain DK1 flagellum was added to the wells in combination with peroxidase-conjugated streptavidin. The antibodies that were specific to the flagellum-conjugate could bind, and excess flagellum conjugate was washed away. Finally, the amount of bound flagella in the wells could be visualised by adding a chromogenic substance and stopping the reaction with an acidic solution. The absorbance of the wells was then measured with a microplate reader at 450 nm. The following formula was used to determine the AI for IgM and IgG separately:

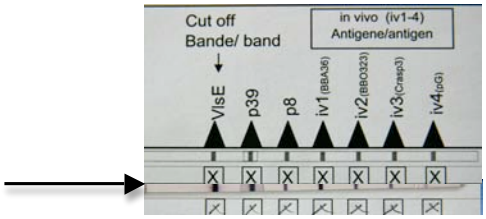
$$AI = \frac{OD_{CSF}}{OD_{Serum}} * (OD_{CSF} - OD_{Serum})$$

AI of ≥ 0.3 for both IgM and IgG was considered positive and AI < 0.3 was considered negative.

Virotech Borrelia Ecoline IgG/IgM Line Immunoblot (WB EL) (REV 040202-01)

This assay was used for paper III. It was also performed on all samples included in paper I, but results have not been published. The proteins of the pathogen-antigen were transferred to the nitrocellulose membrane, and the membrane was cut into strips. These strips were then incubated with patient serum samples, which allowed for detection of specific antibodies. These antibodies formed immune complexes with the antigens fixed on the strips. Unbound antibodies were washed away and alkalik phosphatasis-conjugated anti-human IgG and IgM antibodies, respectively, were added. After removal of unbound conjugated antibodies by washing, the bound antibodies could be visualised by adding a substrate that would induce a blue-violet precipitation in the presence of the bound conjugated antibodies at each antigen site (bands). Washing the strips with deionised or distilled water stopped the enzyme-substrate reaction. Depending on the observed banding pattern, one could discern the presence of specific IgM and IgG antibodies (Figure 3). The following antigens were used for IgM antibodies: OspC, VlsE and borrelial membrane protein A (BmpA or p39). For IgG antibodies the following antigens were included: VlsE, BmpA, p83, BBA36, BBO323, Crasp3 and pG. An additional IgM control band for EBV infection was also included: Epstein-Barr Virus capsid antigen gp125. According to the manufacturer, the IgM- and the IgG-banding pattern should be judged together, and a positive WB EL result was defined as at least two IgM or two IgG bands, at least one IgM and one IgG band or a positive IgM band for OspC. The interpretation was indeterminate in the case of only an IgG VlsE band or one IgM band except when OspC was found and negative, in the case of no bands, or when only one IgG band besides VlsE was found.

Figure 3. Photo of a highly positive IgG Virotech EcoLine Western blot (I. Tjernberg).



R&D Systems Quantikine Human CXCL13 Immunoassay (DCX130)

This assay was used for serum and CSF samples in paper IV and is of quantitative sandwich enzyme immunoassay type. A monoclonal antibody specific to CXCL13 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any CXCL13 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific to CXCL13 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, and colour developed in proportion to the amount of CXCL13 bound in the initial step. The colour development was stopped and the intensity of the colour was measured. By using a standard curve based on known CXCL13 concentrations, the concentrations of CXCL13 in the samples could be calculated. The lowest standard concentration was 7.8 pg/mL and the highest was 500 pg/mL, thereby giving the measuring range of the patient samples without additional dilution.

Other laboratory methods

Cell counting of CSF for samples analysed in papers I, II and IV was performed manually at the hospital clinical chemistry laboratories in Kalmar and Jönköping Counties. CSF samples with pleocytosis were defined as ≥ $6*10^6$ leukocytes/L CSF for papers II and IV and as ≥ $20*10^6$ leukocytes/L CSF for paper I. One patient in paper I had $10*10^6$ leukocytes/L CSF, the only patient in paper I with at least 6 but less than $20*10^6$ leukocytes/L CSF. Concentrations of albumin and total concentrations of IgM and IgG in both serum and CSF were determined for paper IV through rate nephelometry using Beckman Coulter instrument Immage 800. The lowest detection limits in CSF were 0.10 g/L albumin, 0.3 mg/L IgM and 10 mg/L IgG.

Samples from Jönköping County were analysed in paper IV regarding anti-*Borrelia* antibodies using the DakoCytomation Lyme borreliosis ELISA kit, 2nd Generation, K0416. This kit, like the IDEIA Lyme neuroborreliosis kit, also used purified native flagellum as test antigen. This ELISA kit was utilised and results interpreted for serum and CSF separately, according to the manufacturer.

Laboratory formulas (paper IV)

The following formulas were used in paper IV:

$$\text{Albumin ratio} = \frac{\text{CSF – albumin (mg/L)}}{\text{Serum – albumin (g/L)}}$$

$$\text{Total - IgM index} = \frac{\text{CSF - IgM (mg(L) / Serum - IgM (g/L)}}{\text{CSF - albumin (mg/L) / Serum - albumin (g/L)}}$$

$$\text{Total - IgG index} = \frac{\text{CSF - IgG (mg/L) / Serum - IgG (g/L)}}{\text{CSF - albumin (mg/L) / Serum - albumin (g/L)}}$$

$$\text{CXCL13 ratio} = \frac{\text{CSF - CXCL13 (pg/mL)} * 1000}{\text{Serum - CXCL13 (pg/mL)}}$$

$$\text{CXCL13/Albumin ratio} = \frac{\text{CSF - CXCL13 (pg/mL)}}{\text{CSF - albumin (g/L)}}$$

Statistics

The following statistical softwares were used in this thesis: Statistica software from Statsoft versions 6.0 (paper I), 7.1 (papers II and III) and 8.0 (paper IV) and MedCalc from MedCalc Software versions 9.4.2.0 (paper III) and 11.2.1.0 (paper IV). In paper I, where different ELISAs were performed on the same sample with positive, negative or indeterminate results, the exact test corresponding to McNemar's paired chi-squared modification, first proposed by Liddell, was chosen (Armitage et al., 2001). For non-paired measurements, Fisher's exact two-tailed test was chosen, and an exact test was used to calculate confidence intervals for the proportions of positive test results for the various ELISAs using the binomial distribution. The material in paper II was divided into two main groups; it was not paired and not normally distributed. Therefore, Mann-Whitney's U-test was chosen in order to compare age differences between groups and Fisher's exact two-tailed test when comparing proportions between the two groups. For paper III, non-parametric data such as OD values, age and symptom duration were compared between groups using Mann-Whitney's U-test. For proportions, Fisher's exact two-tailed test was used. To compare analytical performance of the various tests, including sensitivity and specificity levels, receiver operating characteristic curve (ROC) analyses were performed, and the areas under curve (AUC) were calculated and compared pairwise using a non-parametrical method in the MedCalc software. Finally, in paper IV, symptom

durations between the four groups were compared pairwise using Mann-Whitney's U-test, and AUC were compared for patients with and without LNB. By performing these ROC analyses, the best performance cut-off values could also be obtained for the various laboratory tests used. A p-value < 0.05 was considered significant.

Ethics

All ethical permissions for papers I-IV were obtained from the Regional Ethics Review Board in Linköping, Sweden: Dnr 03-129 and Dnr M47-06 for paper I, Dnr M47-06 for papers II and III, and, finally Dnr M47-06 and Dnr M83-05 T91-08 for paper IV. Informed consent was obtained for prospectively included individuals in paper I.

RESULTS AND DISCUSSION

Erythema migrans (papers I and III)

Clinical features

Altogether, 148 patients with EM were prospectively included. Serum samples were collected at the time of diagnosis, after 2–3 months and after 6 months and were tested for C6 antibodies, VT IgM/IgG, Li IgM/IgG, IR6 peptides and WB EL. General clinical characteristics of these 148 patients are shown in Table 11 and Figure 4 and with regard to previous self-reported episodes of LB in Table 12. Clinical features such as fever, arthralgia, arthritis, headache, other pain, vertigo, fatigue, lymphocytoma and multiple EM were regarded as associated symptoms.

Table 11. Baseline clinical data for patients with erythema migrans, n=148 (papers I and III).

	EM (n=148)	
Median age in years, all (range)	58 (7–84)	
Female		90
Median age in years (range)	58 (7–84)	
Male		58
Median age in years (range)	56 (11–79)	
Tickbite (yes/multiple/suspected/no)[a]	64/19/48/14	
Associated symptoms		46
Median duration in days (range) of EM at presentation[b]	7 (1–45)	

n = numbers

[a]Unknown for 3 patients

[b]Unknown for 23 patients

Although the sample size is relatively small, this EM study (Figures 4 and 5) is similar to previously reported samples from Sweden, regarding seasonal distribution, age distribution and the predominance of women in the middle-age and older age groups (Bennet et al., 2006b, Berglund et al., 1995). Interestingly, at least one previous episode of LB was reported in as many as 46 (31%) of the EM patients (Table 12). In this study the previous occurrence of episodes of LB was not associated with the presence of EM-associated symptoms (p=0.5676). Another interesting finding was that women under the age of 44 did not report previous LB episodes, which confirms a previous report (Bennet and Berglund, 2002). In addition, women reporting previous LB episodes were older than the men (p=0.012). The cause of these findings is unknown, although immunological

differences have been suggested to explain the gender differences (Jarefors et al., 2006).

Figure 4. The age and gender distribution of patients with erythema migrans, n=148 (papers I and III).

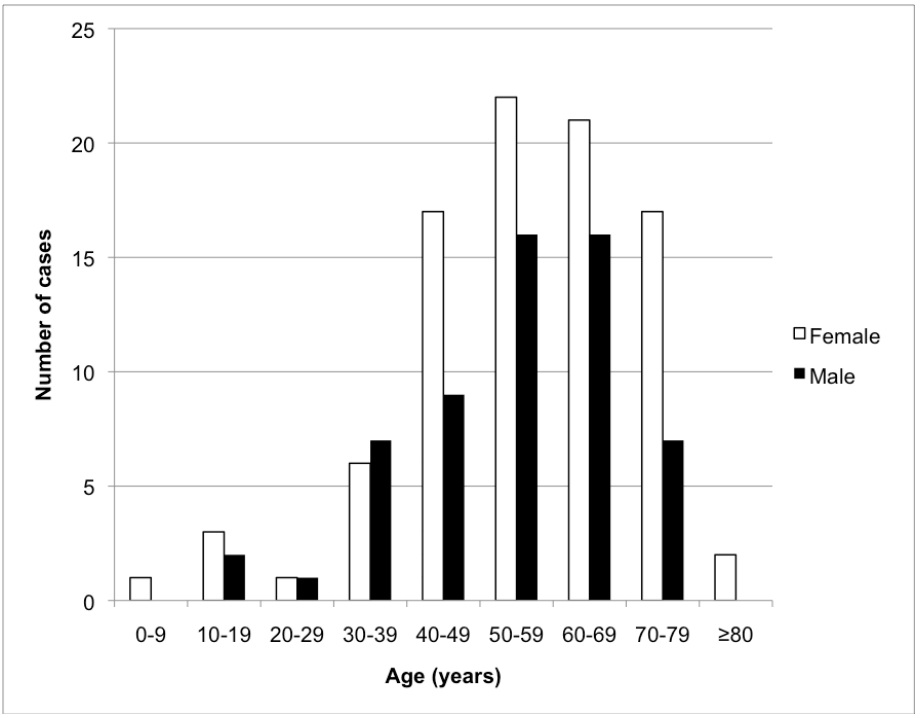


Figure 5. The monthly distribution of diagnosis of patients with erythema migrans, n=148 (papers I and III).

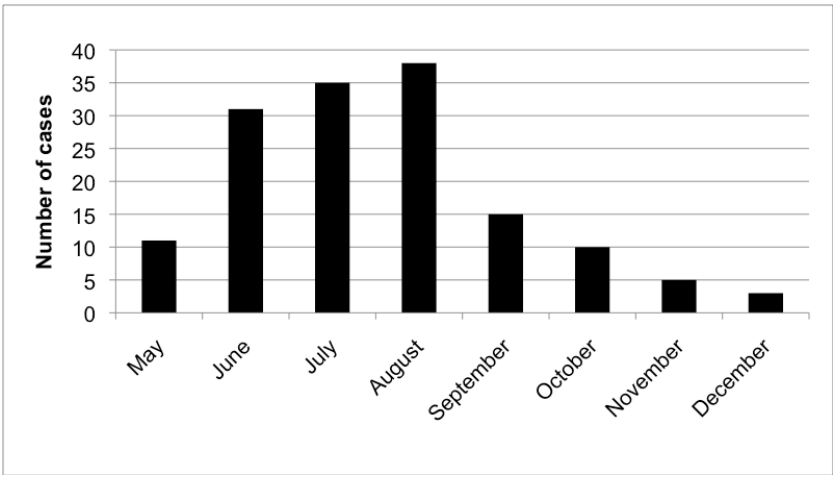


Table 12. Erythema migrans patients sub-grouped, based on self-reported previous Lyme borreliosis, n=143 (papers I and III).

	Previous LB n=46[a]	**%**	**No previous LB n=97[a]**	**%**	**p-value**
Median age in years, all (range)	61 (11–84)		54 (7–83)		0.0100
Female	30	65	58	60	
Median age in years (range)	62 (44–84)[b]		52 (7–83)		
Male	16	35	39	40	
Median age in years (range)	56 (11–69)[b]		56 (18–79)		
Tick bite (yes/multiple/suspected/no)[c]	20/7/14/5		40/11/34/9		
Associated symptoms	13	28	33	34	0.5676
Median duration in days (range)	7 (1–30)		7 (1–45)		0.7663
of EM at presentation[d]					

n = Numbers

[a]Previous episode of LB unknown for 5 patients.

[b]p=0.0120

[c]Unknown for 3 patients

[d]Unknown for 23 patients

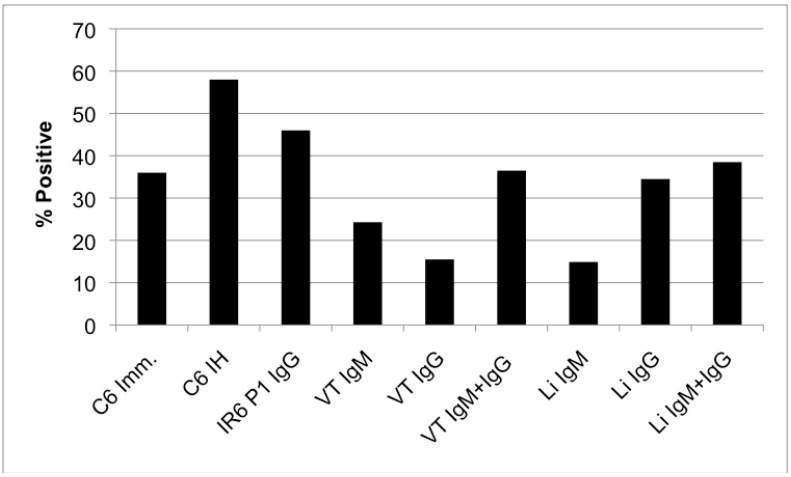
P-values represent comparisons of EM patients with or without previous reported LB.

Serological results

The proportions of positive test results at presentation of EM is shown in Figure 6. Overall, 36–38% of the patients were seropositive for C6 Imm. (IgM/IgG) and the combination (considered positive if either IgM or IgG or both were positive) of VT IgM+IgG and Li IgM+IgG. These results are roughly in line with previously published levels of sensitivity of ELISA tests at presentation of EM, in the range of 20–50% (Bennet et al., 2006a, Hansen and Asbrink, 1989, Wilske et al., 2007). However, in a study by Smismans et al., the seropositivity of the C6 test was 91% in early-localised LB in Dutch patients. This high seropositivity rate could be explained by the patient selection criteria, as all were either positive in IgM and/or IgG immunoblot assay (Smismans et al., 2006). Also, in a study of Finnish and Slovenian patients with EM, the seropositivity of the C6 test varied from 55% to 65% (Sillanpaa et al., 2007). These seropositivty rates are in contrast to the 36% found in paper I. The differences could be explained by differences in patient composition. For instance, in the study by Sillanpää et al. all EM patients from Finland were either culture- or PCR-positive. Time of sampling and differences in the relative distribution of different *Borrelia* genospecies in different geographical locations may also have affected the results.

Usually, the IgM response prevails in EM (Hansen and Asbrink, 1989, Wilske et al., 2007). However, this does not seem to be the case for the VlsE antigen. In a study by Bacon et al., the seropositivity rate was 44% for IgG versus 19% for IgM in acute EM and 59% versus 43%, respectively, in convalescent EM (Bacon et al., 2003). Our finding of a seropositivity rate of 46% for the IgG IR6 peptide P1 at presentation of EM seems to confirm the observation of Bacon et al. (Figure 6, paper III). After adjusting the cut-off for the C6 test to the in-house level, sensitivity increased from 36% to 58% (p=0.002). Thus, it is recommended that clinical laboratories make their own judgement of cut-off levels based on in-house reference materials representative of the catchment area.

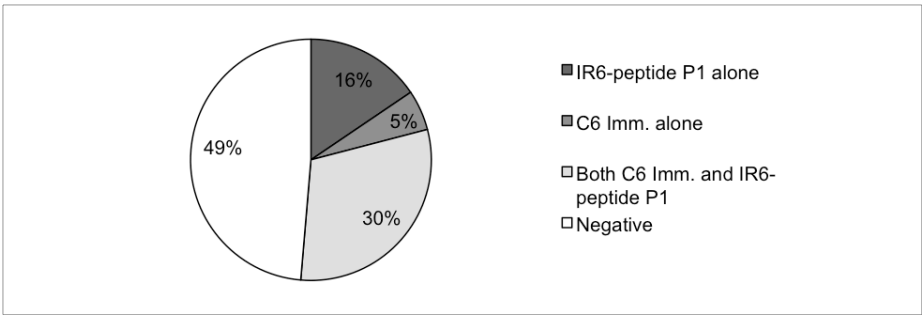
Figure 6. Seropositivity rates in 148 patients with erythema migrans at presentation (papers I and III).



C6 Imm. = C6 Immunetics cut-off, C6 = C6 in-house cut-off, VT = Virotech, Li = Diasorin Liaison, IR6 P1 = invariable region 6, peptide 1, *B. burgdorferi* s.s.

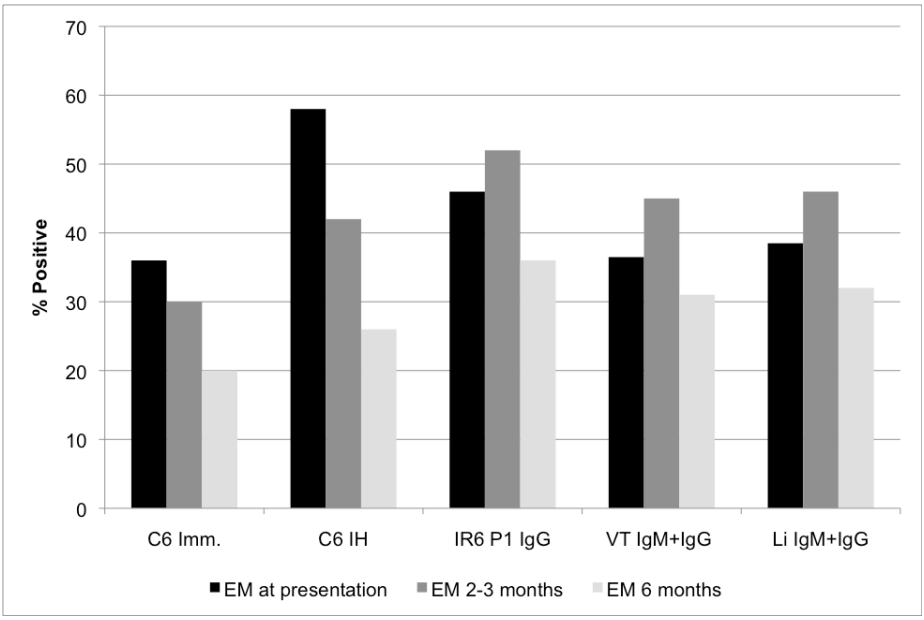
By combining assays based on the same or different antigens from the various European strains of *Borrelia*, one could possibly achieve higher seropositivity. Figure 7 displays the distribution of C6 Imm. (*B. garinii*) and IR6 peptide P1 (*B. burgdorferi* s.s.) seropositivity in 148 EM patients at presentation. Although the majority of seropositive samples were positive for both C6 Imm. and IR6 peptide P1, indicating a reasonable level of cross-reactivity among IR6 peptides, some samples were positive for only one of the assays. Thus, in the light of a positive result for either or both assays, a sensitivity of 51% could be achieved.

Figure 7. C6 Imm. and IR6 peptide P1 seropositivity in 148 patients with erythema migrans at presentation (paper III).



All EM patients were treated with antibiotics, and follow-up sera were sampled 2–3 months and 6 months post-presentation in order to determine to what extent seropositivity persisted (see Figure 8 for the C6 test and IR6 P1 together with the combined interpretation of IgM+IgG for VT and Li).
The seropositivity rates of C6 Imm. were significantly lower than the combined IgM+IgG VT and Li ELISAs, both at 2–3 months ($p<0.002$) and at 6 months ($p<0.005$). Although the C6 test is based on an IR6 peptide, the dynamics of the antibody response in the C6 test following EM differs from the IR6 peptide response to P1 (paper III). Apparently, the antibody dynamics following EM vary depending on the antigen used in the ELISAs. In addition, the commercial C6 test detects both IgM and IgG antibodies, which may explain the difference in dynamics to some extent. The reduced seropositivity rate in the C6 IH between EM at presentation and at 2–3 months ($p=0.0074$), with further reduction at 6 months ($p=0.0069$), is also in line with the rapidly waning IR6/C6 antibody response in both antibiotically treated animals with LB and humans with EM. It has actually been suggested that a quantification of C6 antibodies could be used to assess whether a *B. burgdorferi* infection has been eliminated (Philipp et al., 2001, Philipp et al., 2005). However, the prognosis for pcV-treated EM is very good, with a 98% chance of complete recovery (Bennet et al., 2003), and persistent IR6 antibody responses have been found in successfully treated patients with both early and late manifestations as well as in healthy persons (Peltomaa et al., 2003, Strle and Stanek, 2009). This argues against the use of the C6 test as a marker of active infection.

Figure 8. Seropositivity rates in 148 patients with erythema migrans over time (papers I and III).



C6 Imm. = C6 Immunetics cut-off, C6 = C6 in-house cut-off, IR6 P1 = invariable region 6, peptide 1, *B. burgdorferi* s.s.,VT = Virotech, Li = Diasorin Liaison

Diagnosis of EM in both Europe and North America is generally clinical, based on the patient's history together with the typical clinical skin rash (Nadelman and Wormser, 1998, Stanek et al., 2010, Wilske, 2005, Wormser et al., 2006). Serology in EM is of little use for clinical EM diagnosis, but serological reactions in EM are of interest in determining the early immune response in LB. As diagnosis is typical and EM is the most common manifestation of LB, it is an easy and suitable group of patients to study. Serological reactions in this early phase of the infection, together with serological reactions in other LB manifestations, may also be useful when evaluating the sensitivity of serodiagnostic kits for LB. Serological reactions over time in patients treated for EM are also of interest in understanding to what extent this group of patients might add to the seroprevalence in the healthy population.

In conclusion, serosensitivity in EM at diagnosis is generally low among the C6, VT and Li test kits. However, by adjusting the cut-off level of the C6 test to an in-house level, the sensitivity was increased substantially. One other possible way of increasing serosensitivity in European EM could be to combine ELISAs using different antigens, as exemplified by the combination of the C6 test and an IR6 peptide ELISA from another *Borrelia* strain. Finally, over time, the C6 antibody response differed from the other evaluated tests in EM patients, which may be favourable regarding the usefulness of the test in case of re-infection.

Lyme neuroborreliosis (papers II and IV)

Clinical features

In paper II a retrospective study of LNB patients from Kalmar County was conducted. A total of 124 AI-positive patients for whom CSF cell counts had been performed was identified between 2003–2005. An equal number of AI-negative patients was identified and matched to the AI-positive patients with regard to sex, age and sample year. Paired serum and CSF samples were available from all patients. All 248 patients had been referred to the laboratory to either confirm or rule out LNB. Thus, clinically, they all had symptoms and signs consistent with LNB. The type and duration of symptoms by lumbar puncture were collected from medical records and are shown in Table 13. Symptoms such as fever, dizziness, headache, fatigue, nausea and non-specific pain were regarded as general symptoms, while paralysis, radicular pain and loss of sensation were noted as focal neurological symptoms or signs. Cranial nerve palsy was a common cause of neurological affection in the AI-positive group, reported in 51 out of 124 cases (41%), similar to the 46% reported in a Danish study and 37% in another Swedish study (Henningsson et al., 2010, Hansen and Lebech, 1992). A favourable clinical outcome within 6 months after lumbar puncture was more common in the AI-positive group than in the AI-negative group (p=0.0061).

Table 13. Characteristics of 248 patients sampled to confirm or rule out Lyme neuroborreliosis, grouped based on the results of specific anti-*Borrelia* antibody cerebrospinal fluid index (paper II).

Variable	AI positive, n=124	%	AI negative, n=124	%
Female / Male	55/69	44/56	55/69	44/56
Median age in years (range)	40 (2–87)		40 (2–84)	
General symptoms[a]	91	73	92	74
Median duration of symptoms in days (range)	17 (1 to > 6 months)		14 (0 to > 6 months)[b]	
Neurological symptoms[a]	76	61	71	57
Median duration of symptoms in days (range)	3 (-5 to > 6 months)		3 (0 to > 6 months)	
Cranial nerve palsy	51	41	33	27
Healed within 6 months, n/total known	81/101	80	55/89	62

n = numbers

AI = specific anti-*Borrelia* antibody cerebrospinal fluid index

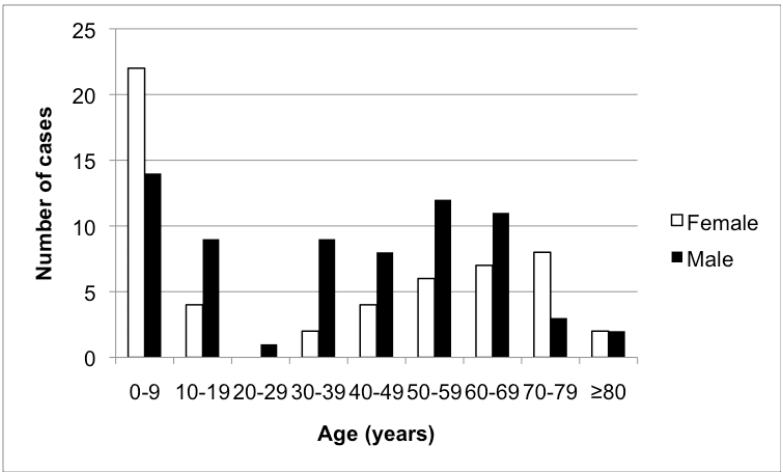
[a] Each patient may have several different symptoms at once

[b] Unknown for one patient

A male-to-female ratio of 1.25 was found in paper II, not quite as high as previously described (1.5) (Hansen and Lebech, 1992). The age and gender distributions among AI-positive cases are shown in Figure 9. A bimodal

distribution was found, with two peaks: one in childhood and one in the 50–70 age range, similar to that shown in other studies (Hansen and Lebech, 1992, Henningsson et al., 2010).

Figure 9. The age and gender distribution in 124 patients with positive specific anti-*Borrelia* antibody cerebrospinal fluid index (paper II).



AI-positive and -negative patients were also sub-grouped according to the presence or absence of CSF pleocytosis. Clinical characteristics of these four groups are shown in Tables 14 and 15. In AI-positive patients, pleocytosis was associated with lower age, occurrence of general symptoms, cranial nerve palsy and clinical outcome. Pleocytosis in AI-positive patients was also associated with the specific type of positive AI: an AI-positive finding of both IgM and IgG was more common in the pleocytosis group, while an IgG-positive AI was more common in the non-pleocytosis group. This could be explained by differences in disease duration between the two groups. AI-positive patients without pleocytosis could represent patients in a later stage, with a lower bacterial burden in the CSF and a correspondingly lower level of inflammatory activity.

In the AI-negative patients, pleocytosis was associated with lower age, occurrence of neurological symptoms, particularly cranial nerve palsy and clinical outcome.

To conclude the characterisation of patients, pleocytosis was associated with lower age, cranial nerve palsy and a favourable clinical outcome in both the AI-positive and AI-negative groups. In the AI-positive group, pleocytosis was also associated with the specific IgM/IgG AI index and the occurrence of general symptoms, while pleocytosis in the AI-negative group was also associated with the occurrence of neurological symptoms.

Table 14. Characteristics of 124 patients positive for specific anti-*Borrelia* antibody cerebrospinal fluid index, sub-grouped according to presence or absence of pleocytosis of cerebrospinal fluid (paper II).

Variable	Pleocytosis (n=93)	%	No pleocytosis (n=31)	%	p-value
Female / Male	45/48	48/52	10/21	32/68	0.1456
Median age in years (range)	30 (2–87)		50 (13–82)		0.0060
General symptoms[a]	74	80	17	55	0.0100
Median duration of symptoms in days (range)	17 (1–74)		30 (3 to > 6 months)		
Neurological symptoms[a]	61	66	15	48	0.0946
Median duration of symptoms in days (range)	3 (-5–80)		14 (1 to > 6 months)		
Cranial nerve palsy	46	49	5	16	0.0013
AI pos IgM	10	11	6	19	0.2266
AI pos IgG	30	32	22	71	0.0003
AI pos IgM and IgG	53	57	3	10	<0.0001
Healed within 6 months, n/total known	71/82	87	10/19	53	0.0023

n = numbers

Pleocytosis = total white blood cell count ≥ $6*10^6$/L cerebrospinal fluid

[a] Each patient may have several different symptoms at once

Table 15. Characteristics of 124 patients negative for specific anti-*Borrelia* antibody cerebrospinal fluid index, sub-grouped according to presence or absence of pleocytosis of cerebrospinal fluid (paper II).

Variable	Pleocytosis (n=31)	%	No pleocytosis (n=93)	%	p-value
Female / Male	11/20	35/65	44/49	47/53	0.2996
Median age in years (range)	8 (2–67)		50 (2–84)		<0.0001
General symptoms[a]	23	74	69	74	1
Median duration of symptoms in days (range)	7 (2–110)		24.5 (0 to > 6 months)[b]		
Neurological symptoms[a]	28	90	43	46	<0.0001
Median duration of symptoms in days (range)	2 (0–110)		8 (0 to > 6 months)		
Cranial nerve palsy	19	61	14	15	<0.0001
Healed within 6 months, n/total known	20/25	80	35/64	55	0.0310

n = numbers

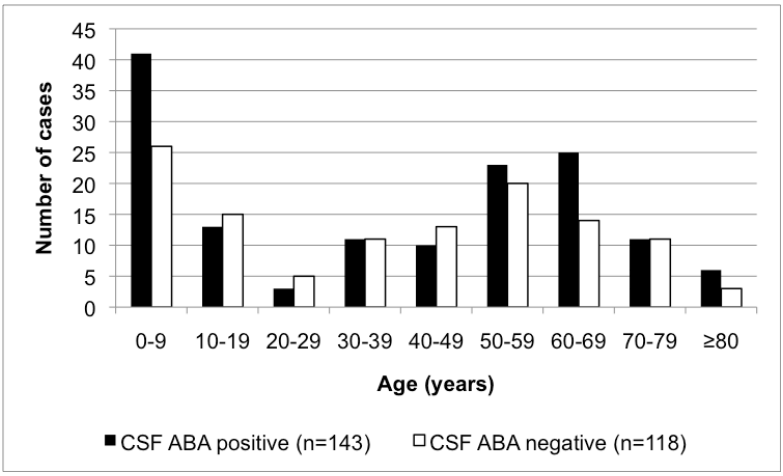
Pleocytosis = total white blood cell count ≥ $6*10^6$/L cerebrospinal fluid

[a] Each patient may have several different symptoms at once

[b] Unknown for one patient

Paper IV included the 248 patients from paper II along with an additional 115 patients from Jönköping County, for a total of 363 patients. Paired CSF and serum samples from all patients were sent to the laboratory to either confirm or to rule out LNB. Paired sample volumes were sufficient in 143 CSF ABA-positive cases and 118 CSF ABA-negative cases, for a total of 261 patients. The age and gender distributions of CSF ABA-positive and -negative patients are shown in Figure 10. Although samples from 102 patients could not be studied further, the remaining 261 patients generally adhere to the bimodal distribution of age, as previously shown in LNB (Hansen and Lebech, 1992); thus it is considered representative.

Figure 10. The age and gender distribution in 261 patients sampled to confirm or rule out Lyme neuroborreliosis, grouped based on the results of cerebrospinal fluid anti-*Borrelia* antibodies (paper IV).

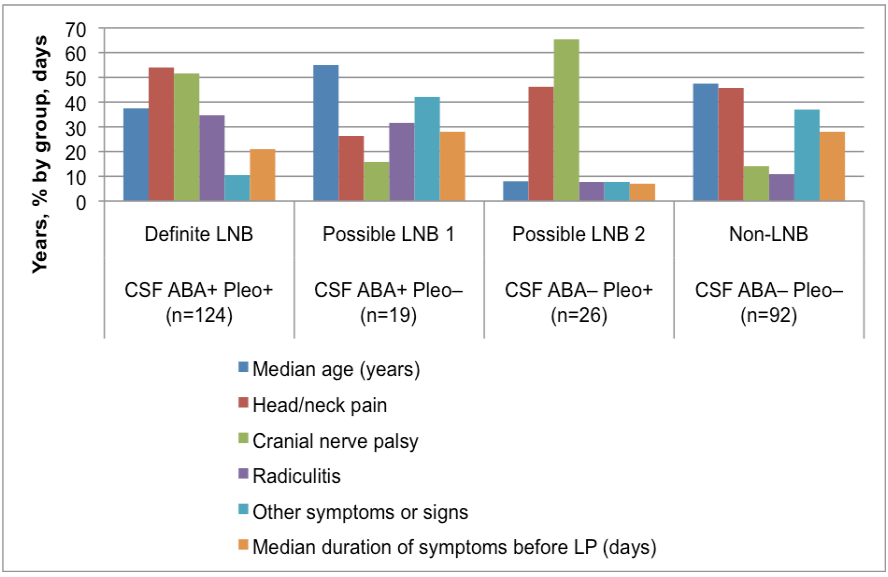


CSF ABA = cerebrospinal fluid anti-*Borrelia* antibodies

The 261 patients in paper IV were also sub-grouped into four separate groups according to the presence or absence of pleocytosis in CSF, in addition to CSF ABA status. The clinical characteristics of these groups are shown in Figure 11. All symptoms or signs other than head/neck pain, cranial nerve palsy and radiculitis were regarded as “other symptoms or signs”. Median age varied. In the group with pleocytosis but negative for CSF ABA (possible LNB 2), the median age was eight years of age. Cranial nerve palsy and short median duration of symptoms (seven days) were also noted in a large proportion (65%) of this group. Considering a reported sensitivity of only 17% for AI in patients with symptom duration of less than eight days (Hansen and Lebech, 1991), a diagnosis of LNB could still be possible. In fact, it was considered likely in 17 of these 26 patients, based on a clinical finding of facial palsy (15) or meningitis (2), sample months, the short duration of symptoms and the status of serum anti-*Borrelia* antibodies. These 17 patients were all children between 2 and 13 years of age.

Also further studied was the other group of possible LNB, group 1, positive for CSF ABA but without pleocytosis. The finding of a positive IgG with negative IgM CSF ABA was common in this group (17/19), and a median symptom duration of four weeks, together with the lack of pleocytosis, suggests that these patients had had previous episodes or possibly prolonged symptoms of LNB. This is supported by the fact that CSF ABA may persist for months and years, even after clinical recovery from LNB (Hammers-Berggren et al., 1993, Hansen and Lebech, 1991). In one of the two remaining patients in possible LNB group 1, ongoing LNB was considered probable due to both IgM- and IgG-positive CSF ABA and a symptom duration of 1.1 weeks. The final patient was positive in IgM CSF ABA only, had a symptom duration of 26 weeks and was not considered to have ongoing LNB.

Figure 11. Clinical characteristics of 261 patients sampled to confirm or rule out Lyme neuroborreliosis, grouped based on cerebrospinal fluid results (paper IV).



CSF ABA = cerebrospinal fluid anti-*Borrelia* antibodies. Pleo = pleocytosis of CSF (total white blood cell count $\geq 6*10^6$/L CSF). All symptoms or signs other than head/neck pain, cranial nerve palsy and radiculitis were regarded as "other symptoms or signs." LP = lumbar puncture.

Results of diagnostic tests in serum (papers II and IV)

The results of the C6 test in serum were examined in relation to the duration of the symptom, with the longest duration at lumbar puncture, in order to gauge the performance of the test and rule out LNB. The results for the 124 AI-positive and 123/124 AI-negative patients from paper II are shown in Tables 16 and 17. Regarding the AI-positive patients, sensitivity of the C6 test in serum increased with increased symptom duration. A serosensitivity of 93% was noted in patients

with symptom duration of more than 30 days. Because immunoglobulin concentrations gradually increase with age, in particular in younger children, we grouped patients using 12 years of age as a cut-off point. We considered patients of at least 12 years of age to have reached adult levels. This cut-off has also been used in determining antibody concentrations in childhood (Schauer et al., 2003b, Schauer et al., 2003a). In AI-positive patients with pleocytosis, the C6 Imm. was positive in 51/54 (94%) patients ≥ 12 years of age and in 32/39 (82%) patients < 12 years of age (p=0.0882, data not shown).

Table 16. Characteristics in relation to symptom duration at lumbar puncture in 124 patients with positive specific anti-*Borrelia* antibody cerebrospinal fluid index (paper II).

	< 8 d		**8–14 d**		**15–30 d**		**> 30 d**	
C6 Imm. positive results (%)								
All AI positive (n=124)	21/31	(68)	19/24	(79)	34/40	(85)	27/29	(93)
AI positive with pleocytosis (n=93)	18/22	(82)	19/22	(86)	31/34	(91)	15/15	(100)
AI positive without pleocytosis (n=31)	3/9	(33)	0/2	(0)	3/6	(50)	12/14	(86)
AI positive age < 12 years (n=39)	6/9	(67)	12/15	(80)	12/13	(92)	2/2	(100)
AI positive age ≥ 12 years (n=85)	15/22	(68)	7/9	(78)	22/27	(81)	25/27	(93)

n = numbers
d = days
C6 Imm. = Immunetics Quick ELISA C6 *Borrelia* assay kit
AI = specific anti-*Borrelia* antibody cerebrospinal fluid index
Pleocytosis = total white blood cell count ≥ $6*10^6$/L cerebrospinal fluid

Table 17. Characteristics in relation to symptom duration at lumbar puncture in 123 patients with negative specific anti-*Borrelia* antibody cerebrospinal fluid index (paper II).

	< 8 d		**8–14 d**		**15–30 d**		**> 30 d**	
C6 Imm. positive results (%)								
All AI negative (n=123)	21/52	(40)	6/12	(50)	7/19	(37)	15/40	(38)
AI negative with pleocytosis (n =31)	9/17	(53)	5/6	(83)	1/5	(20)	1/3	(33)
AI negative without pleocytosis (n=92)	12/35	(34)	1/6	(17)	6/14	(43)	14/37	(38)
AI negative age < 12 years (n=34)	10/18	(56)	5/8	(62)	2/3	(67)	3/5	(60)
AI negative age ≥ 12 years (n=89)	11/34	(32)	1/4	(25)	5/16	(31)	12/35	(34)

n = numbers
d = days
C6 Imm. = Immunetics Quick ELISA C6 *Borrelia* assay kit
AI = specific anti-*Borrelia* antibody cerebrospinal fluid index
Pleocytosis = total white blood cell count ≥ $6*10^6$/L cerebrospinal fluid

The C6 test in serum was positive in 50/124 (40%) AI-negative patients, indicating that the test has a specificity of roughly 60% (calculated data, not shown). However, the specificity may have been underestimated, as there may be falsely AI-negative cases of LNB in this group, which presented with short symptom duration. In fact, 52/123 (42%) actually presented with a symptom duration of less than eight days, compared to 31/124 (25%) in the AI-positive group (p=0.0047).

It has been shown that the serosensitivity of flagella-based ELISA is 100% in patients with LNB and a symptom duration of at least 42 days (Hansen and Lebech, 1991). The high sensitivity in AI-positive patients with a symptom duration of more than 30 days, studied in paper II, confirms these data using the C6 peptide as antigen. The findings of paper II are further confirmed by the 100% serosensitivity of the C6 test in 14 Finnish and 98% in 60 Norwegian LNB cases, together with a specificity of 61% in a clinical control group (Sillanpaa et al., 2007, Skarpaas et al., 2007).

In conclusion, as shown in paper II, a negative C6 test in serum in a patient of at least 12 years of age and with a symptom duration of more than 30 days argues against LNB. A patient with a positive C6 test result should be further investigated with lumbar puncture for a diagnosis of LNB. Lumbar puncture should also be performed in children < 12 years of age in cases of suspected LNB, as the C6 test in serum might be falsely negative.

Levels of serum-CXCL13 were measured in the various groups in paper IV, and ROC analysis was performed using the definite LNB (n=124) and non-LNB (n=92) groups. The use of serum CXCL13 led to a poor differentiation among the groups, with a sensitivity of 47%, a specificity of 80% and an AUC of 0.634 (data not shown). These findings were consistent with previously published studies of serum CXCL13 in LNB (Rupprecht et al., 2009). Therefore, the use of CXCL13 in serum cannot alone be recommended as a diagnostic tool in suspected LNB.

Results of tests in CSF and CSF-to-serum ratios (paper IV)

Concentrations of CXCL13, albumin, total IgM and IgG in CSF and serum were measured together with C6 antibodies in CSF in the 261 patients in paper IV. Patients were categorised as "definite LNB" and "non-LNB," and ROC analyses were performed to enable comparisons of the diagnostic performance of the different parameters and to find the best performance cut-off values. The most interesting results of these ROC analyses are shown in Table 18.

No significant differences were noted regarding the various ways in which AUC expressed CSF CXCL13, either by itself or divided by serum CXCL13 or CSF albumin. Originally, CXCL13 in CSF of LNB patients was reported in ratio to the total protein concentration in CSF in order to compensate for damage to the blood-brain barrier (Rupprecht et al., 2005). However, the CSF-to-serum CXCL13 ratio has been reported to differentiate patients with neurosyphilis from LNB patients more effectively than the CSF CXCL13-to-CSF protein ratio (Rupprecht et al., 2007, Rupprecht et al., 2009). CXCL13 performed very well as a CSF marker of LNB, which is in line with previous reports (Ljostad and Mygland, 2008, Rupprecht et al., 2005, Senel et al., 2010).

Interestingly, performance of the total IgM index was high, with a sensitivity of 100%, a specificity of 92% and an AUC of 0.987 confirming earlier data (Tumani et al., 1995). Regarding the C6 test in CSF, a sensitivity of 99% and a specificity of 88% could be achieved, which was similar to the findings Skarpaas et al. but higher than the sensitivity found by Vermeersch et al., perhaps due to differences in selection criteria (Skarpaas et al., 2007, Vermeersch et al., 2009).

Table 18. Diagnostic performance of various laboratory parameters in cerebrospinal fluid and serum in Lyme neuroborreliosis (LNB), based on definite LNB (n=124) and non-LNB (n=92) cases (paper IV).

Analysis	Cut-off (>)	Sensitivity (%)	Specificity (%)	ROC AUC
Total-IgM Index	0.234	100	92	0.987
CSF-CXCL13 (pg/ml)	142	98	98	0.984
CSF-S CXCL13 ratio * 1000	354	99	96	0.991
CSF-CXCL13/Albumin (ng/g)	163	98	95	0.966
CSF-C6 LI	0.680	94	98	0.989
CSF-C6 mean LI + 3 SD of 92 negative controls[a]	0.602	94	97	N/A
CSF-C6 mean LI + 3 SD of 63 negative controls[b]	0.092	99	88	N/A

ROC = receiver operating characteristic analysis
AUC = area under curve
CSF = cerebrospinal fluid
S = serum
LI = Lyme index, calculated by dividing sample optical density (OD) with calibrator OD plus 0.3
SD = standard deviation
[a] Lacking CSF pleocytosis, intrathecal anti-*Borrelia* antibodies or showing negative anti-*Borrelia* antibody index
[b] As for [a] but also lacking serum anti-*Borrelia* antibodies
n/a = not applicable
The Immunetics C6 Lyme ELISA kit was performed on CSF using a sample dilution of 1:20 if not otherwise specified.

Using the cut-off levels defined by ROC analyses of the definite LNB and non-LNB groups in Table 18, the positivity rates for the two possible LNB groups could be calculated. These rates are shown, together with the positivity rates for the definite LNB and non-LNB groups, in Table 19.

Regarding both possible LNB groups, LNB was considered probable in 1 out of 19 patients in possible LNB group 1, but CSF CXCL13 and the C6 test were negative in this patient. In possible LNB group 2, a diagnosis of LNB was considered likely in at least 17 out of 26 patients. CSF-CXCL13 was positive in 15 of these 17 (88%), the CSF-to-serum CXCL13 ratio was positive in 17/17 (100%) and CSF C6 antibodies were positive in 13/17 (76%) at cut-off 0.092 (data not shown in Table).

Table 19. Proportion of positive (%) per parameter and diagnostic group in 261 patients investigated for suspected Lyme neuroborreliosis, based on cut-off values obtained in Table 18 (paper IV).

	n=124	*n=19*	*n=26*	*n=92*
CSF Anti-*Borrelia* antibodies[a]	+	+	−	−
CSF Pleocytosis	+	−	+	−
	Definite LNB	Possible LNB 1	Possible LNB 2	Non-LNB
Total-IgM Index	100	5.3	73	7.6
CSF-CXCL13	98	0.0	65	2.2
CSF-S CXCL13 ratio * 1000	99	0.0	73	4.3
CSF-CXCL13/Albumin	98	0.0	73	5.4
CSF-C6 cut-off at LI 0.092	99	37	54	12
Both CSF-S CXCL13 ratio and CSF-C6 cut-off at LI 0.092	99	0.0	54	2.2

n = numbers

CSF = cerebrospinal fluid

[a] Either *Borrelia*-specific antibody index or intrathecal anti-*Borrelia* antibodies

Pleocytosis = total white blood cell count ≥ $6*10^6$/L CSF

S = serum

LI = Lyme index, calculated by dividing sample optical density (OD) by calibrator OD plus 0.3.

The Immunetics C6 Lyme ELISA kit was performed on CSF using a sample dilution of 1:20 if not otherwise specified.

Increased levels of CSF CXCL13 have been reported in neurosyphilis as well as in LNB, but CSF-to-serum CXCL13 ratios are higher in LNB compared to neurosyphilis patients, primarily due to higher serum CXCL13 levels in neurosyphilis (Rupprecht et al., 2007, Rupprecht et al., 2009). Also, increased CSF CXCL13 levels have been reported in LNB patients compared to patients with multiple sclerosis, albeit generally at higher levels (Ljostad and Mygland, 2008, Rupprecht et al., 2005, Krumbholz et al., 2006, Sellebjerg et al., 2009). With this in mind, we also evaluated the CSF-to-serum CXCL13 ratio in parallel with CSF C6 antibodies (cut-off at 0.092) in order to gain specificity. Using this combination and considering the result as positive only when both markers were positive, the sensitivity in definite LNB was 99% and the specificity in the non-LNB group reached 98%. This was not statistically different from the specificity of the CSF-to-serum CXCL13 ratio alone, which had a specificity of 96% in the same group.

To date, there is only limited data on the performance of C6 antibodies in CSF of LNB. In paper IV, we reported a sensitivity of 99% and a specificity of 88% similar to that reported by Skarpaas et al. (2007). However, a lower sensitivity of 61-68% has also been reported (Vermeersch et al., 2009). At present there is no officially recommended method for C6 antibodies in CSF and data is limited and somewhat contradicting. Therefore, and because no significant difference in diagnostic performance could be detected comparing a combination of CSF-to-

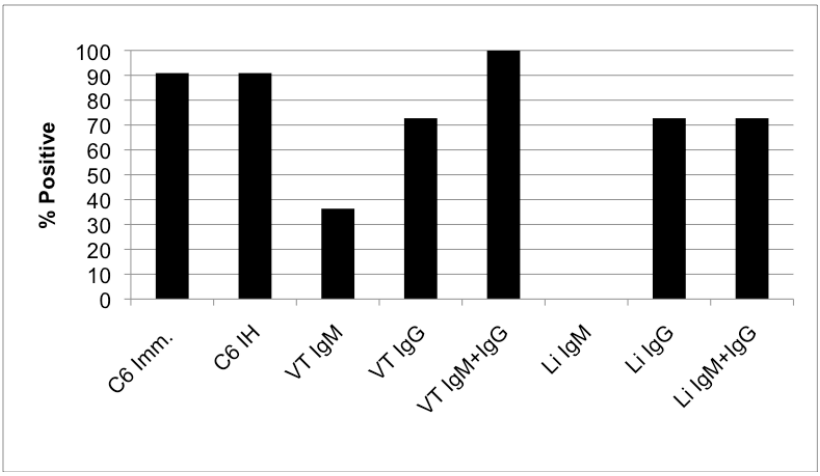
serum CXCL13 ratio and CSF C6 antibodies to the CSF-to-serum CXCL13 ratio alone, further evaluation of the C6 test in CSF is required to adopt it as a standard method for detection of CSF ABA.

In conclusion, CSF CXCL13 performed well as a marker of LNB and also detected highly probable cases of LNB among children with short symptom duration where CSF ABA were still negative.

Acrodermatitis chronica atrophicans and Lyme arthritis (paper I)

Only a few cases of other LB manifestations were investigated in this thesis, and LB manifestations other than EM and LNB are uncommon in Sweden (Berglund et al., 1995). The limited number of patients and available samples makes evaluating serodiagnostic tests difficult. In paper I a total of nine patients with ACA were studied, together with three cases of LA. However, serum from one of the LA cases was negative in all studied tests (C6 Imm., C6 IH, VT and Li tests and WB EL). This is unlikely in a case of LA (Aguero-Rosenfeld et al., 2005), and the case was therefore excluded from the calculations shown in Figure 12. As both ACA and LA are considered stage III chronic LB and the studied number of cases was low, these two groups were combined. As expected, the dominant response was type IgG, as shown by the VT and Li test results (Wilske, 2005). No significant differences in serosensitivity could be determined when comparing the combined IgM and IgG VT and Li tests and the C6 test. Somewhat surprising in stage III LB, 3 of the 11 patients showed a positive VT IgM response, while VT IgG was negative. A serosensitivity of 91% was achieved for the C6 test using either the manufacturer's cut-off or the in-house cut-off, which is in line with the sensitivity of 89% achieved for the C6 test in 56 American and 93% in 14 Finnish LA cases (Sillanpaa et al., 2007). Perhaps the expected 100% serosensitivity could not be achieved by the C6 test in these cases because it is based on *B. garnii*, which does not cause LA in North-America.

Figure 12. Seropositivity rates in nine patients with *acrodermatitis chronica atrophicans*, together with two patients with Lyme arthritis (paper I).

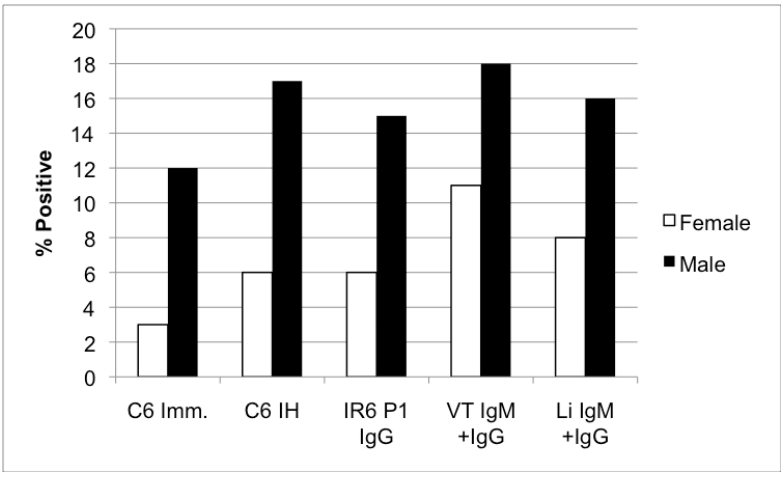


C6 Imm. = C6 Immunetics cut-off, C6 = C6 in-house cut-off, VT = Virotech, Li = Diasorin Liaison

Blood donors (papers I and III)

The seropositivity rates for the combined VT and Li IgM+IgG tests along with C6 Imm. and C6 IH results for the 100 female and 100 male blood donors are shown in Figure 13. General seropositivity rates were 8% for C6 Imm., 12% for C6 IH, 10% for IR6 P1 IgG, 14% for VT and 12% for the Li. The C6 Imm. seropositivity rate was less than the VT and Li tests ($p \leq 0.012$). Using a combination of the C6 Imm. and/or the IR6 P1 IgG test, 21 of the 200 (10%) would be positive. Significantly more men than women ($p \leq 0.029$) were seropositive in the C6 test using both cut-offs. However, there were no differences in sex distribution between the IR6 P1 IgG, the VT and the Li tests. The reason for the variations in seroprevalence in respect to sex in the C6 test is unknown, but higher *Borrelia* antibody seroprevalence in men (23.6%) than in women (16.7%) has also been found in the population of Åland Islands (Carlsson et al., 1998).

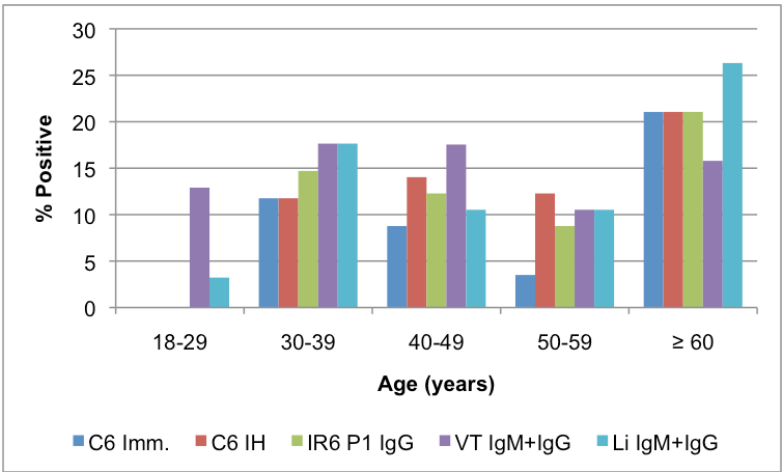
Figure 13. Seropositivity rates in 200 blood donors: 100 female and 100 male (papers I and III).



C6 Imm. = C6 Immunetics cut-off, C6 = C6 in-house cut-off, IR6 P1 = invariable region 6, peptide 1, *B. burgdorferi* s.s., VT = Virotech, Li = Diasorin Liaison

Regarding age, it would be reasonable to expect an increasing seropositivity rate with increasing age, as the accumulated risk of previous exposure to *B. burgdorferi* s.l. would also increase. Figure 14 shows the seropositivity rates for the various tests for blood donors according to age group.

Figure 14. Seropositivity rates in 198 blood donors with respect to age (papers I and III).



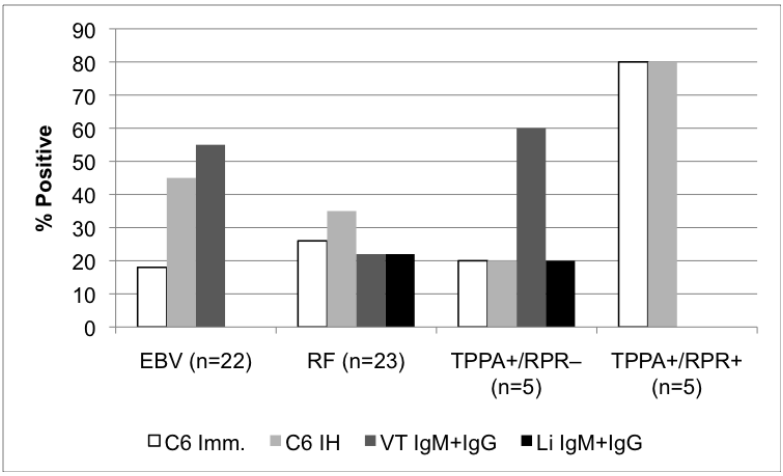
C6 Imm. = C6 Immunetics cut-off, C6 = C6 in-house cut-off, IR6 P1 = invariable region 6, peptide 1, *B. burgdorferi* s.s., VT = Virotech, Li = Diasorin Liaison. Information on age is missing for two blood donors.

Indeed, seropositivity rates were higher among blood donors at least 60 years of age compared with donors 18–29 years of age for the C6 test (both C6 Imm. and C6 IH, p=0.0168), the IR6 P1 IgG (p=0.0168) and the Li test (p=0.0244), but not for the VT test. An increasing seroprevalence with age has also been shown in the Åland Islands (Carlsson et al., 1998). Therefore, age is important when interpreting a positive LB serological test, especially in a highly endemic area. The positive predictive value for ongoing LB would therefore be lower in elderly patients than in children for the C6, the IR6 P1 IgG and the Li tests.

Conditions with potentially cross-reacting antibodies (paper I)

A number of conditions are known to cause cross-reactions in LB serological tests. The seropositivity rates of the various studied tests were therefore assessed in a selection of patients with such conditions (Figure 15).

Figure 15. Seropositivity rates in 55 control patients (paper I).



C6 Imm. = C6 Immunetics cut-off, C6 = C6 in-house cut-off, VT = Virotech, Li = Diasorin Liaison, EBV = Epstein-Barr virus infection, RF = rheumatoid factor positive, TPPA+/RPR– = syphilis healed, TPPA+/RPR+ = syphilis ongoing

Considering EBV infection, the VT ELISA seropositivity was high at 55%, mostly type IgM. In no sample was the IgG VT positive when IgM VT was negative. For the RF-positive a general seropositivity rate of 22–35% was noted. Full concordance for the four tests was achieved in 17 negative and 5 positive samples out of 23 (96%), suggesting previous exposure to *B. burgdorferi* s.l. as a cause of the reactive pattern rather than cross-reactions in these samples: this was confirmed by WB EL (see next section). In sera from a limited study of syphilis patients, cross-reactivity in the C6 test seemed high in patients with laboratory signs of ongoing syphilis. These C6 Imm. seropositivity figures are in contrast to those published by Sillanpää et al., who found that none of the 63 patients had a positive C6 test despite serological signs of syphilis, RF, recent EBV infection, anti-nuclear antibodies or infection with *Salmonella* or *Yersinia enterocolitica* (Sillanpaa et al., 2007). This difference could be caused by different selection criteria for the controls but could also reflect a difference in seroprevalence of C6 antibodies in the general population, as none of the 20 tested Finnish blood donors were positive in the C6 test (Sillanpaa et al., 2007).

Two-tiered testing and Western blot results (previously unpublished)

The current recommendation of LB serological testing in both Europe and the USA is a two-step protocol (Centers for Disease Control and Prevention, 1995, Johnson et al., 1996, Wilske, 2005). However, the development in recent years of more specific ELISAs based on recombinant proteins such as VlsE and the synthetic peptide C6 have raised the question of whether it is possible to abandon this strategy (Stanek et al., 2010, Strle and Stanek, 2009). Therefore, Table 20 compares seropositivity rates for C6 Imm. with a two-tiered testing approach, using C6 Imm. together with WB EL. Table 21 shows the corresponding seropositivity rates of the VT IgM+IgG ELISA and a two-tiered test using the VT ELISA together with WB EL. Patients from paper I were studied. Of the original 26 LNB patients, a selection of 21 AI-positive with pleocytosis (definite LNB) was used for this analysis to ensure that only true LB cases were included. Only results that were either borderline or positive in the first step and subsequently positive in WB EL were considered positive; all others were considered negative in the two-tiered test.

Table 20. Seropositivity rates in C6 Imm. and two-tiered testing using C6 Imm. in combination with Virotech Ecoline Western blot in various LB manifestations and controls (paper I).

Condition	C6 Imm.	Two-tier (C6 Imm. and WB EL)	p-value[a]
EM at presentation (n=148)	36%	33%	0.344
LNB (=21)	95%	67%	0.031
ACA/LA (n=11)	91%	82%	1.000
BD (n=200)	7.5%	6.5%	0.500
EBV (n=22)	18%	4.5%	0.250
RF (n=23)	26%	22%	1.000
TPPA+/RPR– (n=5)	20%	20%	1.000
TPPA+/RPR+ (n=5)	80%	60%	1.000

C6 Imm. = C6 Immunetics cut-off
WB EL = Virotech Ecoline Western blot
EM = erythema migrans
LNB = definite Lyme neuroborreliosis
ACA = *acrodermatitis chronicum atrophicans*
LA = Lyme arthritis
EBV = Epstein-Barr virus infection
RF = rheumatoid factor positive
TPPA+/RPR– = syphilis healed
TPPA+/RPR+ = syphilis ongoing
[a] According to Liddell (Armitage et al., 2001)

As shown in Table 20, there were no significant differences in seropositivity rates among the various ways of determining the positivity of the sample, apart from a lower serosensitivity for the two-tiered test in cases of definite LNB (p=0.031). The two-tiered test reduced the false positive rate from 18% to 4.5% in individuals with EBV, although this difference was not statistically significant.

However, in general, the specificity of two-tiered testing could not be shown to be superior.

Table 21. Seropositivity rates in VT IgM+IgG ELISA and two-tiered testing using VT IgM+IgG ELISA in combination with Virotech Ecoline Western blot in various LB manifestations and controls (paper I).

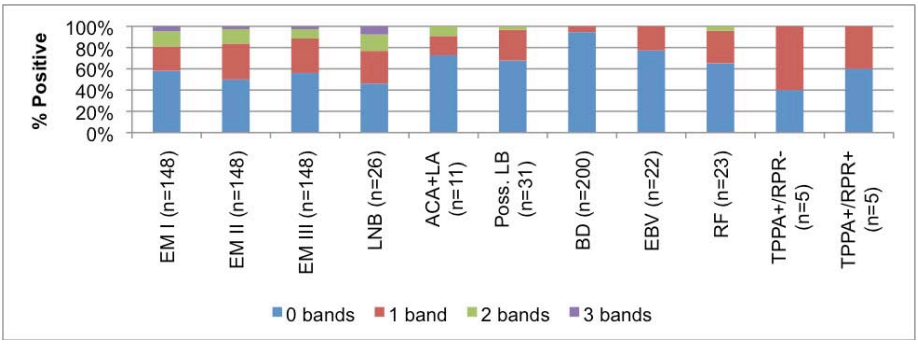
Condition	VT IgM+IgG	Two-tier (VT and WB EL)	p-value[a]
EM at presentation (n=148)	36%	40%	0.332
LNB (=21)	95%	71%	0.062
ACA/LA (n=11)	100%	82%	0.500
BD (n=200)	14%	10%	0.022
EBV (n=22)	54%	14%	0.012
RF (n=23)	22%	26%	1.000
TPPA+/RPR– (n=5)	60%	40%	1.000
TPPA+/RPR+ (n=5)	0%	40%	0.500

VT = Virotech
WB EL = Virotech Ecoline Western blot
EM = erythema migrans
LNB = definite Lyme neuroborreliosis
ACA = *acrodermatitis chronicum atrophicans*
LA = Lyme arthritis
EBV = Epstein-Barr virus infection
RF = rheumatoid factor positive
TPPA+/RPR– = syphilis healed
TPPA+/RPR+ = syphilis ongoing
[a] According to Liddell (Armitage et al., 2001)

In Table 21, increased specificity was noted for the two-tiered test principle in both the blood donors and the EBV-positive patients (p=0.022 and p=0.012). No significant differences were found in the other groups, apart from a tendency toward a lower serosensitivity of the two-tiered test in cases of definite LNB (p=0.062).

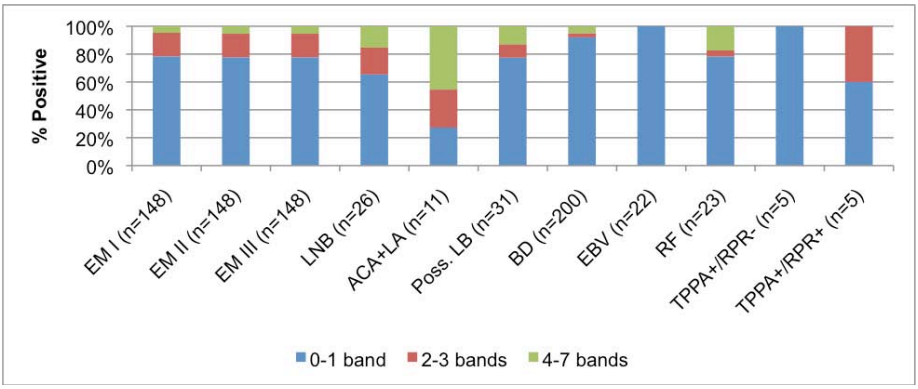
To conclude the evaluation of two-tiered testing, specificity was shown to be higher in a two-tiered protocol than in the VT ELISA alone. However, the newer C6 Imm. test revealed a specificity similar to the corresponding two-tiered test. Thus, the results of the C6 Imm. test support the idea of abandoning the general two-tiered testing protocol; this is further supported by the significantly lower sensitivity of two-tiered testing in LNB.

Figure 16. IgM bands (0–3) in Virotech Ecoline Western blot, according to group (paper I).



EM = erythema migrans I at presentation, II at 2–3 months and III at 6 months post-presentation, LNB = Lyme neuroborreliosis, ACA = *acrodermatitis chronicum atrophicans*, LA = Lyme arthritis, Poss. LB = possible Lyme borreliosis, BD = blood donors, EBV = Epstein-Barr virus infection, RF = rheumatoid factor positive, TPPA+/RPR– = syphilis healed, TPPA+/RPR+ = syphilis ongoing

Figure 17. IgG bands (0–7) in Virotech Ecoline Western blot, according to group (paper I).



EM = erythema migrans I at presentation, II at 2–3 months and III at 6 months post presentation, LNB = Lyme neuroborreliosis, ACA = *acrodermatitis chronicum atrophicans*, LA = Lyme arthritis, Poss. LB = possible Lyme borreliosis, BD = blood donors, EBV = Epstein-Barr virus infection, RF = rheumatoid factor positive, TPPA+/RPR– = syphilis healed, TPPA+/RPR+ = syphilis ongoing

However, WB has other benfits, as it simultaneously provides information regarding both IgM and IgG antibody responses to multiple antigens. This may aid in determining the stage of LB. As shown in Figures 16 and 17, the banding patterns of various LB manifestations and control cases differ. For instance, multiple IgG bands are commonly found in cases of ACA and LA, i.e., late-stage LB, compared with cases of EM and LNB, in which IgM bands are more common and the number of IgG bands is fewer; this is in accordance with previous reports (Craft et al., 1986, Dressler et al., 1993). No obvious diagnostic support for LNB could be found using WB EL. Interestingly, more than three IgG-positive bands were found in 4 out of 23 (17%) RF-positive patients, probably reflecting a previous exposure to *Borrelia* spirochetes, as these 4 cases were also all positive in the C6 Imm., VT and Li tests.

The common finding of multiple IgG bands in WB EL suggests that it may be a valuable diagnostic tool in cases of suspected ACA and LA, although it does not have 100% sensitivity.

General discussion

The use of and interpretation of LB serology is problematic. It is well known that the sensitivity is low in early LB such as EM, a finding that was also confirmed in our investigations. The serosensitivity in LNB of duration shorter than 30 days is also too low to allow a confident rule out use of serological tests. In addition, specificity is a problem in LB serological tests. Positive test results are common in the healthy population in areas highly endemic to LB, due to previous exposure to *B. burgdorferi* s.l. Cross-reactions in LB tests such as the C6 test continue to occur, but specificity seems to improve with VlsE-based testing, especially in IgG tests, according to paper I and Sillanpää et al. (Sillanpaa et al., 2007). The higher specificity could also allow adjustments of cut-off levels, thereby possibly improving sensitivity without great loss of specificity, as shown in paper III. The optimal cut-off depends on the desired level of either sensitivity or specificity or both, which in turn depends on the severity of the disease in question, potential risks of missing a diagnosis in terms of complications and the risk of obtaining a false positive result. Since endemicity of LB and the distribution of different *Borrelia* species vary with geographical location, the laboratory diagnostic routine for LB may benefit from a localised perspective. For instance, a more suitable cut-off level for the *Borrelia* antibody screening ELISA may be achieved by accumulating a local serum bank of samples from patients with LB and control samples from non-LB patients. The recently published European case definitions may be helpful for this purpose and may also permit comparisons among different geographical areas (Stanek et al., 2010).

The assessment of cross-reactivity in conditions such as EBV infection, RF positivity and syphilis is important in order to generally evaluate the performance of an LB serological test. However, these conditions or features may or may not resemble the clinical picture of an LB manifestation. In clinical practice, the most

relevant property of LB tests should be high specificity in patients with conditions clinically resembling an LB manifestation.

Since different LB manifestations lead to different seroresponses, as shown in this thesis, a manifestation-based approach is preferred regarding diagnosis of LB. In patients presenting with multiple manifestations at once (Berglund et al., 1995), the laboratory investigation should be directed to the most advanced manifestation at hand. For instance, a patient presenting with EM followed by three days of headache and neck pain should be investigated by lumbar puncture for a suspected LNB. By knowing which manifestation to focus on, the laboratory may utilize various LB diagnostic tests in a differentiated way that takes advantage of the performance characteristics of each test. Apart from the economical advantages, this also permits the laboratory to deliver more accurate interpretations regarding the probability of an LB manifestation. However, before ordering an LB serological test, a pre-test evaluation of the probability of the suspected manifestation is required, i.e., an estimation of the probability that the patient actually suffers from the suspected manifestation. For a specific LB manifestation, this depends on a number of important parameters: Apparently, the risk of LB and its manifestations varies with tick abundance in various geographical areas across Europe and between Europe and North America. The general risk of LB also varies with season; in Europe, EM, LNB, BL and possibly LC occur mainly in the summer and autumn and more seldom during the winter months (Bennet et al., 2006b, Berglund et al., 1995, Hansen and Lebech, 1992, Strle et al., 1992, Fish et al., 2008). In addition, the various LB manifestations have different relationships with sex and age. EM and ACA seem more common among women, while LNB and LC are more common among men (Asbrink et al., 1986, Bennet et al., 2006b, Fish et al., 2008, Hansen and Lebech, 1992, van der Linde, 1991), and no obvious difference has been reported for BL or LA (Steere et al., 1987, Strle et al., 1992). Regarding age, BL and LNB are more commonly reported among children, while ACA only rarely occurs among children (Berglund et al., 1995, Strle and Stanek, 2009). LA seems to occur both in older children and adults (Stanek and Strle, 2008, Strle and Stanek, 2009). The relative frequencies of the various LB manifestations are also important background information in the diagnosis of LB. The above information thus forms a basis for determining the risk of an LB manifestation, along with the patient history, including previous episodes of LB and a physical examination. Only in light of these clinical parameters may an LB serological test be properly interpreted. Although co-infections are not covered in this thesis, other tick-borne infections, such as human granulocytic anaplasmosis and tick-borne encephalitis virus, may also be transmitted in parallel with *Borrelia.* So far, little is known regarding the possible effects on LB serology in these cases.

EM is the most commonly reported LB manifestation, and in endemic areas during summer and autumn, LB serology does not typically add any useful information. A typically expanding erythema or multiple EM is sufficient to start antibiotic treatment, which cures the infection and effectively reduces the risk of

other disseminated LB manifestations (Bennet et al., 2003). However, atypical EM cases have been reported, in addition to misdiagnosis of EM (Bennet et al., 2006a, Feder and Whitaker, 1995). LB serology, seroconversion and skin biopsies for culture and/or PCR may add information in such cases, as well as in cases of EM occurring in non-endemic LB areas.

Distinct neurological symptoms are typically present in LNB, thereby leading to referral of the patient to a hospital clinic for lumbar puncture. However, there are definite LNB cases lacking classical focal neurological symptoms (see Figure 11). Symptoms in such cases may vary considerably and include pain at various sites of the body, fatigue, decreased appetite, weight loss and sleep disturbances. Such symptoms are common among patients seeking medical advice in primary health care centres, and LNB is therefore one possible differential diagnosis in LB-endemic areas. Lumbar puncture, however, is seldom performed in primary health care centres in Sweden, and it would be unpractical to refer all such patients for lumbar puncture. As shown in paper II, the C6 test may be useful in helping the general practitioner decide which patients should be referred for further investigation. A negative C6 test in a patient over 11 years of age with a symptom duration of more than 30 days argues against LNB and therefore supports the decision not to refer the patient, in cases with no other indications. Conversely, since specificity of a positive C6 test result in a patient with suspected LNB was shown to be 60%, such patients should be further investigated, including lumbar puncture, to obtain a definite diagnosis. In patients with suspected LNB under 12 years of age, or presenting with a symptom duration shorter than 30 days, the need for lumbar puncture should be individually assessed, as the C6 test may be falsely negative. The possibility of using the C6 test in blood as a diagnostic tool in suspected LNB is further supported by the high (98–100%) blood C6/VlsE(IR6) serosensitivity reported by others (Peltomaa et al., 2004, Sillanpaa et al., 2007, Skarpaas et al., 2007, Steere et al., 2008). Additionally, WB EL did not seem to add any useful information regarding the diagnosis of LNB.

The investigation of CSF and serum in patients with suspected LNB has for a number of years been based on the detection of CSF ABA. However, CSF ABA may be negative for the first few weeks, and in addition, a positive AI may persist for several months and even years, despite appropriate treatment (Blanc et al., 2007, Brouqui et al., 2004, Hammers-Berggren et al., 1993, Hansen and Lebech, 1991, Ljostad et al., 2007, Stanek and Strle, 2003, Strle et al., 2006). As shown in previous studies (Ljostad and Mygland, 2008, Rupprecht et al., 2005, Senel et al., 2010), CSF CXCL13 seems to be a reliable marker of LNB, also detecting highly probable cases of LNB among children with short symptom duration (paper IV). Therefore, we recommend CXCL13 as a diagnostic CSF marker of LNB. The main concern for CXCL13 is that elevated levels can also occur in other diseases that can mimic LNB, in particular multiple sclerosis (Ljostad and Mygland, 2008, Sellebjerg et al., 2009). Although levels in multiple sclerosis are typically lower than in LNB, it is necessary to combine CXCL13

with CSF ABA. In CXCL13-positive cases that are negative for CSF ABA, treatment with antibiotics seems reasonable when other data are not in favour of a diagnosis of multiple sclerosis. Other findings supporting LNB include pleocytosis, elevated total IgM index and oligoclonal bands on IEF of CSF (Oschmann et al., 1998, Tumani et al., 1995, Mygland et al., 2010), although these findings are not specific to LNB.

There are limited published data on serosensitivity in cases of BL. In one Slovenian report, 25 of 36 (69%) BL cases were seropositive at presentation, and another 4 patients showed seroconversion a few months later (Strle et al., 1992). Thus, a BL diagnosis is mainly based on clinical findings, such as a lymphocytoma in a typical location, e.g., the earlobe of a child or in the nipple of an adult. Further support comes from presentation in summer or autumn, possibly in combination with EM or another LB manifestation (Strle et al., 1992). Clinically, it may be difficult to distinguish BL from malignant lymphomas. Biopsies may be performed on affected skin in such cases as an additional support, usually showing dense lymphocytic infiltration in BL (Strle et al., 1992, van Vloten and Willemze, 2003).

The diagnosis of LC is difficult, as the condition is rare and serology may be of limited value. Positive serology has been reported in the majority of patients, but the presence of anti-*Borrelia* antibodies does not necessarily prove an ongoing infection, nor does it confirm heart involvement. In reality, a diagnosis of LC therefore relies on heart involvement, such as various types of A-V heart block in combination with or in close proximity to other LB manifestations, such as EM and LNB (Stanek et al., 2010, Strle and Stanek, 2009).

Finally, diagnosing ACA and LA requires laboratory support (Stanek et al., 2010). In stage III LB, these manifestations are usually accompanied by a dominant IgG antibody response with multiple IgG bands on WB (Craft et al., 1986, Dressler et al., 1993). This was also shown in this thesis, although sample size was limited (see Figures 16 and 17). However, our patients included two WB EL-negative cases that were positive in ELISAs; therefore, we recommend performing both the C6 test and WB EL in suspected cases of ACA and LA in order to increase sensitivity. Additional diagnostic support in these cases may be acquired by collecting synovial fluid or skin biopsies for culture and/or PCR. In LA, granulocytic inflammation is commonly found in synovial fluid (Stanek et al., 2010).

CONCLUDING REMARKS

In general, no significant differences were noted between the C6, the VT and the Li *Borrelia* antibody tests in terms of serosensitivity in various manifestations of LB such as EM, LNB, ACA and LA. However, antibody dynamics differed in follow-up samples after EM. The C6 test seropositivity rate was lower than that of the VT and Li tests both at 2–3 and 6 months post-EM diagnosis. In addition, EM patients reporting a previous LB episode had a C6 seropositivity rate similar to those without a previous LB episode, and seroprevalence in healthy blood donors was lower in the C6 test compared with the VT and Li tests. Cross-reactivity was lower in the C6 test compared with the VT test. Taken together, these results support the recommendation of the C6 test as a *Borrelia* serological test, primarily due to its ability to reflect ongoing or recent infection.

Although the majority of patients with EM at presentation showed concordant serological responses to IR6 peptides representing the three main *Borrelia* species and the C6 peptide, there were also clinical EM cases that were negative in the C6 test and could be detected mainly in a seroresponse to a *B. burgdorferi* s.s.-derived IR6 peptide. Thus, an ELISA combining antigens could be of value in the serodiagnosis of LB in Europe. However, more studies are needed to determine the optimal strategy.

The presence of C6 antibodies in serum in cases of LNB was shown to be associated with symptom duration. A serosensitivity rate of 93% for the C6 test was found in AI-positive patients aged ≥ 12 years of age with an LNB symptom duration of more than 30 days. Therefore, in these patients, a negative C6 test in serum argues against an LNB diagnosis.

The chemokine CXCL13 in CSF was confirmed as a reliable marker of LNB. CXCL13 differentiated LNB from other conditions; this study also showed that it detected highly probable cases of LNB in children with short symptom duration where CSF ABA were still lacking.

No significant diagnostic improvement could be shown using a two-tiered approach combining the C6 test and WB EL as opposed to the C6 test alone. However, WB EL may be useful in suspected ACA and LA, usually displaying multiple IgG bands.

Based on the findings in this thesis, the following diagnostic routines are proposed:

PROPOSED DIAGNOSTIC ROUTINES

Most advanced manifestation	Prerequisite	Laboratory investigation	Interpretation	Comment	Additional support
EM		None		Clinical diagnosis	Biopsy for culture/PCR
LNB	Age ≥ 12 years and symptom duration > 30 days	Serology: C6 Imm.	Neg. C6 Imm. argues against LNB.	Investigate CSF in C6 Imm. pos. patients. No additional value of WB EL	
	Age < 12 years and/or symptom duration ≤ 30 days	CSF: Cell count, CSF ABA and CXCL13	Pleocytosis, CSF ABA and elevated CXCL13 argues strongly for ongoing LNB.		Elevated total IgM index, IEF of CSF
LA / ACA		Both C6 Imm. and WB EL	WB EL with ≥ 2 IgG bands is consistent with LA/ACA	Diagnosis unlikely if WB EL is neg.	Synovial fluid or biopsy for culture/PCR
BL / LC		Both C6 Imm. and WB EL		C6 Imm. and WB EL are of unknown value	Seroconversion supports diagnosis

Neg. = negative according to the manufacturer's recommendation
Pos. = positive according to the manufacturer's recommendation
C6 Imm. = Immunetics Quick ELISA C6 *Borrelia* assay kit
PCR = polymerase chain reaction
CSF = cerebrospinal fluid
ABA = anti-*Borrelia* antibodies
IEF = isoelectric focusing
WB EL = Western blot using Virotech *Borrelia* Ecoline immunoblot

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****

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