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Tick-Borne Infections in Humans

Aspects of immunopathogenesis, diagnosis and co-infections with *Borrelia burgdorferi* and *Anaplasma phagocytophilum*

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To my family worldwide

För att man ska kunna flyga
måste modet vara aningen större än rädslan
och en gynnsam vind råda

Ur: Instruktion för skalbaggar/ Margareta Ekstrand

“The *Borrelia* war” by Aaron Nordberg, seven years old 2009. The immune system fighting against the *Borrelia* bacteria.

CONTENTS

ABSTRACT

The tick-borne infectious agents, *B. burgdorferi*, *A. phagocytophilum* and the TBE-virus, can all cause clinical disease in humans and may all initially give rise to myalgia, arthralgia, headache and fever. The clinical manifestations of the infections range from subclinical or mild to severe, in some cases with a post-infectious sequel, and mixed infections may occur, confusing the clinical picture.

The aim of this thesis was to investigate the occurrence and co-existence of these infections in a Scandinavian context. A further aim was to study aspects of the immunopathogenesis of *B. burgdorferi* infection and possible effects on the immune response when previously exposed to *A. phagocytophilum*. Finally, an attempt was made to improve the laboratory diagnosis of Lyme neuroborreliosis (LNB).

In a prospective clinical study, patients were recruited based on two independent inclusion criteria; 1) patients with unspecific symptoms or fever, and 2) patients with erythema migrans (EM). Among 206 patients, we found 186 cases of Lyme borreliosis (LB) (174 with EM), 18 confirmed and two probable cases of human granulocytic anaplasmosis (HGA), and two cases of Tick-borne encephalitis (TBE). Thirteen of the HGA cases presented without fever. Furthermore, 22 of the EM patients had a subclinical co-infection with *A. phagocytophilum*, based on serology. Both TBE cases had co-infections, one *with B. burgdorferi* and one with *A. phagocytophilum*.

In another investigation, IL-12p70 secretion in patients with current LB was compared in patients with or without previous *A. phagocytophilum* infection. Patients with serological evidence of previous exposure to *A. phagocytophilum* had a lower *B. burgdorferi*-induced IL-12p70 secretion. Since IL-12p70 induces the Th1 response, this finding indicates a reduced Th1 response, possibly caused by *A. phagocytophilum*. In a separate study, we showed that patients with LNB had increased levels of cytokines associated with cytotoxicity in cerebrospinal fluid (CSF), including the recently described cytokine IL-17.

Since it is known that the adaptive immune system, especially the T cells, is activated during an infection with *B. burgdorferi*, a modified ELISPOT assay using cells from CSF was evaluated to be a useful complementary test in diagnosing LNB. However, we found that the diagnostic performance was too weak in our setting, and we could not recommend it for use in clinical laboratories at this stage.

In conclusion, tick-borne co-infections are probably quite common in Sweden. Our HGA cases were most often discovered as co-infections with LB and would probably have been missed during a routine consultation. They presented

with mild symptoms and often without fever, which in previous reports has been part of the disease definition.

The immune response in LNB was shown to be compartmentalized to the target organ, also in terms of cytokine response. Furthermore, we found indications of possible long-term effects of *A. phagocytophilum* infection, demonstrated as a reduced IL-12p70 secretion in patients with ongoing LB. This could be a disadvantage when mounting a Th1 response to infection with *B. burgdorferi*. If this is so, the inter-play of these infectious agents in co-infections or consecutive infections may be of importance to clinical outcome.

SAMMANFATTNING PÅ SVENSKA

De fästingburna patogenerna *B. burgdorferi*, *A. phagocytophilum* och TBE-virus, kan alla ge upphov till infektioner hos människa. Kliniskt kan de alla initialt ge sig till känna med allmän sjukdomskänsla med muskelvärk, ledvärk och feber. Infektionerna kan också från fall till fall variera i svårighetsgrad från milda, ibland subkliniska, till dramatiska, och i vissa fall med postinfektiösa restsymtom. Dessutom kan blandinfektioner förekomma vilket kan försvåra den kliniska bilden.

Ett syfte med arbetena i denna avhandling har varit att försöka att ytterligare öka kunskapen om dessa fästingburna infektioners epidemiologi, fr a med avseende på förekomst av blandinfektioner med flera av de aktuella smittämnena. Ett annat syfte var att studera immunpatogenesen vid borreliainfektion, och eventuell påverkan på denna av tidigare genomgången infektion med *A. phagocytophilum*. Förutom detta gjordes även ett försök att förbättra den laborativa diagnostiken av borreliainfektioner i centrala nervsystemet, dvs Lyme neuroborreliosis (LNB).

I en prospektiv klinisk studie (delarbete III) rekryterades patienter baserat på två olika inklusionskriterier; 1) patienter med ospecifika symtom eller feber, och 2) patienter med erythema migrans (EM), i båda fallen efter känt eller misstänkt fästingbett. Bland 206 patienter som fullföljde studien identifierdes 186 fall av Lyme borrelios (LB), varav 174 med EM. Vidare hittades 18 säkra och två sannolika fall av human granulocytär anaplasmos (HGA), även kallad fästingfeber. Dessutom hittades två fall av TBE. Tretton av HGA-fallen hade ingen feber, vilket var anmärkningsvärt eftersom feber i de flesta tidigare rapporter om HGA har varit en del av sjukdomsdefinitionen. Bland patienterna som hade EM utan kliniska symtom hittades 22 patienter med en samtidig, subklinsk infektion med *A. phagocytophilum,* baserat på serologi. Även de båda TBE-fallen hade andra, samtidiga infektioner, den ena med *B. burgdorferi* och den andra med *A. phagocytophilum*.

I delarbete II jämfördes utsöndringen av IL-12p70 hos patienter med en pågående LB med patienter med eller utan tidigare infektion med *A. phagocytophilum.* . Patienter med serologiska belägg för en tidigare genomgången *A. phagocytophilum*-infektion hade lägre *B. burgdorferi*-inducerad IL-12p70 utsöndring. Eftersom IL-12p70 inducerar Th1-svaret indikerar detta fynd att en genomgången anaplasmainfektion möjlighen kan orsaka ett kvarstående, reducerat Th1-svar.

Vi visade även att patienter med LNB hade förhöjda nivåer av cytokiner associerade med cytotoxicitet i cerebrospinalvätska (CSF), inkluderat det nyligen beskrivna cytokinet IL-17.

I delarbete IV utvärderades ett modifierat ELISPOT-test där celler, och då fr a T-lymfocyter, från CSF testades med avseende på cytokinfrisättning efter stimulering med borreliaantigen. Tanken var att ELISPOT skulle kunna vara ett komplement i LNB diagnostiken. Resultaten visade dock att den diagnostiska prestandan var för svag, och vi kunde därför i detta skede inte rekommendera testet för rutindiagnostik.

Sammanfattningsvis så visade sig blandinfektioner med flera samtidiga, fästingburna smittämnen vara vanliga i patientmaterial från sydöstra Sverige. Våra HGA-fall upptäcktes ofta som en parallellinfektion med LB och skulle troligen ha missats under ett rutinmässigt läkarbesök. De hade milda symtom och ofta saknades feber, som i tidigare rapporter har varit en del av sjukdomsdefinitionen.

Immunsvaret vid LNB skedde i målorganet, dvs CSF, också vad gäller cytokinfrisättning. Fynden indikerar en möjlig långtidseffekt på Th1-svaret efter genomgången *A. phagocytophilum* infektion. Detta antagande bygger på att vi kunde visa en reducerad IL-12p70-utsöndring hos patienter med en aktuell LB. Om Th1-svaret på något sätt störs av en anaplasmainfektion så kan det vara till nackdel när Th1-reaktiviteten ska påbörjas som svar på *B. burgdorferi* infektion. Om så är fallet, kan samspelet mellan dessa infektiösa patogener vara av vikt för det kliniska förloppet.

LIST OF PAPERS

I. Jarefors S, **Karlsson (Nordberg) M**, Forsberg P, Eliasson E, Ernerudh J, Ekerfelt C. Reduced number of interleukin-12-secreting cells in patients with Lyme borreliosis previously exposed to *Anaplasma phagocytophilum*. *Clin Exp Immunol*, 2006;143(2):322-328.

II. **Nordberg M**, Forsberg P, Johansson A, Nyman D, Jansson C, Ernerudh J, Ekerfelt C. Cytotoxic mechanisms may play a role in the local immune response in the central nervous system in neuroborreliosis. *J Neuroimmunol*, 2011, 232(1-2):186-93.

III. **Nordberg M**, Forsberg P, Berglund J, Bjöersdorff A, Ernerudh J, Garpmo U, Haglund M, Nilsson K, Eliasson I. Aetiology of Tick-Borne Infections in an Adult Swedish Population – are Co-Infections with Multiple Agents Common? *Manuscript.*

IV. **Nordberg M**, Forsberg P, Nyman D, Skogman BH, Nyberg C, Ernerudh J, Eliasson I, Ekerfelt C. Can ELISPOT Be Applied to A Clinical Setting as A Diagnostic Utility for Neuroborreliosis*? Cells*. 2012; 1(2):153-167.

ABBREVIATIONS

A. phagocytophilum	*Anaplasma phagocytophilum*
ACA	Acrodermatitis chronica atrophicans
APCs	Antigen-presenting cells
B. burgdorferi	*Borrelia burgdorferi sensu lato*
BBB	Blood-brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T cells
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
EM	Erythema migrans
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGA	Human granulocytic anaplasmosis
IFA	Immunofluorescence assay
IFN	Interferon
IL	Interleukin
LA	Lyme arthritis
LB	Lyme borreliosis
LNB	Lyme neuroborreliosis
MHC	Major histocompatibility complex
MNC	Mononuclear cells
NK cells	Natural killer cells
Osp	Outer surface protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cells
PLDS	Post-Lyme disease syndrome
RT-PCT	Realtime reverse transcription - PCR
s.s.	*sensu stricto*
TBE	Tick-borne encephalitis
Th	T helper cells
TLR	Toll-like receptor
Treg	Regulatory T cell

INTRODUCTION

Framework and context

In Scandinavia, ticks are vectors of different zoonotic infections, e.g. *Borrelia. burgdorferi sensu lato (B. burgdorferi)*, *Anaplasma phagocytophilum (A. phagocytophilum)* and tick-borne encephalitis virus (TBE). Lyme borreliosis (LB), the most common tick-borne disease in Europe, is a complex infection that can affect various organs, such as the nervous system, joints and skin.

Human granulocytic anaplasmosis (HGA) is a newly discovered disease in humans, although known in the veterinary medicine since the 1930s. HGA is most commonly manifested by nonspecific fever, chills, headache and myalgia, ranging from asymptomatic to fatal disease. TBE was first described in Ålandic church records of the 18th century. Typically, TBE has a biphasic course with an initial phase consisting of unspecific symptoms that could resolve or continue to a second phase with more neurological symptoms.

Thus, all three infections can initially cause unspecific symptoms and signs such as fever, myalgia, arthralgia and headache. As a clinician I meet patients with unspecific symptoms in association with a suspected tick bite. One interesting question is why some patients develop an array of symptoms while others have a silent subclinical course. Furthermore, how does the immune system deal with the pathogens and what mechanisms are involved? Another problem is that laboratory diagnoses are incomplete and rely mainly on serology. Antibodies can last for years and it is not always an easy task to decide if they are due to a previous or a present infection. Therefore, new tests are needed, especially in the identification of *B. burgdorferi*. Additionally, how common are tick-borne infections in south-east Sweden and are subclinical infections frequent? In the literature, reports indicate that co-infections with more than one tick-borne pathogen would cause a more severe outcome. These are some of the thoughts and questions I have been considering and they are also the background to why I started my doctoral thesis.

In this thesis, prevention and vaccination will not be discussed.

Tick-borne infections

Hard ticks, in Sweden mainly *Ixodes ricinus* (Figure 1), transmit several zoonotic agents, the most well-known being the bacteria *Borrelia burgdorferi sensu latu (B. burgdorferi)* and *Anaplasma phagocytophilum (A. phagocytophilum)* and tick-borne encephalitis (TBE) virus. Tick-borne infections are sylvatic zoonoses affecting both animals and humans (Nieto and Foley, 2009). The pathogens are maintained in cycles between tick vectors and mammalian reservoir hosts, predominantly small rodents. For *B. burgdorferi* tick-bird cycles have also been described, while *A. phagocytophilum* has shown an ability to maintain a tick-ruminant cycle, at least with sheep (Ogden et al., 2003; Olsen et al., 1993). Larger mammals, such as roe deer, may also be involved, not so much as reservoirs but by contributing to an effective spread of large numbers of ticks.

When it concerns the TBE virus, recent findings suggest that the tick is actually the main reservoir, and that co-feeding of larvae and nymphs is necessary to maintain the virus in the tick population. This explains the more patchy prevalence of TBE-infected ticks. They can only exist in areas where larvae and nymphs are host-seeking, at least partly during the same time period (Labuda and Randolph, 1999; Randolph, 2011) . In all tick-borne infections humans are considered as dead-end hosts. The incidence of tick-borne diseases is increasing in Europe, and there is speculation that this could partly be caused by climate change. (Gray et al., 2009).

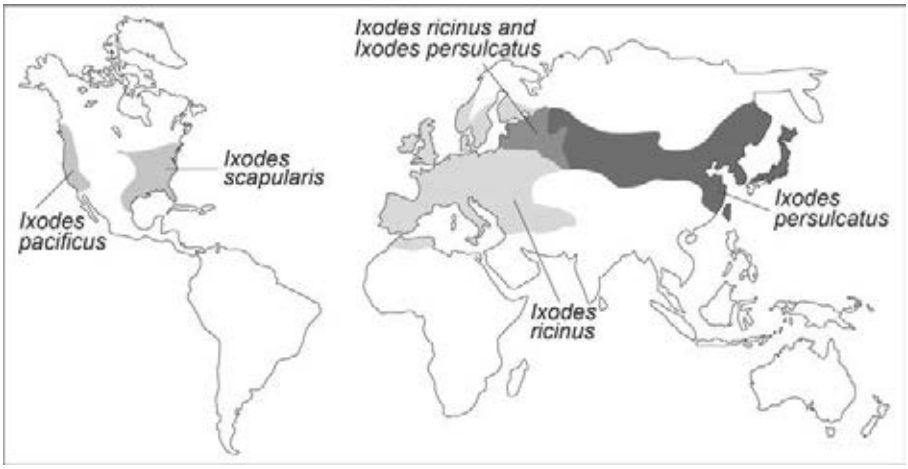

Figure 1. Distribution of the vectors, *Ixodes ricinus* species complex of Lyme borreliosis. (Courtesy of EUCALB, European Union Conserted Action on Lyme Borreliosis).

Tick – the vector

Ticks are classified into two families, the soft ticks (*Argasidae*) and the hard ticks (*Ixodidae*). The *Ixodes (I) ricinus* species complex is distributed throughout Europe while *I. persulcatus* is found in Russia and Asia and *I. scapularis* and *I. pacificus* are found in eastern North America and western North America respectively (Gray, 2002) (Figure 1). Typically the *Ixodes* ticks have a three-host life cycle that is usually completed in 2-3 years where each stage of the tick feeds only once (Parola and Raoult, 2001) (Figure 2). After mating, which usually takes place on the host prior to blood feeding, the female lays between 1,000-5,000 eggs and dies (Suss, 2003).

After attachment, the tick pierces the host's skin with its scalpel-like mouthparts and inserts a hypostome (Figure 3), (Gray et al., 2009; Parola and Raoult, 2001), which explains why it might be difficult to remove the tick from the skin.

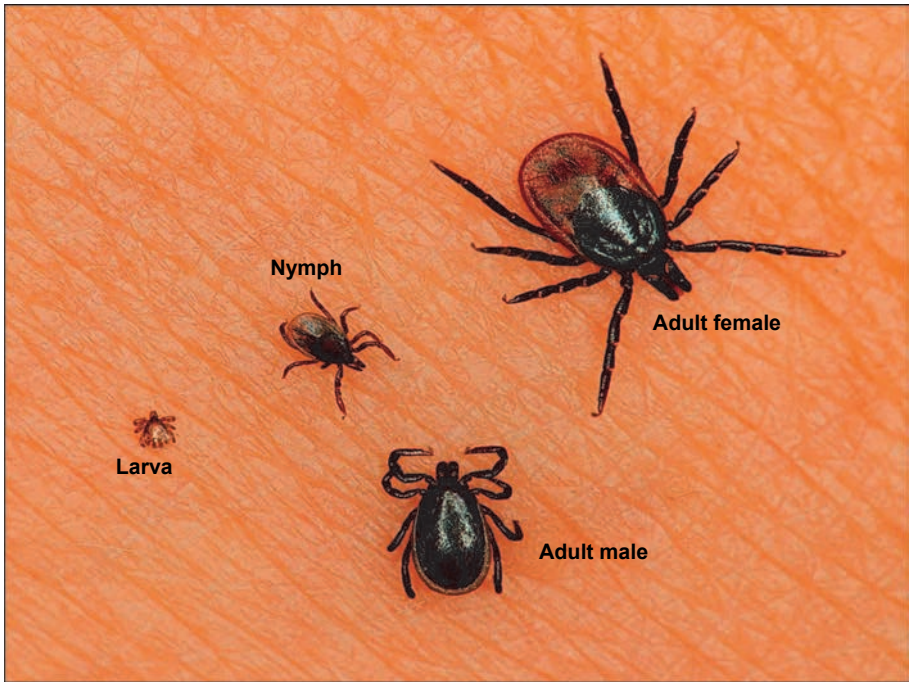

Figure 2. Tick stages of *Ixodes ricinus*. (Courtesy of Ulf Garpmo and Frank B Widlind, Kalmar County Hospital).

Various substances are produced by the tick salivary gland and include a cement to anchor the mouthparts to the skin further, enzymes, vasodilators, anti-inflammatory, antihemostatic, and immunosuppressive substances (Parola and Raoult, 2001). It is suggested that the tick is blind, but senses carbon dioxide, temperature, odours, ammonia and movements (Suss, 2003). They have a variety

of sensory organs, e.g. Haller`s organ, that are located on the final segment of the first pair of legs, hair-like structures on the legs, body and mouth parts. These sensory organs allow the tick to locate the host and also to communicate with other ticks. (Parola and Raoult, 2001).

Three ticks have been described as being vectors for *B. burgdorferi s.l.* in Europe, *I. ricinus*, *I. hexagonus* and *I. uriae. I. ricinus* is the main vector and has a wide geographical distribution throughout Europe and also in North Africa (Piesman and Gern, 2004). *I. ricinus* feeds on over 300 vertebrate species (Anderson, 1991). In addition, *I. ricinus* is also the main vector in Europe for *A. phagocytophilum* and TBE virus (Suss, 2003; Wormser et al., 2006).

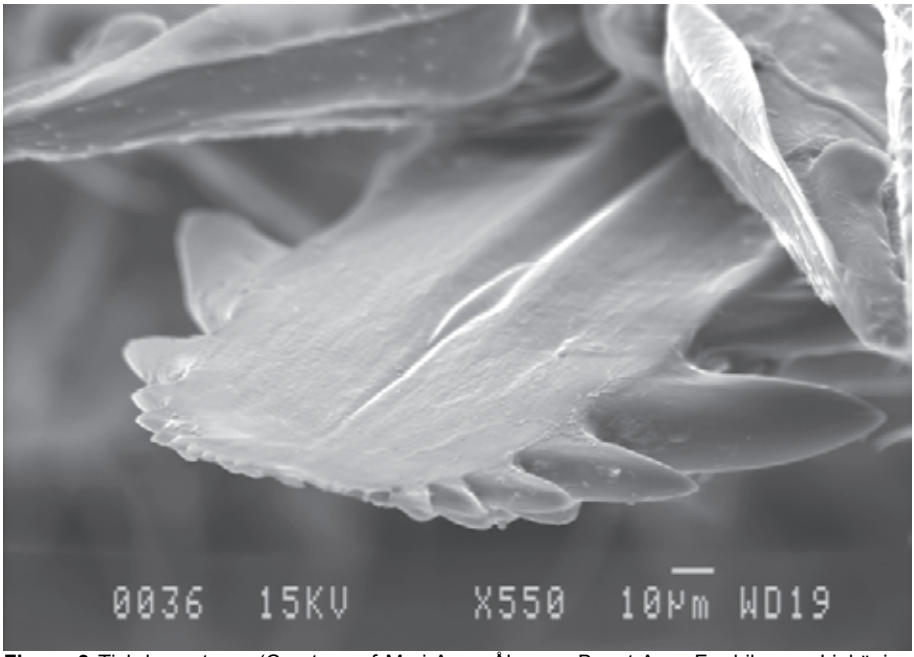

Figure 3 Tick hypostoma (Courtesy of Mari-Anne Åkeson, Bengt-Arne Fredriksson, Linköping University, Sweden).

Lyme borreliosis

Lyme borreliosis (LB), is caused by the obligate tick-borne spirochete *Borrelia burgdorferi sensu lato* complex. Since it was discovered in 1982 by Burgdorfer et al. at least 18 different genospecies have been described (Burgdorfer et al., 1982; Stanek and Reiter, 2011). LB is the most common tick-borne disease in the Northern hemisphere (Stanek and Strle, 2003). Clinically, LB has been known for over a hundred years and the first clinical manifestations were described in 1883 by Buchwald (Buchwald, 1883). However, it was in1975 in Old Lyme, Connecticut, USA, that Steere and colleagues started a surveillance system

among residents, mainly children that had developed arthritis. The arthritis was first believed to be caused by juvenile rheumatoid arthritis among the children, but a connection was made between an erythematous lesion and a previous tick bite (Steere et al., 1977a; Steere et al., 1977b). This finding led to the discovery of LB and soon the spirochete was isolated, first from ticks, blood, skin and cerebrospinal fluid (CSF) (Benach et al., 1983; Burgdorfer et al., 1982; Steere et al., 1983b).

Borrelia burgdorferi sensu lato – the pathogen

Borreliae are spirochetes and share with other spirochetes several structural characteristics such as a spiral or wavelike body and flagella (organs of motility), enclosed between the outer and inner membranes (Figure 4). The outer cell membrane surrounds the protoplasmic cylinder complex, consisting of cytoplasm, the inner cell membrane and the peptidoglycan (Barbour and Hayes, 1986; Tilly et al., 2008). Other spirochetes include *Treponema pallidum*, which causes syphilis, and *Leptospira interrogans*, which causes leptospirosis (Tilly et al., 2008).

The *B. burgdorferi sensu lato* complex consists of several genotypes, and today 18 genospecies have been described. Of these, *B. afzelii*, *B. garnii* and *B. burgdorferi s.s*. have been confirmed to cause localized, disseminated or chronic manifestations of LB. Recently, other genospecies have been reported to possess pathogenic potential to humans (Rudenko et al., 2011; Stanek and Reiter, 2011). For example, *B spielmanii* has been cultured from European patients with erythema migrans (EM) (Fingerle et al., 2008; Foldvari et al., 2005; Maraspin et al., 2006) while *B. Bisetti* was detected in CSF isolate (Fingerle et al., 2008). The clinical role of *B. Lusitaniae* is still unclear (Stanek and Reiter, 2011) and remains to be further investigated.

The *Borrelia* spirochetes are 10-30 µm in length and 0.18-0.25 µm in diameter, corkscrew-shaped and considered as extracellular (Burgdorfer et al., 1982; Pal and Fikrig, 2003). The composition of the cell envelope is similar to gram-negative bacteria but with some differences, such as absence of lipopolysaccharide and an abundance of lipoproteins in the outer cell membrane (Fraser et al., 1997; Takayama et al., 1987). The outer membrane is rich in lipoproteins, including the highly immunogenic outer-surface proteins (Osps) A-F (Fraser et al., 1997; Guerau-de-Arellano and Huber, 2005). Many of the lipoproteins in the outer membrane are on the bacterial surface where they act as adhesins, targets for bactericidal antibodies, or receptors for various molecules (Samuels and Radolf, 2010).

B. burgdorferi is able to alter its surface molecules during life. This is called antigenic variation, and facilitates transmission of *B. burgdorferi* from vector to host. It also helps the spirochete to escape from the immune reactions of the host (Rupprecht et al., 2008).

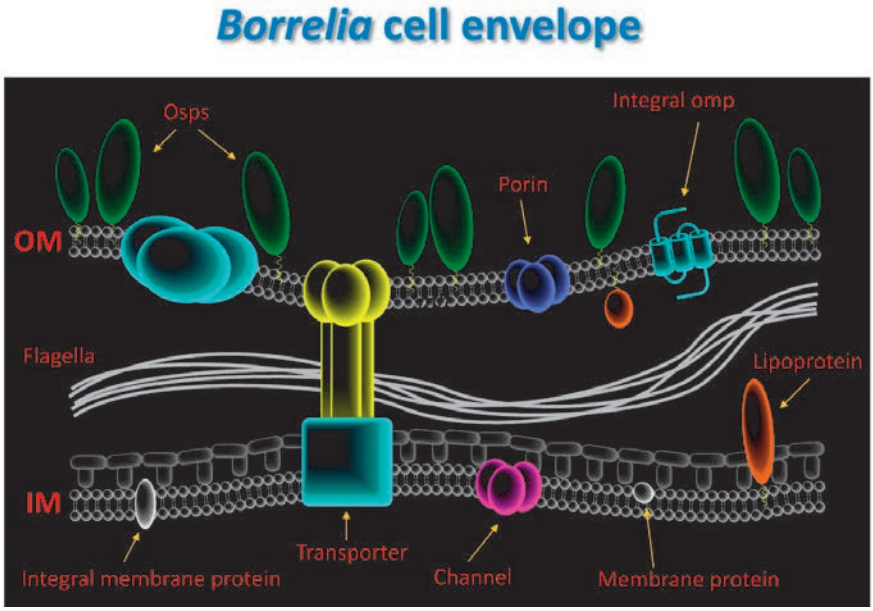

Figure 4. Cell envelope of B. *Burgdorferi*. (Courtesy of Sven Bergström, Umeå University, Pinne et al. Porins of Borrelia, in Molecular Biology of Spirochetes. IOS Press NATO Science Series Vol 373, 2006).

Epidemiology

The incidence of LB in Europe varies. A German study showed an annual incidence of 111 cases per 100,000 inhabitants, the highest rate occurring in children and elderly adults (Huppertz et al., 1999). This age distribution is in line with other studies, where a bimodal age distribution usually occurs with most LB cases in children and older adults (Berglund et al., 1995; Carlsson et al., 1998). It is difficult to estimate the incidence rates in Europe, since the surveillance strategies vary and also because LB is not a notable disease in most countries. However, in south Sweden the overall incidence of LB was 69 infections per 100,000 inhabitants per year (Berglund et al., 1995) while the incidence in the county of Blekinge has been reported to be 464/100,000/year (Bennet et al., 2006b). The incidence is decreasing from south to north in Scandinavia and from north to south in south Europe (Stanek et al., 2011a).

The risk of developing LB in an area depends on several factors, such as the density of the tick population, the number of ticks infected with *B. burgdorferi* and the frequency of human contact with tick biotopes (Huegli et al., 2011). However, the risk of contracting an infection with *B. burgdorferi* from infected ticks increases with the duration of tick attachment, although transmission of spi-

rochetes may occur after only 24 h (Crippa et al., 2002; Kahl et al., 1998). Hugeli et al. reported that the risk of developing asymptomatic infections or clinical symptoms of LB after an infected tick bite was 8.2% (Huegli et al., 2011). Interestingly, in Sweden, Fryland et al. reported that the risk of acquiring an infection with *B. burgdorferi*, even after a bite from an infected tick, was small (6%) (Fryland et al., 2011) while Stjernberg et al. reported the risk to be as low as 0.5% (1/221 tick bites) (Stjernberg and Berglund, 2002).

The reported prevalence of *B. burgdorferi* in Swedish ticks varies in different studies from 3% to 23% (Fraenkel et al., 2002; Gustafson et al., 1995; Wilhelmsson et al., 2010) with a higher prevalence in adult ticks (33%) than nymphs (14%) (Wilhelmsson et al., 2010).

Tick-borne infections on the Åland Islands

The Åland Islands in Finland, with a population of 28,000, are known to be endemic for tick-borne diseases. Åland is an archipelago consisting of Main Åland, which is group of larger islands, and more than 6,000 smaller islands. The islands have a rich ecology of foliage, woods and fields, except for the rocky sea border. The climate is maritime, with a usually mild autumn and a relatively short winter. The incidence of LB on Åland is reported to be 1.700 cases/100 000 inhabitants/year in 2011 compared to main Finland with 30 cases/100 000 inhabitants/year (Finland´s National institute for health and welfare).

On the Åland Islands the prevalence of seropositivity rises with age, the highest being seen in men (44.7%) and women (37 %) over 70 years of age (Carlsson et al., 1998). Wahlberg et al. conducted an epidemiological study that showed that 85% of the adult population had been bitten by ticks (Wahlberg, 1990). The first clinical case of serologically verified disseminated LB was found in 1984. The most common manifestation of LB on Åland is EM, with about 250-300 cases annually, followed by disseminated manifestations (personal communication Dag Nyman).

Concerning human granulocytic anaplasmosis, no published data from the Åland islands exist. In a recent study investigating ticks with PCR, the prevalence of *A. phagocytophilum* in ticks was 3.1% on Åland compared to 1.0% in Sweden. The prevalence in nymphs was 2.8%, and 6.9% in adult ticks (Kozak et al. unpublished). Tick-borne encephalitis, also called Kumlinge disease, was first described in Ålandic church records of the 18th century (Kunz 2003, Vaccine). Furthermore, the causative virus was isolated in 1959 from ticks on Kumlinge, in the Åland archipelago (Oker-Blom, 1956) .

In conclusion, the Åland islands are hyper-endemic for tick-borne diseases, with several clinical cases every year,

Clinical manifestations of Lyme borreliosis

Lyme borreliosis is a multi-organ infection with a wide spectrum of clinical manifestations (Table 1, 2) affecting parts of the body including the skin, nervous system, joints, heart and eyes (Stanek et al., 2011a). The different genospecies of *B. burgdorferi* are associated with different clinical features (van Dam et al., 1993). *Borrelia burgdorferi s.s* is the only known genospecies in North America, while several are found in Europe, e.g. *B. afzelii*, *B. garinii*, *B. burgdorferi s.s*. and occasionally *B. spielmanii and B. bavariensis* (Fingerle et al., 2008; Stanek and Strle, 2003; Stanek et al., 2011b; Wang et al., 1999)

The genospecies of *B. burgdorferi* possess different organotropisms. Thus, *B. afzelii* is associated with skin manifestations, *B. garinii* seems to be mostly neurotropic, while *B. burgdorferi s.s* is mostly associated with arthritis (Stanek et al., 2011b), although there is an overlap between the genospecies and the clinical features of LB (Balmelli and Piffaretti, 1995).

The clinical manifestations are divided into early localized, early disseminated and late disseminated stages (Mullegger, 2004; Wilske, 2005) (Table 1). However, it is important to keep in mind that the disease does not necessarily follow such stages.

Table 1. Staging of the different clinical features of Lyme borreliosis.

Early localized **Days – weeks**	**Early disseminated** **Weeks – months**	**Late disseminated/Persistent** **Months – years**
Erythema migrans	Early Lyme neuroborreliosis	Late Lyme neuroborreliosis
Borrelia lymphocytoma	Multiple erythema migrans	Lyme Arthritis
	Borrelia lymphocytoma	Acrodermatitis chronica atrophicans
	Lyme carditis	

Adapted from Wilske et al. 2005 and Műllegger et al. 2004.

Table 2. Frequency of clinical manifestations of Lyme borreliosis.

	Berglund et al. 1995 Sweden	Priem et al. 2003 Germany	Christova et al. 2004 Bulgaria	Strle et al. 2009 Slovenia
Number	**n=1471 %**	**n=3935 %**	**n=1257 %**	**n=1020 %**
Erythema migrans	77	51	69	82
Lyme neuroborreliosis	16	18	19	9
Lyme arthritis	7	24	8	3.3
ACA	3	2	0.3	4.8
Lymphocytoma	3	5	0.3	0.8
Lyme carditis	<1	NR	1%	0.2

n= number, NR= not reported

Erythema migrans

Localized *Borrelia* infection is typically manifested by an EM skin lesion, the hallmark of acute LB. In Europe, EM is the most common clinical presentation of LB (Berglund et al., 1995; Huppertz et al., 1999; Strle and Stanek, 2009), affecting people of all ages and both genders (Stanek and Strle, 2003). The lesion is usually an expanding maculae or papule that forms a red or bluish-red patch, with or without a central clearing (Stanek et al., 2011a) (Figure 5, 6, 7). The EM lesion usually appears at the site of the tick bite after an incubation period of 10-30 days (range, a few days to six months). EM is typically "annular" with a central clearing, or "homogeneous", but atypical forms do exist (Mullegger, 2004) . In Europe the EM lesions are usually caused by *B. afzelii* or *B. garinii*, whereas *B. burgdorferi s.s*. is the cause in North America (Bennet et al., 2006a; Strle et al., 1999; Strle et al., 2011).

Along with EM, non-specific symptoms, such as fatigue, malaise, headache, fever, arthralgia and myalgia may occur (Stanek et al., 2011a). Systemic symptoms with a solitary EM might indicate dissemination of spirochetes. (Oksi et al., 2001; Stanek and Strle, 2003). However, the dissemination may occur without generalized symptoms (Oksi et al., 2001). Interestingly, patients in North America are reported to develop systemic symptoms more often than European patients (Mullegger, 2004; Strle et al., 1999). Differences in the clinical picture may be explained by the differences in the geographical distribution of the various genospecies of *B. burgdorferi s.s. (*Steere, 2001).

Erythematous lesions that occur within a few hours after a tick bite do not qualify as EM and are usually hypersensitivity reactions. Furthermore, differential diagnoses of EM include e.g. local tick bite reactions, erysipelas, insect bites, tinea and contact dermatitis (Hengge et al., 2003; Stanek et al., 2011a). EM may disappear spontaneously after few weeks or months. However, it is not definite that the infection has disappeared since spirochetes can be cultured from skin biopsy specimens several months after the disappearance of the EM (Hytonen et

al., 2008). As a consequence of the haematogenous spread of the spirochete, EM lesions may occur as multiple EM. This is more common in American patients, occurring in up to 25% of cases, while European patients have multiple EM in about 4-8% of cases (Hytonen et al., 2008).

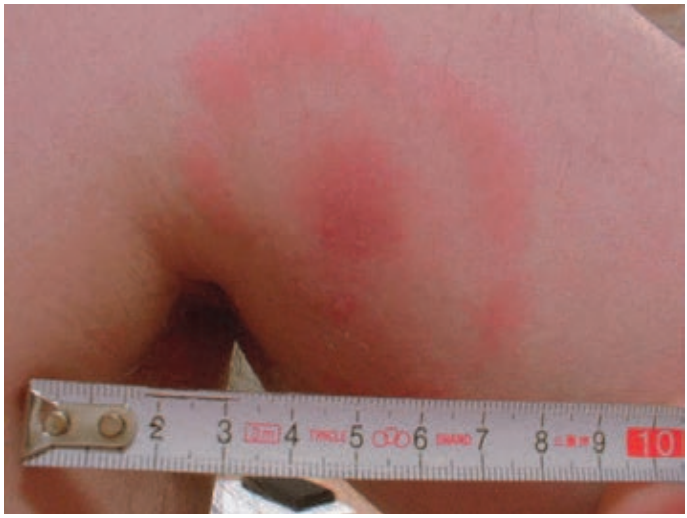

Figure 5. Erythema migrans. (Courtesy of Susanne Olausson, Borelia Group, Åland Islands, Finland)

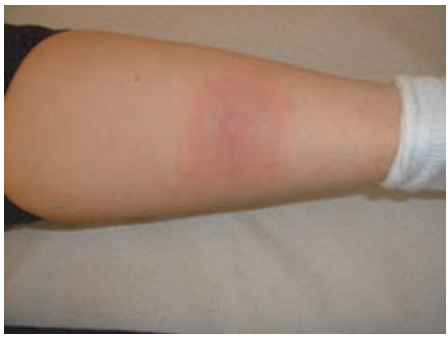

Figure 6. Erythema migrans caused by *B.afzelii*. (Courtesy of Sten-Anders Carlsson, The Borrelia group, Åland).

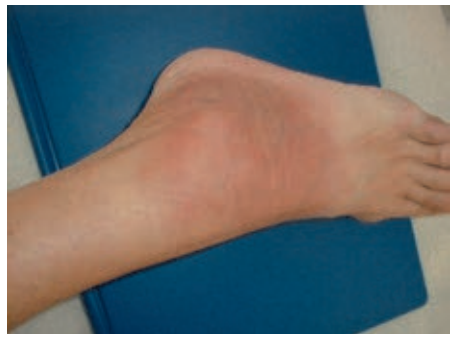

Figure 7. Erythema migrans caused by *B. garinii*. (Courtesy of Sten-Anders Carlsson, The Borrelia group, Åland).

Lymphocytoma

Borrelia lymphocytoma (*Lymphadenosis benigna cutis*) is a bluish-red nodule with a size between 1-5 cm, typically located on the earlobe (Figure 8). It can also be located on the breast nipples/aerola, scrotum or the nose (Franz and Krause, 2003; Mullegger, 2004). Lymphocytomas are soft, painless and occur more frequently in children than in adults. The incubation time after tick bite is usually longer than in EM and lymphocytoma is usually defined as a manifestation of early disseminated LB. However, it may also occur at the site of a tick bite and is then defined as early localized infection (Mullegger, 2004).

Lymphocytoma is considered to be a benign B-cell lymphoproliferative process that reacts to the presence of Borrelia in the skin (Asbrink and Hovmark, 1988). Histopathologically it is a dense lymphocytic infiltrate in subcutaneous tissue or dermis (Colli et al., 2004). Clinical differential diagnoses are, for example, cutaneous lymphoma, insect bite, cutaneous metastasis or keloid (Mullegger, 2004).

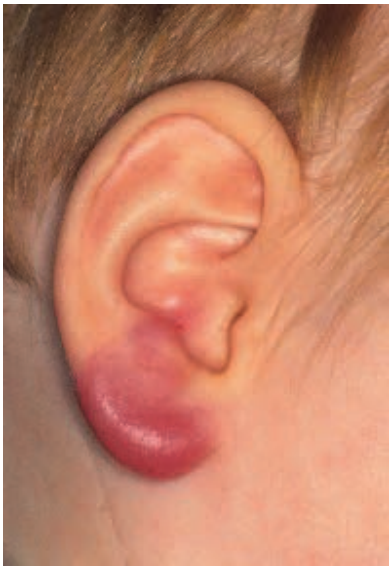

Figure 8. Earlobe lymphocytoma (Courtesy of Barbro Hedin-Skogman, Department of Pediatrics, Falu General Hospital, Sweden).

Acrodermatitis chronica atrophicans

Acrodermatitis chronica atrophicans (ACA) is a cutaneous manifestation of late LB, almost exclusively seen in Europe (Strle and Stanek, 2009). It has mainly been observed in patients older than 40 years, and more often in women. A previous EM on the same location as ACA is reported in about 20% of patients (Stanek and Strle, 2003). It is usually located on the extensor sites of the hands and feet, but also on the lower leg. ACA typically begins with a bluish-red discoloration and oedema. Gradually it progresses to an atrophic phase over months

to years due to persistence of *B. burgdorferi* in the skin. It does not resolve spontaneously. The skin becomes thin, wrinkled, and violet. About 60% of patients with ACA also develop peripheral neuropathy (Mullegger, 2004; Stanek and Strle, 2003).

Neuroborreliosis

Lyme neuroborreliosis (LNB) is the most common manifestation of disseminated borreliosis in Europe and also in Sweden and on the Åland islands. About 14-34% of patients with borreliosis are diagnosed with LNB (Berglund et al., 1995; Cimmino, 1998; Stanek et al., 1996).

LNB can arise at any time during the course of borreliosis but a history of EM is common (Pachner and Steiner, 2007; Stanek and Strle, 2003). Neurological symptoms usually occur 1-12 (mostly 4-6) weeks after the infecting tick bite (Mygland et al., 2010) More than 95% of those with LNB can be classified as having early LNB, defined as signs and symptoms lasting for less than six months. Less than 5% have a symptom duration exceeding six months, classified as late LNB (Mygland et al., 2010).

LNB can affect all parts of the nervous system and in Europe, manifestations such as lymphocytic meningitis, radiculoneuritis and cranial nerve palsies are seen (Kaiser, 1998). This classic triad of symptoms can occur alone or in combination (Garcia-Monco and Benach, 1995). The most common manifestation of early LNB among European patients is painful meningoradiculitis, affecting the peripheral nervous system (PNS) (Mygland et al., 2010). Pain is one of the most pronounced clinical symptoms as a result of radiculoneuritis. The pain is usually severe and the intensity and localization may vary over time. Typically it is most pronounced during the night (Mygland et al., 2010; Stanek and Strle, 2003). Paresis is seen in about 60% of patients with early LNB (Hansen and Lebech, 1992).

Facial nerves are most involved, but any cranial nerve can be affected in early LNB and this may result in unilateral or bilateral peripheral nerve facial palsy (Stanek and Strle, 2003). LNB is estimated to cause 2-20% of peripheral facial nerve palsy in settings endemic for LB (Bjerkhoel et al., 1989; Olsson et al., 1988; Peltomaa et al., 2002). In a Swedish study investigating patients with peripheral facial nerve palsy caused by LNB and Bells´ palsy, the LNB patients had more neurological symptoms outside the paretic area of the face. They also had more sensibility disturbances and pareses (Bremell and Hagberg, 2011).

Borrelial meningitis usually presents with mild or intermittent headache. Fever, nausea and vomiting are frequently absent (Stanek and Strle, 2003). Other symptoms such as fatigue, muscle/joint pain, neck pain, vertigo and concentration difficulties may occur in patients with LNB (Henningsson et al., 2010).

In Europe most cases of LNB are caused by *B. garinii*, followed by *B. afzelii*, and rarely by *B. burgdorferi s.s* (Ornstein et al., 2002; Strle and Stanek,

2009). In a Slovenian study with *B. garinii* and B. afzelii isolated from CSF, the patients with *B. garinii* showed typical clinical features of LNB i.e., painful meningoradiculitis, whereas symptoms and signs associated with *B. afzelii* were more unspecific and harder to diagnose. Only 59% of the patients with B. garinii had intrathecal borrelial antibody production, and in the case of the *B. afzelii* group the figure was 10% (Strle et al., 2006). This could partly be explained by short duration of illness since it is known that intrathecal antibodies may be absent during the initial phase of LNB (Cerar et al., 2010). However, the authors speculate that B. afzelii might be able to pass through the BBB but has restricted capability to initiate a substantial inflammation of the CNS (Strle et al., 2006).

Both early and late manifestations of the CNS are rare, but may include myelitis, encephalitis, dementia, paraparesis and cerebral vasculitis (Mygland et al., 2010; Stiernstedt et al., 1988).

The clinical presentation in children is diverse, with peripheral facial nerve palsy and meningitis as the most common symptoms (Broekhuijsen-van Henten et al., 2010; Stanek et al., 2011b). Small children may present with unspecific symptoms, i.e. fatigue and loss of appetite (Mygland et al., 2010). In a Swedish study, children diagnosed with confirmed LNB (n=72) presented with fatigue 86%, headache 69%, facial nerve palsy, 60% and loss of appetite 60% (Skogman et al., 2008).

Lyme arthritis

In Europe Lyme arthritis (LA) is a quite rare feature of LB and the reported frequency seems to vary from 2-7 % (Berglund et al., 1995; Strle and Stanek, 2009). In the United States, LA is a prominent disseminated manifestation of LB due to the sole genospecies *B. burgdorferi s.s.*, which seems to be the most arthriogenic, but not the only *Borrelia* species involved in LA (Stanek et al., 2011b; Strle and Stanek, 2009).

Months after onset of illness, approximately 60% of untreated patients in North America develop intermittent attacks of joint swelling and pain, usually in large joints, but especially the knee (Steere, 2001). The spectrum of LA manifestations can be divided into musculoskeletal pain, without objective findings, arthritis (intermittent or chronic) with objective findings, or chronic joint and bone involvement under affected skin in ACA (Strle and Stanek, 2009). Serological testing usually reveals high specific IgG *Borrelia* antibodies in serum (Stanek et al., 2011b).

Other Lyme borreliosis manifestations

Lyme carditis is a rare manifestation of LB.in Europe, and has been reported to occur in less than four percent of untreated patients with LB (Strle and Stanek, 2009). Cardiac involvement appears as an acute onset of fluctuating degrees of disturbances in the atrio-ventricular conduction (Semmler et al., 2010). It usually

occurs within two months after onset of infection (four days – seven months). Lyme carditis may occur together with other features of LB, such as EM, LNB, or Lyme arthritis (Strle and Stanek, 2009).

Eye involvement is very rare and is a result of inflammation, e.g. conjunctivitis, keratitis, retinal vasculitis and optic neuritis or as a result of extra ocular manifestations of LB, including paresis of cranial nerves (Strle and Stanek, 2009)

Laboratory methods of detecting *B. burgdorferi*

The diagnosis of LB is based on a combination of patient history, clinical examination and the detection of anti-*Borrelia* antibodies in serum/CSF. Furthermore, it is also important to know if the patient lives in or has visited an area endemic for LB. A typical EM is a clinical diagnosis and no serology testing is needed. For all other *Borrelia* manifestations laboratory support is required (Strle and Stanek, 2009). Both direct and indirect methods are used for detection of *B. burgdorferi* infection.

Direct detection

Direct detection of *B. burgdorferi* is possible by microscopy, culture and PCR (Aguero-Rosenfeld et al., 2005; Wilske, 2005).

Microscopy

The diagnostic value of microscopy in the clinical laboratory is limited since the number of *Borrelia* spirochetes in clinical samples is very small. Furthermore, the spirochete morphology is variable, making them difficult to distinguish from host tissue structures. Spirochetes may be visualized after Giemsa, carbol-fuchsin and silver staining in human tissues (Aberer and Duray, 1991; Samuels and Radolf, 2010).

Culture

Culture of *B. burgdorferi* provides the best diagnostic evidence of LB and is the gold standard in diagnosing infectious diseases (Tugwell et al., 1997). However, culturing *B. burgdorferi* has limitations such as the low number of viable spiro-

chetes present in patient biopsies and the slow growth of the borreliae (Aguero-Rosenfeld et al., 2005; Stanek et al., 2011a). *B. burgdorferi* can be cultured in BSK II (Barbour-Stoenner-Kelly II) medium in vitro or other modifications of the original Kelly medium. Cultures are incubated for up to 12 weeks, because of the slow growth (Aguero-Rosenfeld, 2008; Mygland et al., 2010). The sensitivity of the culture is highly variable, ranging from less than 1% in arthritis, 10-30% in early LNB, 50-70% in skin biopsies and less than 10% in blood (EM) (Mygland et al., 2010; Stanek et al., 2011a). Thus, this method is not used as a first line diagnostic tool. However, culturing may be helpful in certain cases (Stanek et al., 2011a; Wilske, 2003)

Polymerase chain reaction (PCR)

Gene amplification by PCR is the most sensitive method for detection of *B. burgdorferi*, especially in tissues and in synovial fluid (Dumler, 2001; Wilske et al., 2007).

The reported sensitivity of PCR in CSF from patients with LNB is low, 10-30%, although it is higher when disease duration is less than two weeks (Karlsson et al., 1990; Wilske, 2005). PCR has the best sensitivity (50-70%) in skin samples (EM, ACA) and in synovial fluids (and is even better when used with synovial tissue) (Asbrink and Hovmark, 1985; Eiffert et al., 1998; van Dam et al., 1993; Wilske, 2003; Wilske, 2005).

PCR detects borrelial DNA of both viable and non-viable spirochetes, making it impossible to distinguish if an infection is active or not (Strle and Stanek, 2009). Another limitation is that no standardization of targets, primers or methods has currently been established (Stanek et al., 2011a).Thus, PCR is not a routine method in diagnosing LB, but in some circumstances it can be valuable as an additional diagnostic tool (Aguero-Rosenfeld, 2008; Brouqui et al., 2004).

Indirect detection

A number of methods have been used for detection of antibodies to *B. burgdorferi* e.g. indirect immunofluorescent-antibody assay (IFA), enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) (Aguero-Rosenfeld et al., 2005).

Antibody analyses in serum

The recommendation today in both Europe and the US is to follow the principles of a two-step approach (CDC, 1995; Wilske et al., 2007). The first step is a serological screening assay such as a sensitive enzyme-linked immunosorbent assay (ELISA). If a positive or equivocal result is obtained, a confirmatory Western blot (WB), follows (CDC, 1995; Wilske, 2005; Wilske et al., 2007).

ELISA is the most frequently used method for testing antibodies to B. burgdorferi, and several commercial kits exist. Since there is no standardization of serological testing in Europe, commercial kits show highly varied performances. Furthermore, variations exist between assays in the antigenic composition and in the detection of specific antibodies (Aguero-Rosenfeld et al., 2005; Ekerfelt et al., 2004). The advantages of ELISA include the possibility for large scale testing, an automated procedure, and the objective determination of antibodies using a numeric value (optical density OD, Index) (Samuels and Radolf, 2010).

In the WB assay the antigens are separated by molecular size, using electrophoresis. This makes it possible to determinate which antigens of *B. burgdorferi* are immunodominant at different stages of LB. The disadvantages of using WB are the subjective interpretation of band intensity, and lack of standardization of the antigen source (Aguero-Rosenfeld et al., 2005).

Today, several assays with various antigens are available, for example, whole-cell sonicate, purified intact flagella antigen, recombinant antigens and synthetic peptides. The limitations of whole-cell antigens are the lack of specificity because of the presence of cross-reacting antigens of *B. burgdorferi* (Aguero-Rosenfeld, 2008; Aguero-Rosenfeld et al., 2005).

The search for better serological tools has led to the development of recombinant and peptide antigens, used in ELISA or immunoblots (Aguero-Rosenfeld, 2008). Examples of recombinant antigens are, OspC, p100, p58, P41i (internal portion of flagellin), DbpA and others (Aguero-Rosenfeld, 2008; Wilske, 2005).

The C6 antigen is based on the sixth invariable region of the VlsE (variable major protein-like sequence expressed) lipoprotein (van Burgel et al., 2011b). The VlsE sequence is highly immunogenic and also has cross-reactivity among different genospecies of *B. burgdorferi* (Aguero-Rosenfeld, 2008). This *in vivo* antigen is important in modern serological tests as it has been proven to be both specific, and sensitive in detecting antibodies early in the course of infection (Nyman et al., 2006).

Detection rates for antibodies differ during the course of LB. During early infection the sensitivity of antibodies is 20-50%, with a predominance of IgM, in the early disseminated stage 70-90% and nearly 100% during late infection (months or years after tick bite) (Wilske, 2005).

Antibody analyses in CSF

The diagnosis of LNB is based on a combination of patient history, clinical examination, analysis of CSF and the detection of anti-*Borrelia* antibodies in serum and CSF. Spirochetes are difficult to grow (Brouqui et al., 2004) and the sensitivity of culture and the PCR is low in CSF (Aguero-Rosenfeld et al., 2005).

European LNB is associated with elevated cell count in CSF (Mygland et al., 2010). Typical CSF findings are lymphocytic pleocytosis (mainly helper T cells) and plasma cells, elevated CSF protein and oligoclonal IgG bands and also in-

trathecal synthesis of specific antibodies, (Garcia-Monco and Benach, 1995; Mygland et al., 2010).

For diagnosis of LNB an antibody index (AI) is used to measure *B. burgdorferi* specific antibody titres in paired CSF and serum, calculated to measure intrathecal antibody production. In some cases the level of intrathecal antibodies may be high when serum antibodies are not present, especially if the duration of symptoms is short (Pachner and Steiner, 2007; Wilske, 2003). However, this method has its limitations, considering that intrathecal antibodies may persist for years after resolution of the infection (Hammers-Berggren et al., 1993; Kruger et al., 1989). Furthermore, intrathecal *Borrelia* antibody production may be absent for some weeks in the initial phase of LNB (Cerar et al., 2010).

The current European recommendations in cases of suspected LNB are to obtain paired CSF and serum samples for analysis of specific anti-*Borrelia* antibodies, (ELISA and immunoblot) and determination of CSF inflammation parameters (Mygland et al., 2010; Stanek et al., 2011a).

Additional diagnostic methods

The B-cell chemoattractant CXCL13 has been suggested as a biomarker for LNB (Rupprecht et al., 2005; Senel et al., 2010). Elevated levels of intrathecal CXCL13 have been found in LNB patients and the sensitivity was 88% and specificity was 89% (van Burgel et al., 2011a). Tjernberg et al. showed that CXCL13, expressed as CSF-Serum CXCL13 ratio, reached a sensitivity of 99% and a specificity of 96% (Tjernberg et al., 2011), which is in line with other reports (Ljostad and Mygland, 2008; Rupprecht et al., 2005). This has to be interpreted with caution, since patients with autoimmune diseases could also present with elevated levels of CXCL13 intrathecally (Sellebjerg et al., 2009; van Burgel et al., 2011a), although at lower levels in general (Ljostad and Mygland, 2008).The ELISPOT method, based on an ELISA technique, is used to detect cytokine secretion in T cells on a single cell level in response to antigen stimulation. It is a highly sensitive method, which visualises *Borrelia*-specific cytokine secretion as a spot on a nitrocellulose-bottomed microtitre plate (described in detail in “Methods”) (Czerkinsky et al., 1988; Ekerfelt et al., 1997a; Forsberg et al., 1995).

Treatment

Antibiotic therapy is beneficial for all clinical manifestations of LB (Stanek and Strle, 2003). *B. burgdorferi* has been reported to be susceptible in *in vitro* to tetracyclines, most penicillins, second-generation and third generation cephalosporins and macrolides (Stanek et al., 2011b). The antibiotic treatment strategies vary between different countries (EUCALB, 2009; Wormser et al., 2006). The current Swedish treatment recommendations are presented in table 3

(Swedish Medical Products Agency, 2009). Phenoxymethylpenicillin, amoxicillin, doxycycline, cefuroxime axetil and azithromycin are all effective against EM and Borrelia lymphocytoma (EUCALB, 2009; Stanek et al., 2011b). However, macrolides, such as azithromycin are less effective than other oral antibiotics and therefore used as a second-line drug (Stanek et al., 2011b).Oral doxycycline seems to be as efficient as intravenous ceftriaxone for treatment of European patients with LNB (Dotevall and Hagberg, 1999; Ljostad et al., 2008).

Persisting symptoms after treatment

Subjective symptoms such as, fatigue, musculoskeletal pain, paraesthesias and complaints of cognitive difficulties that persist for > six months after a documented episode of LB and standard treatment indicate post-Lyme disease syndrome (PLDS) (Mygland et al., 2010; Wormser et al., 2006). However, lack of standardized case definitions or a biological marker makes it difficult to investigate and identify patients with these symptoms (Wormser et al., 2006). Another thing to consider is that some patients diagnosed with LB and following an appropriate treatment, can take several months to recover completely (Stanek et al., 2011a).

Other systemic infections, for example Epstein-Barr virus, may show post-infectious symptoms and a slow resolution (Hickie et al., 2006; Stanek et al., 2011a). Furthermore, also in the general population, nonspecific symptoms can occur in more than 10%, even in endemic areas for LB (Feder et al., 2007).

Table 3. Swedish treatment recommendations for Lyme borreliosis in adults. (Adapted from the Swedish Medical Products Agency, 2009).

Diagnosis	Antibiotic	Dosage	Duration
EM, single	PcV	1 g x 3	10 days
Pregnancy	PcV	2 g x 3	10 days
Penicillin allergy	Doxycycline	100 mg x 2	10 days
	Azithromycin	500 mg x 1 (day 1), 250 mg x1 (day 2-5)	5 days
EM, multiple	Doxycycline	100 mg x 2	10 days
EM + fever			
Pregnancy	Ceftriaxone	2 g x 1 IV	10 days
Borrelia lymphocytoma	Doxycycline	100 mg x 2	14 days
	PcV	1 g x 3	14 days
ACA	Doxycycline	100 mg x 2	21 days
	PcV	2 g x 3	21 days
Lyme neuroborreliosis	Doxycycline	200 mg x 1	14 days
		200 mg x 2	10 days
	Ceftriaxone	2 g x 1 IV	14 days
Lyme arthritis	Doxycycline	100 mg x 2	14 days
	Ceftriaxone	2 g x 1 IV	14 days
Lyme carditis	Doxycycline	100 mg x 2	14 days
	Ceftriaxone	2 g x 1 IV	14 days

EM, erythema migrans, PcV, phenoxymethylpenicillin, ACA, acrodermatitis chronica atrophicans, IV, intravenous

Human granulocytic anaplasmosis (HGA)

Granulocytic ehrlichiosis *(Ehrlichia phagocytophila)* has been recognized as a veterinary pathogen since the 1930s and is the causative agent for the tick-borne fever, affecting small ruminants in Europe (Bjoersdorff et al., 1999b; Gordon et al., 1932; Parola et al., 2005)

A. phagocytophilum was formerly called the HGE agent, but after molecular phylogenetic studies in 2001 the HGE agent, *Ehrlichia phagocytophila* and *Ehrlichia equi* were reclassified to *A. phagocytophilum* (Dumler et al., 2001).

Human granulocytic anaplasmosis (formerly human granulocytic ehrlichiosis) is an acute tick-borne infection (Dumler et al., 2001). The first human cases of HGA were reported from the US in 1994 (Chen et al., 1994). The first serological cases of HGA in Europe were described in Switzerland in 1995 (Brouqui et al., 1995), while the first confirmed human cases were reported in Slovenia in 1997 (Petrovec et al., 1997). In Sweden, the first HGA cases were reported in 1999 by Björsdorff and colleges (Bjoersdorff et al., 1999a).

Anaplasma phagocytophilum – the pathogen

Anaplasma phagocytophilum is an intracellular pathogen of the family *Anaplasmatacae* in the order Rickettsiales that causes disease in humans and animals (Brown, 2012) The family *Anaplasmataceae* includes five-well known genera, *Ehrlichia*, *Anaplasma*, *Neorikettsia*, *Aegytianella* and *Wolbachia*, while "*Candidatus Neoehrlichia*" and "*Candidatus Xenohaliotis*" are two genera that are less studied (Rikihisa, 2011) (Table 4).

To date *A. phagocytophilum, Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia canis, Neorickettsia sennetsu* and eventually *Ehrlichia ruminantum* infect humans (Rikihisa, 2010) *Ehrlichia chaffeensis* infects monocytes and causes human monocytic ehrlichiosis, *Ehrlichia Ewingii* causes human ewingii ehrlichiois while *A. phagocytophilum* infects neutrophil granulocytes, resulting in HGA (Dumler et al., 2007). Although granulocytes are the target cells of *A. phagocytophilum*, bacterial inclusions have been described in other cells, e.g. endothelial cells (Rikihisa, 2011).

A. phagocytophilum is a small (0.2-1.3 μm in diameter) gram-negative, obligate intracellular bacterium that propagates within phagosomes of neutrophils. Even though they have a gram-negative cell wall, they lack lipopolysaccharide biosynthetic machinery (Dumler et al., 2005; Lin and Rikihisa, 2003; Rikihisa, 2011). *A. phagocytophilum* is enveloped by two membranes, and the outer membrane is often ruffled, creating an irregular periplasmic space. There is no capsule layer. Within the bacteria, fine DNA strands and ribosomes are distinctly seen. *A. phagocytophilum* bacteria replicate in membrane-bound phagosomes within the cytoplasm of host cells (Rikihisa, 2011). They reside within the phagosome after active inhibition of phagosome-lysosome fusion that is mediated by proteins.

How this inhibition is established remains to be further elucidated (Woldehiwet, 2008). Several *A. phagocytophilum* strains have been reported in nature, suggesting genetic variations of *A. phagocytophilum*. Interestingly, the susceptibilities of mammalian species to *A. phagocytophilum* strains also vary (Rikihisa, 2011).

Table 4. *Anaplasmataceae*,hosts and distribution. (Adapted from Rikihisa et al. 2011.)

Genus	Species	Hosts	Host cells	Distribution
Ehrlichia	*E. canis*	Canids, human	Monocytes/macrophages	Global
	E. chaffeensis	Human, deer, dog	Monocytes/macrophages	United States, South America, Asia
	E. ewingii	Human, dog, deer	Granulocytes	United States
	E. muris	Human, rodents	Monocytes /macrophages	Japan, Russia United States
	E. ruminantium	Human, ruminants	Granulocytes, endothelial cells	Africa, Caribbean
Anaplasma	*A. phagocytophilum*	Human, horse, ruminants, rodents, dog, cat, deer	Granulocytes, endothelial cells	Europe, United States, Asia
	A. marginale	Bovine	Erythrocytes	Global
	A. bovis	Bovine, deer, Rabbit	Monocytes	United States, Africa, Japan
	A. platys	Dog	Platelets	Global
Aegyptianella	*A. pallorum*	Birds	Erythrocytes	Global
Neorickettsia	*N. risticii*	Horse	Monocytes/macrophages intestinal epithelial cells, mast cells	North and South America
	N. sennetsu	Human	Monocytes/macrophages	Japan, Southeast Asia
	N. helminthoeca	Canids	Monocytes/macrophages	North and South America
Wolbachia	*W. pipientis*	Arthropds, nematodes	Syncytial lateral cord cells, ovary	Global
Candidatus Neoehrlichia	*Ca. Neoehrlichia Mikurensis*	Human, rodents	Endothelial cells	Europe, Japan, China
	Ca. Neoehrlichia Lotoris	Racoon	Unknown	United States
Candidatus Xenohaliotis	*Ca. Xenohaliotis Californiensis*	Abalone	Gastrointestinal epithelial cells	United States

Epidemiology

HGA is a tick-borne zoonotic infection, the bacteria being maintained through enzootic cycles between ticks and animals.(Parola et al., 2005). Humans are involved as accidental "dead-end" hosts (Blanco and Oteo, 2002). To date, transovarial transmission of *A. phagocytophilum* is not known or occurs only at very low frequency (Bakken and Dumler, 2006).

In Europe, clinical manifestations of *A. phagocytophilum* have been shown in e.g. horse, cattle, sheep, goat, dog and cat and human (Engvall and Egenvall, 2002; Stuen, 2007). Wild rodents, sheep, roe deer and foxes are suggested to be reservoirs (Dumler et al., 2005; Dumler et al., 2007; Stuen, 2007; Thomas et al., 2009). Today, HGA is the third most common tick-transmitted infection in Europe (Dumler, 2012). Most cases of HGA are seen between April and October, reaching a peak in July (Brouqui et al., 2004). More infections have been reported in males than females, and in the US, male patients outnumber females by a factor of 3 to 1 (Bakken and Dumler, 2006; Blanco and Oteo, 2002; Brouqui et al., 2004).

HGA cases have been reported not only from the USA, but also from several countries in Europe and Asia (Dumler et al., 2007). Since HGA is not a nationally reportable disease in Sweden or in most European countries, it is not easy to estimate the true numbers of HGA cases. In Sweden, several HGA cases have been reported since the first case reports in 1999 (Bjoersdorff et al., 1999a). In the USA, HGA is reportable and the disease has increased every year with 1,161 cases reported in 2009 (Dumler et al., 2007).

In Europe the seroprevalence rates range from very low to 28% (Table 17, Results and discussion). Generally the proportion of seropositive persons increases with age, in patients with LB or TBE, tick-exposed persons and is also higher in forestry workers (Strle, 2004). Furthermore, the seroprevalence of IgG antibody titres for *A. phagocytophilum* is rather high in tick-exposed human populations in Sweden, ranging between 8-28 %. Some of the patients have also been found to have a co-infection with LB (Bjoersdorff et al., 1999b; Dumler et al., 1997; Wittesjo et al., 2001).

The antibody response after an *A. phagocytophilum* infection is probably rather long-lasting. European data show that two years after onset of acute illness nearly half of the patients still have antibodies (Lotric-Furlan et al., 2001).

A. phagocytophilum has been detected in *Ixodes* ticks by PCR in most European countries and the prevalence ranges from 0.4% to 66.7% (Brouqui et al., 2004). In Sweden, studies have shown a prevalence of *A. phagocytophilum* infected ticks of between 1.3 and 15.0%. (Severinsson et al., 2010). HGA is most commonly acquired from tick bites. However, HGA cases have also been reported from perinatal transmission (Horowitz et al., 1998), transfusion transmission (Annen et al., 2012), and also from infection due to deer blood infected with *A. phagocytophilum*, affecting butchers or hunters (Bakken et al., 1996b). Zhang

et al. reported a possible nosocomial transmission of *A. phagocytophilum* in China and in that case, the first report of human-to-human transmission (Zhang et al., 2008).

Clinical manifestations of HGA

The clinical presentation of HGA is generally an acute non-specific febrile illness that consists of high fever (>39ºC), headache, malaise, generalized myalgia and or arthralgia (Bakken and Dumler, 2000; Brouqui et al., 2004). Other symptoms, less commonly reported, include nausea, abdominal pain, diarrhoea and cough (Brouqui et al., 2004). Non-specific rash is not considered typical for HGA, as for LB, but is infrequently reported as a rash varying from erythematous to pustular (Bakken and Dumler, 2000). Atypical pneumonitis is reported in some cases (Lotric-Furlan et al., 2003; Remy et al., 2003).

The incubation period is 5-21 (median 11) days (Brouqui et al., 2004). The transmission of *A. phagocytophilum* from ticks to mammal hosts is estimated to occur not earlier than 24 h after attachment, based on mouse studies (Katavolos et al., 1998). The duration of fever ranges from 2-11 days (median 10 days) (Brouqui et al., 2004).

Usually HGA is mild and clinical symptoms and signs resolve in most cases within 30 days, even without antibiotic treatment (Bakken and Dumler, 2000; Wormser et al., 2006) . However, HGA can be severe, including a fatal outcome (Bakken et al., 1996a; Hardalo et al., 1995). In Europe the clinical course of patients is usually favourable. In the USA, mortality has been estimated to be 0-5% (Blanco and Oteo, 2002). Reported complications associated with HGA are e.g. acute respiratory distress syndrome (Dumler et al., 2007), disseminated intravascular coagulation (Bakken et al., 1994), rhabdomyolysis (Boateng et al., 2007) and severe opportunistic infections (Dumler et al., 2007).

Bakken and colleagues showed that patients who were immunocompromised or who took immunosuppressive medication were five times more likely to be hospitalized (Bakken et al., 2002). Different studies show a hospitalization rate of between 28-50% (Aguero-Rosenfeld et al., 1996; Bakken et al., 2002; Dumler et al., 2007). Approximately 5-7% of the patients require intensive care, based on reports from the USA (Dumler et al., 2005). Only rarely are CNS infections seen in HGA, with meningoencephalitis reported in only about 1% of cases (Dumler et al., 2007). In contrast, a number of neurological manifestations have been reported, including cranial nerve palsies, brachial plexopathy and demyelinating polyneuropathy (Dumler et al., 2007; Horowitz et al., 1996).

No chronic forms of HGA have so far been reported in Europe (Brouqui et al., 2004; Wormser et al., 2006). In a two-year follow-up study conducted in Slovenia, none of the HGA cases were found to have a long-term clinical sequel. Interestingly, neither were differences seen in clinical outcome between patients

who had been treated with doxycycline and those not given antibiotics (Lotric-Furlan et al., 2001).

Laboratory tests can be helpful for diagnosis of HGA, but the findings are non-specific (Brouqui et al., 2004). Typical laboratory abnormalities are thrombocytopenia (71-90%), leukopenia (49-70%), elevated hepatic transaminase levels (71%) and anaemia (37%) (Brouqui et al., 2004; Dumler et al., 2005). Also, elevated concentrations of C-reactive protein and erythrocyte sedimentation rate can be found (Blanco and Oteo, 2002; Brouqui et al., 2004). In European patients, all laboratory abnormalities that are present initially, usually resolve within 14 days. Furthermore, Bakken and Dumler also report that leukopenia and thrombocytopenia, seen in American patients, normalize by the end of the second week after onset (Bakken and Dumler, 2006).

Methods of detecting *A. phagocytophilum*

Direct detection

Culture

Successful cultivation of *A. phagocytophilum* in HL-60 cells, a promyelocytic leukaemia cell line, was described in 1996. (Goodman et al., 1996). The HL-60 leukaemia cell line is the most used cell line for growing *A. phagocytophilum* (Brouqui et al., 2004). These cells are maintained in antibiotic-free RPMI-1640 medium (Goodman et al., 1996). At days 3-7 the infection is usually visual as morulae (Bjoersdorff et al., 2002a). *A. phagocytophilum* can also be cultured, using a tick cell line culture (Munderloh et al., 1999; Munderloh et al., 2003).

Blood smear

A. phagocytophilum infect circulating granulocytes, causing formation of intracellular morulae (Figure 9) in the cytoplasm (Bakken et al., 2001) . By using EDTA blood, morulae can be visualized on peripheral blood smears stained with Romanowsky-type stain (Wright, Giemsa) (Aguero-Rosenfeld, 2003). Morulae in blood smears are rarely found in European patients (van Dobbenburgh et al., 1999) but they are present in 25 to 68% of patients in the USA (Aguero-Rosenfeld, 2003; Aguero-Rosenfeld et al., 1996; Bakken et al., 2001; Bakken et al., 1996a; Belongia et al., 1999).

The success of finding morulae is based on the experience of the microscopist (Blanco and Oteo, 2002) but also on the duration of illness since the morulae tend to be less frequent after the first week of illness (Bakken and Dumler, 2000). The success of finding for morulae may also be limited if there are only a few infected granulocytes. However, false-positive results may also be found due to other cytoplasmatic inclusion bodies (Ijdo et al., 1997).

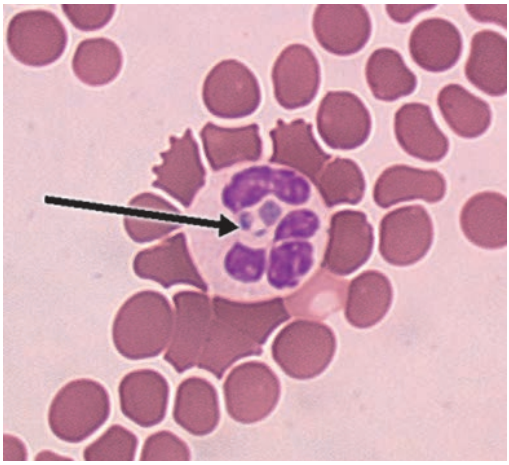

Figure 9. Inclusion of *A. phagocytophilum* in a neutrophil, also called a Morula. (May-Grünwald/Giemsa stain). (Courtesy of Anneli Björsdorff, Kalmar).

PCR- polymerase chain reaction (PCR)

The method based on PCR gene amplification is a rapid process which is of great value to the treating physician. The sensitivity of PCR detection is between 67-90% for *A. phagocytophilum* (Bakken and Dumler, 2000; Dumler et al., 2007). However, the sensitivity for PCR is high only during the first week because the bacteraemic phase of the infection rapidly wanes (Bakken and Dumler, 2006; Thomas et al., 2009). Thus, the PCR technique is of limited value when a patient is presenting with symptoms or signs lasting longer than one week. It is also important to obtain blood samples before treatment since the sensitivity of PCR is affected (Dumler et al., 2007).

Numerous PCR amplifications assays have been described for detection of *A. phagocytophilum,* and the PCR methods are not yet standardized (Blanco and Oteo, 2002; Brouqui et al., 2004; Massung and Slater, 2003). Discrepancies in PCR sensitivity are possibly related to the length of the amplicon and the primer used (Brouqui et al., 2004). Several PCR-based assays are available and there are also different types of primers, such as 16S rRNA, groESL, epank1 and P44 (msp2) (Aguero-Rosenfeld, 2003; Brouqui et al., 2004). Based on the European recommendations by Brouqui et al., whichever DNA target is used, sequencing of PCR products is required to confirm their identity (Brouqui et al., 2004).

Indirect detection

Serology

The most commonly used serological method is an indirect immunofluorescence antibody (IFA) test. This assay detects antibodies reactive to *A. phagocytophilum antigen*. Earlier assays included antigen derived from horse, known as *E. equi*

(Dumler et al., 1995). Today, human isolates are used as antigen in IFA preparations (Aguero-Rosenfeld, 2003). Different antigens are used in the assays, and to date no standardization exists (Brouqui et al., 2004). *A. phagocytophilum* antigens are coated onto glass slides and bound antibodies are detected after incubation with anti-human IgG conjugates (Aguero-Rosenfeld, 2003; Brouqui et al., 2004). The principles of IFA are presented in the Materials and Methods section.

At least two sera should be collected, one at the acute phase and one after 15-21 days during the convalescent phase (Brouqui et al., 2004). An antibody titre ≥80 is considered positive and reflects recent or past exposure to *A. phagocytophilum* (Bakken and Dumler, 2000; Olano and Walker, 2002). Antibodies usually develop within two weeks after onset of illness and may be detectable for several years after successful treatment (Aguero-Rosenfeld, 2003; Bakken et al., 2002). Antibiotic treatment does not prevent a serological response and there is no relationship between the severity of HGA and the absolute IFA titre (Bakken et al., 2002).

Criteria for confirmed HGA cases include seroconversion or fourfold or greater titre rise when comparing acute and convalescent samples (Bakken and Dumler, 2008; Brouqui et al., 2004). Sensitivity ranges from 82-100 % for IgG *A. phagocytophilum* antibodies (Walls et al., 1999), while specificity is estimated at 83-100% (Dumler et al., 2007).

Although IFA is a diagnostic tool with high sensitivity it suffers from limitations, such as a need for acute and convalescent phase testing (Aguero-Rosenfeld, 2003). Other shortcomings are lack of standardization and also possible cross-reactions (Dumler et al., 2007).

Treatment

HGA responds well to treatment with tetracycline antibiotics (Bakken and Dumler, 2006; Dumler et al., 2007), which leads to clinical improvement within 24-48 h (Aguero-Rosenfeld et al., 1996; Bakken and Dumler, 2000). An alternative diagnosis should be considered if the symptoms persist after 48 h of tetracycline therapy (Bakken and Dumler, 2000). Doxycycline is preferred over tetracycline because of its twice-daily oral dosing (or in the case of Sweden, doxycycline 200 mg, once daily) (Bjoersdorff et al., 1999a). Furthermore, it offers better patient tolerance and a relatively lower risk of adverse drug effects (dental staining) for children <8 years (Bakken and Dumler, 2006; Dumler et al., 2007).

In vitro studies have shown that rifampin and quinolones are active against *A. phagocytophilum* (Horowitz et al., 2001; Maurin et al., 2003). A small number of pregnant women and paediatric patients diagnosed with HGA were treated successfully with rifampin (Bakken and Dumler, 2006; Krause et al., 2003). According to Bakken et al, the optimal duration of doxycycline therapy has not yet been established. If a co-infection with *B. burgdorferi* is suspected, doxycycline

therapy for 14 days is recommended. In patients treated for HGA for 7-10 days, the infection has resolved completely. A shorter course of doxycycline (five to seven days) had been suggested for paediatric patients (Bakken and Dumler, 2006).

Differences between Europe and the USA

The clinical picture of LB differs somewhat in Europe compared to the USA, even though there are many similarities. First, *B. burgdorferi s.s* is the only known strain to cause LB in North America, while several genospecies are human pathogenic in Europe (Stanek and Strle, 2009; Wang et al., 1999).

EM is the most common feature of LB in both Europe and North America; however, European patients seem to have less systemic symptoms (Strle and Stanek, 2009). In the US, the skin lesion is frequently accompanied by fever, headache, arthralgia, malaise and myalgia, suggestive of dissemination of the spirochete (Steere et al., 1983a). Strle et al. compared culture-confirmed EM caused by *B. afzelii* in European patients and *B. burgdorferi s.s* in the US and reported systemic symptoms in 50.6% and 68.9% respectively (Strle et al., 1999). In addition, multiple EM are more common in North America compared to Europe (Pachner and Steiner, 2007).

Lymphocytoma and ACA are two dermatological manifestations of LB seen in Europe but not in North America (Samuels and Radolf, 2010). It is believed that early neurological involvement is much more common in Europe, probably because of the occurrence of several neurotropic genospecies, particularly *B. garinii* and occasionally *B. afzelii* (Hengge et al., 2003; Strle et al., 2006).

Some differences can be seen between patients diagnosed with HGA in North America and Europe. Clinical features and laboratory findings are similar, but HGA seems to be milder in Europe and also seems to resolve sooner. Furthermore, the presence of morulae in neutrophils is uncommon (Brouqui et al., 2004).

One explanation for the differences in the clinical pictures of LB and HGA could be the variation in strains of *Borrelia* and *Anaplasma* species between the two countries (Massung et al., 2000; van Dam et al., 1993). Infection with TBE has so far not been found in the USA.

Tick-borne encephalitis (TBE)

Tick-borne encephalitis virus (TBEV) belongs to the genus flavivirus in the flaviviridae family and is related to the viruses causing yellow fever, Japanese encephalitis, dengue and West Nile viruses (Gould and Solomon, 2008; Heinz, 2003). The first clinically description of the disease was given in 1931 by Schneider in Austria (Schneider, 1931), and Russian scientists were able to iso-

late virus strains in 1937 (Kunz and Heinz, 2003). TBEV is a RNA virus and has been divided into three subtypes, Western European, Far-Eastern and Siberian. The vector of the European subtype is mainly *I. ricinus* and *I. persulcatus* (taiga) ticks for the two other subtypes (Jaaskelainen et al., 2011; Lindquist and Vapalahti, 2008).

Epidemiology

Most TBE viruses use rodents as maintenance and amplifying hosts, e.g. mice, thus the mice is a transient bridge for virus transmission (Randolph, 2011; Suss, 2003). Because of its long lifespan, the tick is the main reservoir of TBEV (Lindquist and Vapalahti, 2008). Co-feeding is the major factor in virus transmission, i.e. an uninfected larva feeds next to an infected nymph on the same host and thereby acquires the virus (Donoso Mantke et al., 2011; Labuda and Randolph, 1999; Randolph, 2011). It is assumed that less than <0.5% of the transmission is transovarial, from infected females to eggs (Lindquist and Vapalahti, 2008).

TBEV is transmitted within minutes after a tick bite from the saliva of an infected tick. Occasionally, TBEV can be transmitted after intake of unpasteurised milk, blood transfusions, breast feeding and laboratory work (Lindquist and Vapalahti, 2008).

TBEV is believed to cause about 3,000 cases of TBE in Europe and up to 10,000 human cases in Russia annually (Gritsun et al., 2003; Suss, 2011). In Sweden the European subtype is present and TBE is endemic in the coastal regions, around the big lakes in Southern Sweden and on the west coast (Lundkvist et al., 2011). Recently, new geographic areas of TBE occurrence have been identified in southern Sweden (Fält et al., 2006).

The incidence varies annually and in different regions, and it is believed to depend on several factors, such as the density of ticks and the concentration of virus within individual ticks. Also important is the number of humans encountering infected ticks and the duration of tick feeding on the host (Gritsun et al., 2003). Unlike *B. burgdorferi*, TBEV is not found throughout the range of *I. ricinus* but is found in patchy foci from a few square meters to several square kilometres (Lindquist and Vapalahti, 2008). The rate of environmental temperature increase in spring determines the extent to which larvae and nymphs are host-seeking at the same time period. Co-feeding of larvae and nymphs on the same host, has been shown to be critical for maintaining the virus in the tick population and may explain to the focal distribution of TBEV (Randolph, 2011).

The number of TBE cases has increased during the last decade in Europe and also in Sweden (Donoso Mantke et al., 2011; Lundkvist et al., 2011; Suss, 2011). During 2011, 287 cases were reported in Sweden (Jaenson et al., 2011). The in-

crease probably has several reasons, such as climate changes, enlarged tick populations, or human behaviour (Lundkvist et al., 2011).

Clinical manifestations of TBE

Tick-borne encephalitis has an incubation period of 4-28 days (median eight days) after tick bite. Typically, the disease is biphasic in 72-87% of the patients (Kaiser, 1999; Lindquist and Vapalahti, 2008). The first stage of illness ranges from 2-10 days (median five days) with symptoms such as fever (99%), fatigue (63%), general malaise (62%), headache and body pain (54%) (Lindquist and Vapalahti, 2008). After a symptom-free interval of a median of eight days (range 1-33 days, a meningoencephalitic phase presents in up to 20-30% of the infected patients (Gustafson et al., 1992). However, in the second phase the symptoms range from mild meningitis to severe meningoencephalomyelitis.

CSF analyses reveal initially a dominance of polymorphonuclear cells, but after a few days this changes to nearly 100% mononuclear cells (Haglund and Gunther, 2003). During the first phase, leukopenia, thrombocytopenia and moderate elevated liver transaminases can be seen. Increasing age is correlated to severity of illness (Lindquist and Vapalahti, 2008) and sequelae are reported in 39.8-46.2% after 12 months (Gunther et al., 1997; Mickiene et al., 2002). The case fatality rate is about 1-2% in the European subtype (Gritsun et al., 2003).
No treatment exists, but TBE can be prevented by immunization. In this thesis immunization will not be further discussed.

Co-infections and other tick-borne infections

Ixodes ricinus (Figure 15) transmits several zoonotic agents, the most well-known being bacteria *B. burgdorferi,* and *A. phagocytophilum* and the TBE virus. Each of these causative agents can initially induce fever and other unspecific symptoms, including headache, myalgia and arthralgia (Brouqui et al., 2004; Chen et al., 1994; Lotric-Furlan et al., 2000; Stanek and Strle, 2003; Wahlberg et al., 1964; Walker and Dumler, 1996). *Ixodes ricinus* is distributed mainly in the south and central parts of Sweden, and along the east coast up to Umeå. This coincides with the distribution of infection of *B. burgdorferi* and *A. phagocytophilum*, while the TBE virus is distributed in a more patchy, geographically localised way (Holmgren and Forsgren, 1990).

Co-infections in humans with one or several tick-borne pathogens have been reported both from Europe and North America (Arnez et al., 2003; Lotric-Furlan et al., 2005; Steere et al., 2003). Furthermore, dual infections with *B. burgdorferi* and *A. phagocytophilum* have been documented in e.g. humans, wild mammalian rodents, and in experimental mice (Grzeszczuk et al., 2006; Johnson et al., 2011; Zeidner et al., 2000). Other tick-borne pathogens that could cause unspecific symptoms are *e.g. Rickettsia helvetica, Babesia divergens and Candidatus*

Neoehrlichia micurensis (Parola and Raoult, 2001; Steere et al., 2003; Thompson et al., 2001; Welinder-Olsson et al., 2010). It has been suggested that co-infected patients may experience more unspecific symptoms and have more severe illness (Krause et al., 2002; Steere et al., 2003; Swanson et al., 2006). *Rickettsia helvetica*, a strict intracellular, gram-negative bacterium, has been detected in Swedish *Ixodes* ticks (Nilsson et al., 1999a; Parola and Raoult, 2001; Severinsson et al., 2010). Some data are suggestive of clinical infections, usually mild, with symptoms like fever, headache, muscle pain and rash, but more severe symptoms such as meningitis and periomyocarditis have been reported (Nilsson et al., 1999b; Nilsson et al., 2011; Parola and Raoult, 2001).

Babesiosis, caused in Europe mainly by *Babaesia divergens*, is an intracellular parasite of the erythrocytes. The spectrum of disease is broad, ranging from a silent infection to a fulminant, malaria-like disease with symptoms such as high fever, chills, myalgia and fatigue (Homer et al., 2000). Only a few cases of babesios have been reported in Sweden. (Uhnoo et al., 1992).

Candidatus Neoehrlichia micurensis (*Ca. Neoehrlichia. mikurensis*) is a member of the family Anaplasmataceae, an obligate intracellular bacterium, with endothelial cell tropism (Richter and Matuschka, 2012; Rikihisa, 2011). This novel bacterium was isolated from wild *Rattus norvegicus* and *Ixodes ovatus* ticks in Japan. At least in rats, it appears to reside within the cytoplasm of endothelial cells lining the venous sinuses (Kawahara et al., 2004). DNA of *Ca. Neoehrlichia mikurensis* has been found in European *I. ricinus* ticks and rodents (Richter and Matuschka, 2012) and also in ticks in Sweden in a recent study (Kozak et al. upublished) The first human case, an immune-compromised man with febrile episodes, presenting an erysipelas-like rash and thromboembolic complications, was reported in 2010 in Sweden (Welinder-Olsson et al., 2010). In addition, two human cases of *Ca. Neoehrlicha mikurensis* have been reported from Germany, with severe thrombotic events in patient 1 and aneurysm in patient 2 (von Loewenich et al., 2010).

Co-infections and immunology

Most studies on co-infections with *B. burgdorferi* and *A. phagocytophilum* are based on animal studies, however, human studies exist (Nieto and Foley, 2009). It is suggested that a co-infection would make the clinical outcome worse, and experimental studies have revealed modified immunological responses to both pathogens associated with higher bacterial burdens, longer persistence, and worsened disease (Holden et al., 2005; Zeidner et al., 2000). As *A. phagocytophilum* infects neutrophils and impairs phagocytosis this could lead to an increased availability of *B. burgdorferi* to transmigrate (Garyu et al., 2005).

Furthermore, an experimental study has shown that mice co-infected with *B. burgdorferi* and *A. phagocytophilum* have a decreased T-helper type 1 (Th1) response, with diminished levels of IFN-γ, IL-12 and TNF-α compared to mice

single-infected with *B. burgdorferi*. The co-infected mice also developed a more severe form of arthritis and showed an increased pathogenic load of both bacteria types (Thomas et al., 2001). The immunological aspects associated with TBE are not studied in this thesis.

Immunology

The responses our body makes against infection by potential pathogens are called immune responses. These responses consist of an immediate response (innate immunity), the first line of defence, while the adaptive immune response is a specific response that occurs later (hours-days) (Murphy, 2012; Mölne and A., 2007). Our immune system works in order to protect us effectively from disease. To be able to do this the immune system must fulfil four main tasks: 1. immunological recognition, 2. immune effector functions, 3. immune regulation, and 4. immunological memory (Murphy, 2012). Figure 10 illustrates the cells of the immune system that originate in the bone marrow.

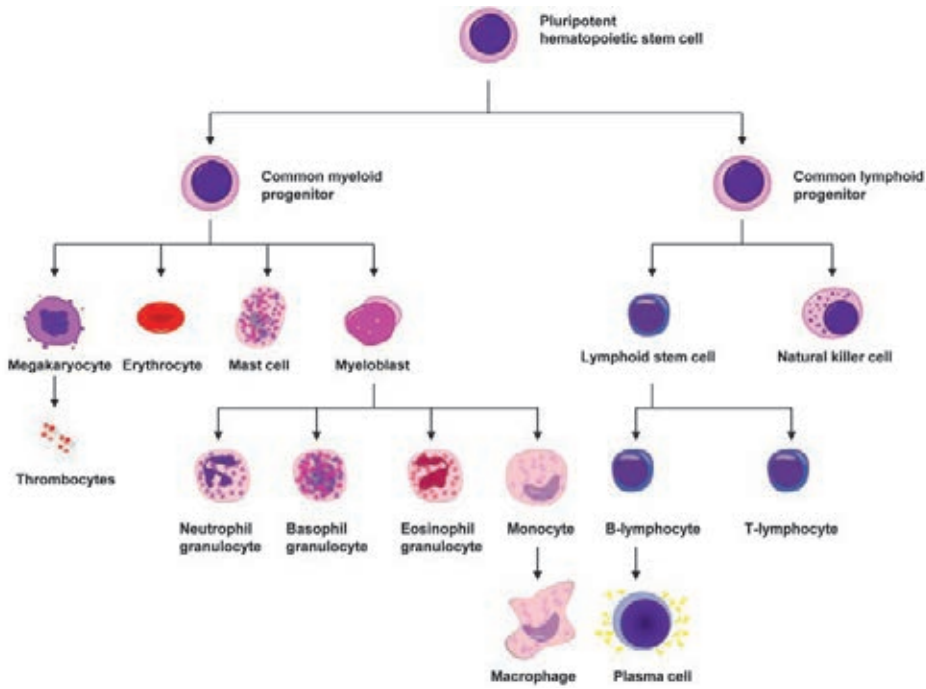

Figure 10. The cells of the immune system, development from a hematopoietic stem cell to mature and specialized blood cells. Cartoons used in the picture were obtained from Clker.com – clip art – public domain royalty free clip art.

Innate immune system

The innate immune response enables the host to differentiate self from pathogens, provides a rapid inflammatory response, and further shapes the adaptive

immune response (Turvey and Broide, 2010). This is the crucial first line of defence and consists of different types of barrier; skin and mucous membranes, physiological (temperature, low pH, bacteriolytic lysozyme in tears and saliva), phagocytic (dendritic cells (DCs), macrophages, neutrophils) and inflammatory responses (Mölne and A., 2007; Turvey and Broide, 2010).

The innate immune system is triggered by lipids and other pathogen-associated molecular patterns (PAMPs) that are recognized by pattern-recognition receptors (PRRs) on the surface of phagocytic cells. Toll-like receptors (TLRs) are the most widely studied PRRs, and currently at least 10 TLRs have been identified in humans (Honeyman and Harrison, 2004). (Kumar et al., 2011).

After recognition of pathogens, signalling pathways are induced, leading to phosphorylation and activation of NF- κβ (nuclear factor κβ), which promotes expression of various cytokines and chemokines (Abbas and Lichtman, 2011). Complement pathways represent a complex part of the first line of defence, consisting of the alternative, classical and lectin pathways. Activation of the complement cascade results in opsonization and formation of a lytic membrane attack complex on the bacterial cell membrane. This results in destruction of the pathogen (Abbas and Lichtman, 2011).

The main cells in the innate defence system are DCs, macrophages, natural killer (NK) cells, polymorphonuclear leukocytes (i.e. neutrophils, eosinophils and basophils) and mast cells. DCs are professional antigen-presenting cells (APCs) and the most potent stimulators of the adaptive T cell response. Macrophages and neutrophils are phagocytes that engulf pathogens and destroy them in intracellular vesicles. In addition, macrophages have the ability to present antigens to T lymphocytes and activate them (Murphy, 2012). NK cells are large, non-T, non-B lymphocytes that lack antigen-specific receptors. NK cells kill self cells that have downregulated MHCI expression (Chaplin, 2010). They are rapid responders to pathogens and destroy the target cell by releasing perforin and granzymes from the granula. NK cells produce IFN-γ following stimulation by IL-12 and TNF, thus NK cells may enhance Th1 responses (Honeyman and Harrison, 2004).

Infection stimulates macrophages to release cytokines and chemokines that initiate the inflammatory response. Briefly, this results in dilatation of small blood vessels and changes in the endothelial cells in their walls, leading to the movement of neutrophils and monocytes into the infected tissue. The blood vessels are also more permeable, allowing plasma proteins and fluid to leak into the tissues, thus causing the classical inflammatory signs of *rubor* (redness), *calor* (heat), *tumor* (swelling) and *dolor* (pain) (Murphy, 2012; Mölne and A., 2007).

Adaptive immune system

The adaptive immune system is composed of T lymphocytes, which mature in the thymus, and antibody-producing cells, B lymphocytes, which arise in the bone marrow. Thus, T and B cells are effector cells of the cellular immune response (Bonilla and Oettgen, 2010). The adaptive immune system has several features that are essential for defence, e.g. specificity, diversity, clonal expansion, memory and non-reactivity to self (Abbas and Lichtman, 2011)

After developing in primary lymphoid organs, the T and B lymphocytes move to secondary lymphoid organs (lymph nodes, spleen), which serve to capture circulating antigens from the lymph and blood, respectively (Bonilla and Oettgen, 2010).

Both T and B cells have a unique receptor that recognizes antigens. T lymphocytes are divided into T helper cells (Th), which carry CD4+ proteins, and cytotoxic T cells (CTLs), which carry CD8+. T cells recognize antigens after they have been processed by APCs and presented on a major histocompatibility complex (MHC) complex. CD4+ T cells recognize MHC II, while CD8+ T cells recognize MHC I (Murphy, 2012). Regulatory T (Treg) cells form a subset of CD4+ T cells that either develop in the thymus and are known as natural Treg (nTreg), or are differentiated from naïve T cells in the presence of TGF-β, following T-cell receptor stimulation. This latter group of Tregs are called induced Tregs (iTreg) (Sakaguchi et al., 1995; Wan, 2010).

B cells constitute about 15 % of the peripheral blood leucocytes. They differentiate in response to antigen into plasma cells that produce antibodies and resting memory cells, sometimes without any help from APCs and sometimes with stimulation from Th cells (Abbas and Lichtman, 2011; Chaplin, 2010; Murphy, 2012). Antibodies can participate in host defence in three main ways, i.e. neutralization of toxins, opsonization by coating antigens, and complement activation (Murphy, 2012).

T helper cell subpopulations

Naïve CD4+ T cells can differentiate into several effector T cells after activation by antigen, e.g. Th1, Th2, Th17 and Tregs (Figure 11). Additionally, CD4+ T cells can differentiate into Th9, Th22 and T-follicular effector cells (Akdis et al., 2011). In 1986, Mosmann and Coffman identified two subsets of T helper cells, Th1 and Th2 (Mosmann et al., 1986). The naïve T helper cells are supposed to have pluripotent capacity, thus they can develop into one of several distinct T helper effector lineages, each which has specific cytokine profiles and promotes different effector functions (Janson et al., 2009).

The two subsets acts as antagonistic subpopulations, thus, Th1 secretes e.g. IFN-γ, TNF, Lymphotoxin (LT) and IL-2, whereas Th2 secretes e.g. IL-4, IL-5, IL-9, IL-10 and IL-13(Awasthi and Kuchroo, 2009; Wan, 2010). However, in

humans, IL-10 is not a typical Th2 cytokine, since both Th1 and Th2 cells are capable of producing IL-10 (Akdis et al., 2011).

Th1 cells promote cell-mediated immunity against intracellular pathogens and extracellular bacteria. They are also responsible for phagocytic activation and the production of opsonizing and complement-fixating antibodies. Unregulated pro-inflammatory activities of Th1 may cause tissue damage (Annunziato and Romagnani, 2009; Wan, 2010).The Th2 response is associated with humoral response, allergy, and defence against extracellular pathogens (parasites). In addition, Th2 cells stimulate B cells to produce IgM, IgE and IgG_4 (Annunziato and Romagnani, 2009; Zhu and Paul, 2008).

A third subset, Th17, was discovered some years ago (Harrington et al., 2005; Park et al., 2005) and the Th17 cells seems to produce e.g. IL-17A, IL-17F, TNF, IL-22, IL-26, IFN-γ, IL-6 and IL-8 (Akdis et al., 2012; Tesmer et al., 2008). Indeed, cells co-expressing IL-17 and IFN-γ have been isolated from human blood (Acosta-Rodriguez et al., 2007; Crome et al., 2010).

Th17 cells are mainly associated with autoimmune tissue diseases, such as rheumatoid arthritis, psoriasis and multiple sclerosis. The functions of Th17 cells and Treg cells work opposite to each other. TGF-β is important for the generation from naïve T cells, for both of them. However, Treg cells are immunosuppressive, inhibit tissue inflammation and maintain self-tolerance, while Th17 cells are essential for the inflammatory process (Awasthi and Kuchroo, 2009; Wan, 2010). Treg cells produce inhibitory cytokines such as IL-10 and TGFβ, thus suppressing immune responses and inflammation (Murphy, 2012). Furthermore, Th17 cells are also involved in the human adaptive response against *C. albicans* (Acosta-Rodriguez et al., 2007), and have also been found to promote production of IgM, IgG and IgA thus, modulating B cell functions (Crome et al., 2010).

Several additional subsets have recently been discovered, e.g. Th9 cells, producing IL-9. IL-9 is produced also by Th2 and Th17 cells and appears to be a key molecule that affects the differentiation of Th17 cells and Treg function. However, the pathophysiological role is not clear (Annunziato and Romagnani, 2009). Finally, two other subsets are Th22 cells, producing IL-22, important in tissue inflammation and follicullar helper T cells (Th FH), that provides a helper function to B cells (Akdis et al., 2012).

Cytokines are important stimulators of Th cell differentiation, but also act as regulators by suppressing the development of other Th lineages. Thus, it is believed that cytokines regulate each other´s signalling by crosstalking (Zhu and Paul, 2010).

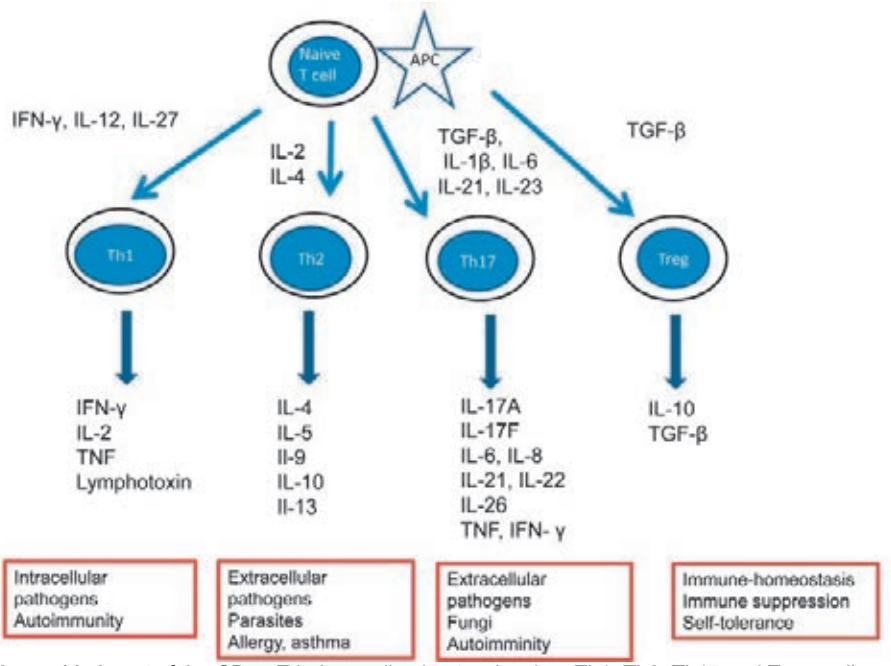

Figure 11. A part of the CD4+ T helper cell subsets, showing, Th1, Th2, Th17 and T reg cells. Adapted from Annunziato et al. 2009, Akdis et al 2012.

Cytotoxic mechanisms

There are two main types of cytotoxic cells - cytotoxic T lymphocytes (CTLs) and NK cells – and these create perform the cell-mediated cytotoxicity. This is carried out by two pathways, e.g. the granule exocytosis and the Fas/Fas ligand (FasL) pathway in order to kill infected cells (Russell and Ley, 2002). CTLs (CD8+ cells) recognize and eliminate tumour cells, virus-infected cells and intracellular pathogens, via MHCI, expressed on the target cells (Murphy, 2012). The naïve CD8+ cells require stimulation by APCs and co-stimulation, an activation process requiring 1-3 days for maximal activity. The presence of CD4+ Th cells is probably also needed in most CTL responses, though this is not completely clear (Figure 12) (Bevan, 2004; Russell and Ley, 2002).

CTLs circulate throughout the body until they encounter foreign antigens in secondary lymphoid tissues (Nolz et al., 2011). The CTL response to acute infection can generally be divided into four phases: 1) During the effector phase, naïve CTLs are primed, undergo expansion, acquire effector functions and travel to sites of infection. This is followed by killing of infected target cells and secretion of effector cytokines. 2) During the contraction phase most effector CTLs die, leaving about 5-10% as memory cells. 3) During the memory maintenance phase, memory CTLs are maintained at stable levels for many years. 4) Re-exposure to

the pathogen drives memory CTL populations away from lymph nodes and towards peripheral tissues (Nolz et al., 2011; Williams and Bevan, 2007).

The generation of effector CTLs requires several signals, the first via the T cell receptor/MHC interactions and antigen, the second via a variety of co-stimulatory molecules expressed by activated DCs and CTLs. B7 and 4-1BB are two molecules that appear to play a role in the activation. B7 molecules interact also with CD28 (Commins et al., 2010). Furthermore, B7 expressed by DCs first activates the CD4+ cells to express e.g. IL-2 and CD40 ligand. CD40 ligand binds to CD40 on DCs, thereby delivering an additional signal that increases the expression of B7 and 4-1BBL by the DCs. This provides additional co-stimulation to the naïve CD8+ T cell (Murphy, 2012; Williams and Bevan, 2007). The cytokines IL-1 and IL-6 induce simultaneous secretion of IL-2, which acts as a growth factor to promote CTL differentiation (Commins et al., 2010; Murphy, 2012).

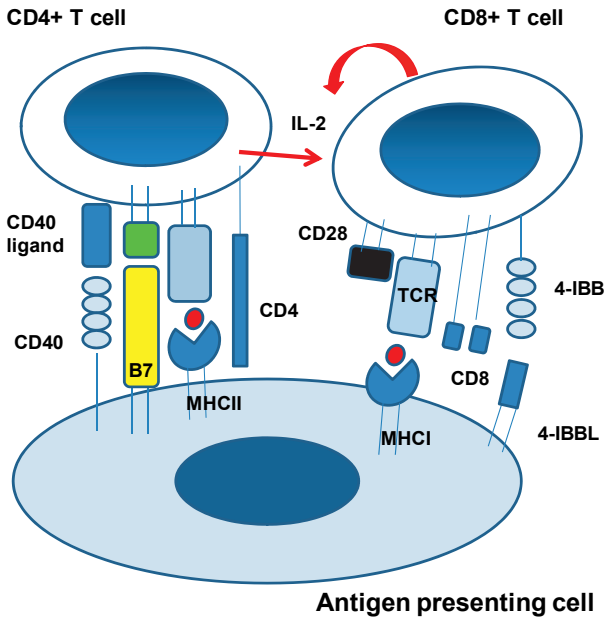

Figure 12. Principles of activation of CD8+ T cells by antigen presenting cell, CD4+ T cell and co-stimulatory molecules. Adapted from Murphy 2012.

When CTLs are activated they start to secrete perforin and granzymes that promote apoptosis. In contrast, NK cells are armed with granules and can start killing within minutes of the first stimulation (Russell and Ley, 2002). Furthermore, the Fas/Fas ligand pathway leads to interaction between Fas L on the surface of the CTLs and Fas on the target cells, which triggers apoptosis via activation of

caspases. CTLs kill target cells with great precision in order to minimize tissue damage (Murphy, 2012).

The third signal for optimal development of CTLs is suggested to be derived from IL-12, produced by activated DCs and phagocytes, or from inflammatory signals supplied by Toll-like receptor ligands and type I IFNs (Williams and Bevan, 2007).

Cytokines and chemokines

Cytokines are small proteins made by various cells in the body that act as signal substances that affect the behaviour of other cells. They induce responses through binding to specific receptors. Murphy (2012). Cytokines are involved in almost every aspect of immunity and inflammation, e.g. innate immunity, antigen presentation and bone marrow differentiation (Borish and Steinke, 2003).They can act in an autocrine manner, affecting the cell that releases the cytokine, or in a paracrine manner, meaning affecting adjacent cells. Some cytokines can also act in an endocrine way, affecting the behaviour of distant cells (Murphy, 2012).

Studies on cytokines and their effector functions are often characterized using murine models. Therefore, it is important to remember that there are similarities but also differences, when comparing e.g. the different T helper subsets and the secreted cytokines.

The **IL-12** family is composed of cytokines including, IL-12, IL-23, IL-27 and IL-35. IL-12 is composed of two subunits, p35 and p40. When combined together, a bioactive form, IL12p70, is formed. IL-23 consists of p19 and shares the p40 subunit with IL-12. Their receptors also share the IL-12Rβ1 subunit (Commins et al., 2010; Gee et al., 2009). Both IL-12 and IL-23 are mainly produced by DCs and macrophages. IL-12 induces differentiation of naïve T cells into Th1 cells, stimulates IFN-γ production by NK cells and T cells, and enhancement of cytotoxity of NK cells and CTL. IL-23 stimulates IFN-γ production and is also suggested to play a crucial role in the generation of Th17 cells (Duvallet et al., 2011; Gee et al., 2009).

IL-10 is a multifunctional cytokine with anti-inflammatory effects; i.e. limitation of the inflammatory response and prevention of tissue damage (Dennis et al., 2006). IL-10 is a product of numerous cells, e.g. T cells, NK cells macrophages and DCs (Akdis et al., 2011). Furthermore, IL-10 directly affects APC functions by downregulating the expression of MHC class II and costimulatory molecules on the surface of macrophages and monocytes (Akdis et al., 2011). It also exerts immunostimulatory effects, i.e. enhancement of antibody production and cytotoxicity, and acts as a growth factor to stimulate proliferation of CD8+T cells (Borish, 1998; Groux et al., 1998; Mosser and Zhang, 2008).

IL-17A (here called **IL-17**) is a pro-inflammatory cytokine that is secreted by cells of both the innate and the adaptive systems, e.g. neutrophils, NK cells, NKT cells, and eosinophils, suggesting a bridging function (Korn et al., 2009). It

stimulates recruitment and activation of neutrophils (Commins et al., 2010). IL-23 appears to have a central role in Th17 cell development. Other cytokines that probably induce Th17 development are e.g. LT, IL-1β, IL-6 and IL-21 (Crome et al., 2010; Di Cesare et al., 2009).

Table 5. Cytokines analyzed in this thesis.

Cytokine	Producer cells	Actions	Paper	Reference
IL-2	CTL cells, CD4+ T cells, DC, NK, NKT cells	Activation of CTL cells. B-cell growth factor. Stimulation of antibody synthesis Development of Treg cells. Proliferation of NK cells, enhances their cytolytic functions.	II	Akdis et al. 2011
IL-4	Th2 cells, NKT cells, Mast cells	Stimulation of Th2 differentiation. IgE class switch. Up-regulation of class II MHC on B cells, activation of B cells. Suppression of Th1 development.	I	Akdis et al 2011, Borish et al. 2003
IL-5	Th2 cells, mast cells, Activated eosinophils	Stimulation of B cells. Stimulation of eosinophil proliferation.	I	Akdis et al 2011 Borish et al. 2003
IL-7	DC, B cells, monocytes, Macrophages, epithelial cells	Development of B, T cells, mature T cells, NK cells. Homeostasis of CD8+ cells.	II	Akdis et al 2011 Schluns et al 2000
IL-10	Monocytes, B cells, Treg, NK cells, macrophages, DC, mast cells, Th1, Th2, CTL cells, Th17 cells, CD8+ T cells	Anti-inflammatory. Prevention of tissue damage. Inhibition of Th1, TH2, mononuclear phagocytes Inhibition of antigen presentation. Promotion of B cell proliferation, differentiation. Proliferation of CD8+ T cells.	II	Akdis et al. 2011 Borish et al. 2003 Dennis et al. 2006 Mosser et al 2008
IL-12p70	DC, macrophages, B cells, monocytes	Induction of Th1 differentiation. Activation of CTL cells, NK cells. Stimulation of IFN-γ production by NK cells and T cells.	I, II	Gee et al. 2009, Commins et al. 2010
IL-13	T cells, NKT cells, Eosinophils	IgE class switch. Parasite defence activation of eosinophils, mast cells	I	Borish et al. 2003 Akdis et al 2011
IL-15	Mononuclear cells,	T cell growth factor. Chemoattractant for T lymphocytes Activation, proliferation of NK cells. Homeostasis of CD8+ memory cells, NK cells, NKT cells. Development of γδ T cells, Enhances differentiation of Th2.	II	Commins et al. 2010, Akdis et al. 2011

Cytokine	Producer cells	Actions	Paper	Reference
IL-17	Th17 cells, neutrophils, eosinophils, NK cells, CD8+T cells, NKT cells, γδ T cells	Pro-inflammatory. Recruitment of neutrophils.	II	Commins et al. 2010 Akdis et al. 2011
GM-CSF	T cells, macrophages, endothelial cells, Bone marrow stromal cells, CTL	Hematopoietic growth factor. Recruitment of neutrophils, monocytes, lymphocytes. Activation of DC maturation, functions. Activation of macrophages	II	Shi et al. 2006 Murphy et al. 2012
IFN-γ	Th, CTL, NK cells, Macrophages	Activation of macrophages. Increased MHC I, II expression. Suppression of IL-4, Th2 effects	I, IV	Borish et al. 2003, Commins et. al 2010

CTL; cytotoxic T cell, DC; dendritic cell, GM-CSF; granulocyte-macrophage colony stimulating factor, IL; interleukin, MHC; major histocompatibility complex, NK; natural killer cell, NKT; natural killer T cell, Th cell; T helper cell, Treg; Regulatory T cells.

Immunology of Lyme borreliosis

Borrelia spirochetes possess sophisticated mechanisms to survive in both the tick vector and the host. After the spirochetes are injected into the skin they need to escape the innate immune system. Furthermore, once a disseminated infection has been established, the spirochetes have to evade adaptive host immune responses.

Transmission of B. burgdorferi from tick to human

The tick bite is usually not painful and may go unnoticed. During feeding the spirochetes are transmitted to the host through the tick saliva. *B. burgdorferi* changes the expression of lipoproteins when it transfers from the tick to the mammalian environment (Singh and Girschick, 2004).

In unfed ticks the spirochetes express OspA and OspB in the tick midgut. OspA is synthesized when the spirochetes enter the tick from an infected host, and continue to produce OspA within the resting tick (Schwan and Piesman, 2000). OspA mediates attachment to a receptor, TROSPA, on the gut cells (Pal et al., 2004a). When an infested tick engorges, *B. burgdorferi* multiplies within the tick gut and downregulates OspA (de Silva et al., 1996). At the same time the spirochetes start producing OspC. Moreover, OspC expression seems to help the spirochetes to exit the midgut and enter the salivary glands of the tick (Pal et al., 2004b). This process takes several days, which delays the transmission (Stanek et al., 2011b). OspC is upregulated during transmission and binds to Salp15 (tick salivary protein) in the salivary gland. Salp15 appears to facilitate the infection of the host via its immunosuppressive properties, including inhibition of antibody-mediated killing and inhibition of CD4+ T-cell activation (Ramamoorthi et al., 2005).

Evasion strategies of B. burgdorferi

When the spirochete enters the host, Salp15 possibly provides protection from innate and adaptive immune responses. The binding of Salp15 to OspC on the spirochetes might serve to mask the immunogenic OspC from being seen by the host. This OspC-Salp15 binding probably provides protection from the host, involving macrophages, naïve CD4+ T cells and anti-*Borrelia* antibodies (Fikrig and Narasimhan, 2006; Ramamoorthi et al., 2005). Salp15 inhibits activation of T lymphocytes by binding to the CD4+ co-receptor on the surface of T lymphocytes (Garg et al., 2006).

In addition, the complement system of the host is important as a first line of defence. *Borrelia* spirochetes display their own complement evasion strategies. The Erp family of proteins and the CRASPs help inactivate the alternative pathway of the complement by binding to complement regulator molecules Factor H and Factor-H-like (FHL)-1/reconectin (Fikrig and Narasimhan, 2006). Furthermore, the *Borrelia* spirochetes differ in their susceptibility to complement activation (Kraiczy et al., 2001; van Dam et al., 1997).

In the innate immune response, spirochetal lipoproteins are recognized by TLR-2 (Hirschfeld et al., 1999). The ligation leads to activation of monocytes/macrophages, neutrophils, lymphocytes, endothelial cells and fibroblasts. In addition, inflammatory cytokines are secreted, such as TNF, IL-1β, IL-6 and IL-12 (Hirschfeld et al., 1999; Radolf et al., 1995). The skin is rich in DCs, potent APCs, that are reported to engulf and process *B. burgdorferi* and after activation they secrete pro-inflammatory cytokines, e.g. IL12p70 and IL-8. They also activate *Borrelia* specific T cells by presenting *B. burgdorferi* antigens on MHC class II (Filgueira et al., 1996; Suhonen et al., 2003).

Once a disseminated infection has been established, the spirochetes need to evade the adaptive immune system of the host (Hovius et al., 2007).

Immune responses to B. burgdorferi

Previous studies on *Borrelia*-induced *in vitro* cytokine secretion in mononuclear cells from patients with LB have shown predominating Th1-responses (Ekerfelt et al., 1998; Forsberg et al., 1995; Oksi et al., 1996; Widhe et al., 1998), most pronounced within the target organ, i.e. in the CSF or joint fluid (Ekerfelt et al., 1997a; Gross et al., 1998; Grusell et al., 2002; Wang et al., 1995). Widhe et al. showed early increased secretion in CSF of *Borrelia*-induced IFN-γ and subsequent upregulation of IL-4 in LNB patients with recovery within six months. In contrast, LNB patients with symptoms lasting >6 months had a persistent IFN-γ response. Furthermore, patients with ACA had *Borrelia*-induced IFN-γ but no IL-4 secretion in blood. In patients with EM, increased IFN-γ was observed in blood early during the disease, whereas increased IL-4 was observed after clearance (Widhe et al., 2004).

This supports the hypothesis that, a strong, initially Th1 response is to clear the infection, and after that, a counterbalanced Th2 response is needed in order to limit tissue damages. In addition, an upregulated IFN-γ response is associated with persistent symptoms (Widhe et al., 2004). In line with this, mice with LA and lacking an early Th1 response had more severe symptoms, while it was favourable to first mount a Th1 response and then switch to a Th2 response (Kang et al., 1997).

Involvement of cytotoxic cells in the immune response against *B. burgdorferi* has been suggested based on in vitro studies (Beermann et al., 2000), and activated CD8+ T cells have been shown to accumulate in the CNS during the early phase of LNB (Jacobsen et al., 2003). Furthermore, cells expressing CD8+ or TCRγδ, which both have cytolytic properties, have been reported to be the main phenotypes of IFN-γ-secreting cells from patients with LNB and persisting symptoms (Ekerfelt et al., 2003).

Antibodies are important in the clearance of *B. burgdorferi,* and the spirochetes have been shown to be killed extracellularly (Montgomery and Malawista, 1994; Samuels and Radolf, 2010). Once bound to the antigen, antibodies have different effector mechanisms, including complement activation, neutralization and opsonisation, leading to phagocytosis (Connolly and Benach, 2005; Montgomery and Malawista, 1994). IgG antibodies to OspA and OspB are found later during the course of infection (Ekdahl et al., 2007).

Reports of the role of IL-17 in LB are scarce, but B. burgdorferi lipoproteins induce production of IL-17 in T-cells (Infante-Duarte et al., 2000) and IL-17 has been suggested as a major contributor to the pathogenesis of LA in mice (Burchill et al., 2003; Nakae et al., 2003). Furthermore, increased levels of IL-17 have been reported to be secreted by *in vitro*-stimulated synovial fluid T cells from patients with LA (Codolo et al., 2008).

Interestingly, in a study from south-east Sweden, IL-17 was found in patients with confirmed LNB, suggesting a hitherto unknown role for Th17 in LNB (Henningsson et al., 2011).

Immunology of human granulocytic anaplasmosis

Phagocytic leukocytes are involved in the first-line of host defence against bacteria. *A. phagocytophilum* invades granulocytes, mainly neutrophils, and is found as a cytoplasmic inclusion “morula” (Bakken et al., 2001; Goodman et al., 1996) Immunologic responses of *A. phagocytophilum* include both humoral and cellular responses. However, the immune control of *A. phagocytophilum* is still not fully understood (Dumler, 2012). It is known that these bacteria lack the genes that encode lipid A of lipopolysaccharide and peptidoglycan (Lin and Rikihisa, 2003), known in other gram-negative bacteria to be important triggers of the innate immune system.

Transmission of A. phagocytophilum from ticks to humans

It is unclear where the bacteria replicate after entering the dermis following tick bite before the development of bacteraemia, 4-7 days later. It is also unknown if the pathogen remains at undetectable levels in the blood or replicates in some other cells before bacteraemia develops (Woldehiwet, 2010).

A. phagocytophilum resides within the host neutrophils and enters the tick gut with the blood meal. The transmission from ticks to mammals is estimated to occur in the first 24 h after attachment in mice studies (des Vignes et al., 2001). Within 24-48 h of feeding *A. phagocytophilum* infects the salivary glands of the tick (Hodzic et al., 1998). It is not fully understood how the bacteria migrate from the tick gut to the salivary glands. Liu et al. have however reported that a secreted salivary tick protein, P11, is important in this process. P11 enables *A. phagocytophilum* to infect tick haemocytes (blood cells), which are probably required for the migration of *A. phagocytophilum* from the gut to the salivary glands (Liu et al., 2011).

Evasion strategies of A. phagocytophilum

During *A. phagocytophilum* infection the TLR2 inflammatory signalling system is activated in human and murine macrophages. It is suggested that a lipoprotein or the glycolipid component of *A. phagocytophilum* membranes is an important trigger of the innate immune response and immunopathology (Choi and Dumler, 2007).

The function of neutrophils, the main target of *A. phagocytophilum* infection, is altered by unknown mechanisms. Alterations such as diminished adhesion to activated endothelium, diminished transendothelial cell migration, diminished antimicrobial response (including resistance to initial phagocyte oxidase responses) are established, followed by downregulated expression of key phagocyte oxidase component proteins. Furthermore, there is also the occurrence of defective phagocytosis, prolonged degranulation of pro-inflammatory mediators or chemokines and delay of spontaneous apoptosis, allowing the pathogen sufficient time (>24h post-infection) to develop morulae (Bakken and Dumler, 2008; Dumler et al., 2005; Rikihisa, 2010).

Interestingly, *A. phagocytophilum* induces IL-8 secretion by the host cell, a neutrophil chemokine attractant, recruiting naïve neutrophils to the infection site. This facilitates bacterial dissemination of *A. phagocytophilum* in the host (Akkoyunlu et al., 2001).

Animal models and immunopathogenicity

IFN-γ is produced during murine *A. phagocytophilum* infection and helps to control early pathogen burden (Akkoyunlu and Fikrig, 2000; Martin et al., 2001). In addition, it may also have a role in human infection (Dumler et al., 2000).

There is a discrepancy between pathogen load and tissue injury. In murine models, the degree of histopathologic severity does not correlate with bacterial load, possibly explained by immunopathologic trigger by the pro-inflammatory response (Lepidi et al., 2000; Martin et al., 2001). Furthermore, Scorpio et al. reported that in a murine model of HGA, the host immune response plays a more important role in histopathologic lesions than does pathogen load (Scorpio et al., 2005).

HYPOTHESIS

Co-infections with several tick-borne infectious agents are common and often misdiagnosed.

One consequence of a co-infection with *B. burgdorferi* and *A. phagocytophilum* is a dysregulation of the immune response, with the risk of a worse clinical course.

Cytotoxic mechanisms and the cytokine IL-17 are involved in the local response in Lyme neuroborreliosis and may cause persisting symptoms, despite treatment.

ELISPOT-test is a useful test for diagnosing Lyme neuroborreliosis.

AIMS

- To estimate the extent to which tick-associated fever in the south-east part of Sweden could be attributed to *B. burgdorferi, A. phagocytophilum,* or TBE virus, or any combination of these agents. In addition, we wanted to reveal co-infections in patients with localised *Borrelia* infection, *i.e*. erythema migrans (EM).

- To investigate the cytokine secretion in patients exposed previously to *A. phagocytophilum* and currently infected with *B. burgdorferi* compared with patients infected with *B. burgdorferi* and seronegative for *A. phagocytophilum*.

- To investigate if cytokines associated with cytotoxicity (IL-2, IL-7, IL-10, IL-12p70, IL-15 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and the Th 17-associated cytokine IL-17 are increased in CSF from patients with LNB, and if cytotoxic cells from patients with LNB and persistent symptoms post-treatment kill *B. burgdorferi*-infected target cells.

- To investigate the diagnostic performance of *B. burgdorferi*-induced IFN-γ secretion detected by ELISPOT modified for use by local clinical laboratories as a supplementary test to the laboratory diagnosis of LNB used as a part of a clinical routine in a large prospective study in an endemic setting in which clinically suspected cases are admitted.

MATERIALS AND METHODS

Subjects (papers I-IV)

Patients and controls

In total, 808 patients and controls were included in this thesis, 643 patients (Table 6) and 165 healthy controls (Papers I-IV,). In **Papers II and IV** the patients were included with a clinically suspected LNB at the Åland Central Hospital on the Åland islands. Fourteen of the LNB patients and three of the patients of the non-Borrelia controls were included in both studies (Table 6).

Table 6. Subjects analyzed in this thesis. Seventeen of the 660 analyzed patients were included in both Papers II and IV, thus the study group consisted of 643 patients.

	Paper I Erythema migrans + Ap+/Ap-	Paper II Cytotoxic study in LNB	Paper III Epidemiological study	Paper IV ELISPOT study in LNB
Symptoms and signs				
Erythema migrans	23		174	
Unspecific symptoms			32	
Diagnosis				
Confirmed LNB		12*		14
Probable LNB		7**		5°
Non-CNS borrelia infection		43#		19°
Non-Borrelia controls		228##		103
	23	290	206	141

LNB: Lyme neuroborreliosis;
Unspecifc-symptoms = Headache, myalgia, arthralgia and or fever
*= Eleven of the confirmed LNB patients are included in Paper II and IV.
**= Three of the probable LNB patients are included in Paper II and IV.
#= 23 of the 43 patients were matched for sex and age. Three of the patients of the non-Borrelia controls are included in Paper II and IV.
##= 22 of the 228 non borrelia controls were matched for sex and age.
°= Not included in the statistical calculations, see under subjects, Paper IV.

In Paper I, 23 patients and 15 healthy controls were included in the study, which was conducted from 2001 to 2003. Twenty-three patients with EM were recruited from three health-care centres in the County of Östergötland. They consulted a general practitioner about suspected tick-borne disease. A questionnaire was completed at the initial visit. All patients and controls were screened for antibodies to *A. phagocytophilum* in serum in the acute phase and after 6-8 weeks. A

general blood status analysis was carried out. In addition, antibodies *to B. burgdorferi* were analysed in the healthy control group.

In Paper II, a total of 290 patients who had been referred to Åland Central Hospital for symptoms and signs of suspected clinical LNB were investigated (Figure 13). The study group consisted of 146 women and 144 men (mean age 57 years). They were classified according to laboratory findings in serum and CSF into different diagnostic groups. Nineteen patients were diagnosed as suffering from LNB, of whom 12 were classified as confirmed LNB and seven as probable LNB (Table 7, 10). A group of 43 patients out of the 290 had other *Borrelia* manifestations but without CNS involvement (non-CNS Bb infection); no CSF lymphocytic pleocytosis, no intrathecal production of specific IgG antibodies, and no blood-brain-barrier damage. Twenty-three of these were selected in order to match age and sex in LNB-patients, and were analysed for cytokines. All of them showed seroconversion or increasing *Borrelia*-specific antibody titres in serum and favourable response to therapy.

The remaining 228 patients did not suffer from any proven *B. burgdorferi* infection (non-Borrelia controls): absence of intrathecal production of specific IgG antibodies, seroconversion or increasing titres of serum Borrelia antibodies. Twenty-two of these were selected to match the age and sex in the groups above, and served as controls. The non-Borrelia controls had the following diagnosis or symptoms: other CNS diseases (n= 7, i.e. dementia, Alzheimer´s disease, pseudotumor cerebri, status post tick-borne encephalitis, arachnoid cyst, cerebellar atrophy, visual field loss), cardiovascular disease (n=5), dizziness/headache/fatigue/depression (n= 4), folic acid or iron deficiency or hypercalcaemia (n= 3), orthopaedic diagnosis (n= 1) and additionally, two patients had an unknown diagnosis. The controls had no indication of an ongoing infection or inflammatory disorder in the CNS.

Eleven of the confirmed LNB cases and three of the probable LNB cases were included in both **Papers II** and **IV**. Three of the patients in the non-Borrelia controls were included in **Papers II** and **IV**. They had the following diagnosis, status post TBE (1), folic acid and iron deficiency (1) and unknown diagnosis (1).

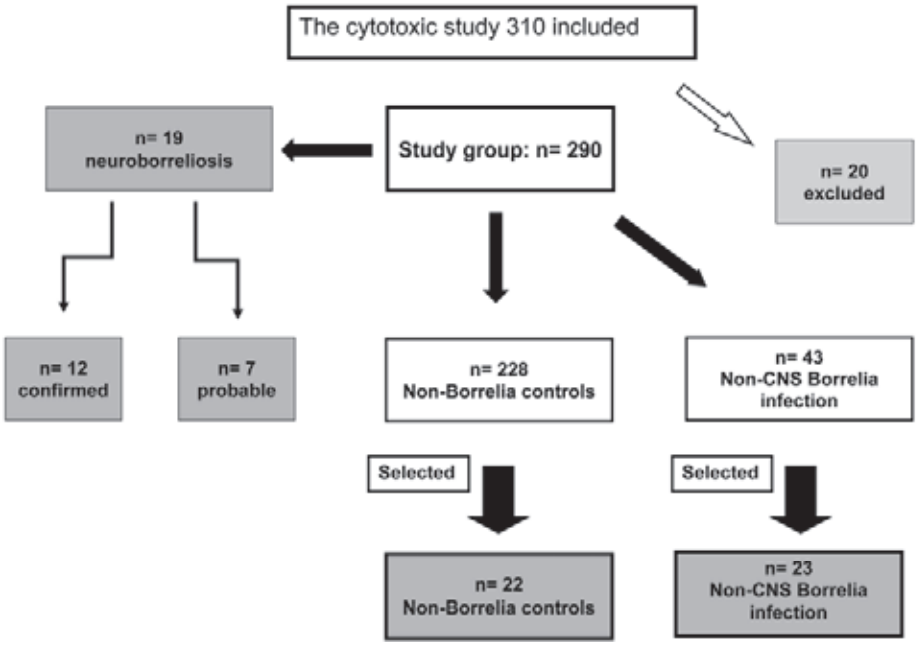

Figure 13. Participant flow in Paper II, "The cytotoxic study", investigating cytokines associated with cytotoxic mechanisms in patients with Lyme neuroborreliosis.

In Paper III, 206 patients were included from the County of Blekinge (n= 102), Kalmar (n= 38) and Östergötland (n= 66). The study group consisted of 110 women and 96 men. Thirty-two were recruited on the basis of unspecific symptoms in conjunction with a tick bite, while 174 were recruited on the basis of EM (Figure 14). The patients were categorized into four age groups, 15-20, 21-40, 41-60 and older than 60 years. At enrolment, serological investigations (*B. burgdorferi*, *A. phagocytophilum*, TBE virus) and polymerase chain reaction (PCR) (*A. phagocytophilum*) were carried out. Blood samples for serological follow-up were collected at 6–8 weeks and six months after enrolment. For patients with positive *Anaplasma*-serology an additional serology was performed 12 months after enrolment. At enrolment, the including physician used a standardized questionnaire to record the patient history, as well as clinical and epidemiological findings. Two to three weeks after the initial visit, the patients were interviewed over the phone by a clinical research nurse, using a standardized follow-up questionnaire.

Sera from 150 healthy blood donors, 50 from each county were investigated for the presence of antibodies against *B. burgdorferi* and *A. phagocytophilum*.

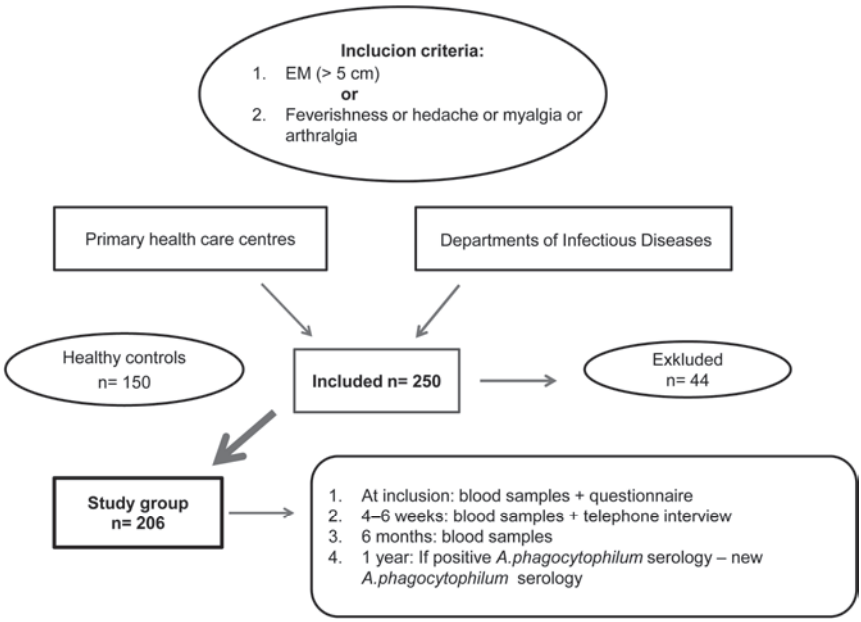

Figure 14. Participant flow in Paper III conducted in the south east of Sweden, investigating the extent *of B.burgdorferi*, *A.phagocytophilum* and TBE-virus in association with a tick-bite. Four Departments of Infectious Diseases and 31 Primary health care centres participated.

In Paper IV, 141 patients with clinically suspected Lyme neuroborreliosis were enrolled in the study, which was conducted at the Åland Central Hospital. Collection of EDTA plasma and lumbar puncture were performed on all patients at inclusion. They could only be included during daytime and during the weekdays, mostly from Monday to Wednesday, due to the ELISPOT test. Fourteen patients were diagnosed as having confirmed LNB: CSF lymphocyteic pleocytosis and intrathecal production of specific anti-*Borrelia* IgG antibodies. Five patients were classified as probable LNB (Table 7). Nineteen had other *B. burgdorferi* manifestations (with seroconversion or increasing levels of serum *B. burgdorferi* antibodies) but without CNS involvement (non-CNS *Bb* infection); no CSF lymphocytic pleocytosis, no intrathecal production of specific IgG antibodies, and no blood-brain-barrier (BBB) damage. The probable and non-CNS groups were not included in the statistical evaluation since they did not show any evidence of infection in the CNS.

The remaining 103 patients (non-LNB) did not suffer from any proven ongoing *Bb* infection; showed absence of intrathecal production of specific IgG antibodies, no seroconversion or increasing levels of serum Borrelia antibodies. In the non-LNB group fourteen patients had pleocytosis, which was compatible with another diagnosis, i.e. tick-borne encephalitis (n =4), other CNS disease (n=4, i.e. multiple sclerosis, dizziness, numbness), orthopaedic diagnosis (n =3,

i.e. spinal stenosis, spondylarthritis), hypothyroidism (n =1), tonsillitis (n =1) and unknown diagnosis (n =1).

The remaining 89 non-LNB patients had no pleocytosis and had the following diagnoses or symptoms; pain/myalgia/arthralgia/fatigue (n =33), other CNS disease (n =18, i.e. epilepsy, Parkinson´s disease, fibromyalgia, Alzheimer´s disease, dementia, status tick-borne encephalitis, depression, facial nerve palsy), autoimmune disorder (n =13, i.e. Sjögren´s syndrome, Crohn´s disease, kollagenosis, polymyalgia rheumatica), other infection (n =13, i.e. pneumonia, pyelonephritis, herpes keratitis, status post herpes zoster), orthopaedic diagnosis (n =7, i.e. arthritis, spinal stenosis, vertebral compression), endocrine disorder (n =3, i.e. diabetes mellitus, hypothyroidism) folic acid and iron deficiency (n =1) and lymphoma (n =1).

Thus, the study-group (n = 117) used in the calculations for the diagnostic performance of the ELISPOT test consisted of 65 women and 52 men (median 58 years, range: 6-87 years).

Case definitions used in this thesis

Erythema migrans (Papers I, II, III)

Cases of EM were clinically diagnosed by a physician and based on preceding risk of exposure to ticks, patient´s history, and physical examination, i.e., an expansible skin rash > 5 cm in diameter (Stanek et al., 1996).

Lyme neuroborreliosis (Papers II, IV)

All the classifications of LNB were done at the Åland Central Hospital by the co-authors (DN, PF together with MN). The patients were diagnosed as having confirmed NB or probable LNB (Table 7).

In addition to laboratory findings, they were also required to have suspected clinical LNB i.e. symptoms or signs compatible with meningitis, radiculitis, facial nerve palsy, other cranial nerve affections, pain, arthralgia, fatigue or dementia. Our criteria are in line with the current European guidelines (Mygland et al., 2010), except that we used the term ‘confirmed LNB’ instead of ‘definite LNB’ and ‘probable’ instead of ‘possible’. Our criteria for ‘probable’ were stricter than the criteria in the European guidelines, since we required seroconversion or increasing titres of specific anti-*Borrelia* IgG antibodies in serum. In CSF the findings were lymphocytic pleocytosis or intrathecal *Borrelia* antibody production or significant damage to BBB. We also required adequate response to therapy. Due to the high seroprevalence (19,5%) for *Borrelia* infections on the Åland islands (Carlsson et al., 1998), these criteria are used by the clinicians and co-authors on Åland.

Table 7. Case definitions of Lyme neuroborreliosis.

Confirmed	Probable
1. Clinical findings compatible with disease 2. CSF lymphocytic pleocytosis ≥5 MNC /µL 3. Intrathecal production of specific anti-*Borrelia* IgG antibodies	1. Clinical findings compatible with disease 2. Adequate response to therapy 3. Detectable seroconversion *or* increasing titres of specific anti-*Borrelia* IgG antibodies in serum 4. CSF lymphocytic pleocytosis ≥5 MNC/µL *or* intrathecal production of specific anti-*Borrelia* IgG antibodies *or* significant damage to BBB

CSF = cerebrospinal fluid; BBB = blood-brain-barrier; MNC = mononuclear leucocytes; IgG = immunoglobulin G

Non-CNS Borrelia infection (Papers II, IV)

Patients with other *Borrelia* manifestations (with seroconversion or increasing levels of serum *B. borrelia* antibodies) but without CNS involvement showed no CSF lymphocytic pleocytosis, no intrathecal production of specific IgG antibodies, and no BBB damage.

Anaplasma phagocytophilum infection (Papers I, III)

HGA *i.e.*, clinical disease caused by *A. phagocytophilum*, was defined as laboratory evidence of infection (Table 8) in combination with concurrent clinical symptoms and signs, i.e. myalgia, arthralgia, headache and/or fever.

Subclinical infection with *A. phagocytophilum* in this thesis was defined as laboratory evidence of infection but without any clinical signs or symptoms.

In **Paper I**, patients with previous exposure were tested for *A. phagocytophilum*, i.e. cases with *A. phagocytophilum* seropositive titre but no signs or symptoms of acute HGA. They also had a stabile titre (≥ 1:80) and no seroconversion between acute and convalescent sera.

Figure 15. Ixodes ricinus tick, adult male. (Courtesy of Pontus Lindblom, Linköping University**).**

Table 8. Case definitions of Human granulocytic ehrlichiosis.

Case definitions	
Confirmed HGA	Clinical disease corresponding to the definition of HGA, e.g. fever, headache, myalgia and/or arthralgia with an acute or sub-acute onset with a history of tick bite or tick exposure And 1. Demonstration of *A. phagocytophilum* infection serologically by IgG antibody seroconversion, i.e. from negative to positive titre or a four-fold or higher change in antibody titre, using an indirect IFA with *A. phagocytophilum*-infected cells as antigen or 2. positive PCR result with subsequent sequencing, demonstrating *A. phagocytophilum*-specific DNA in blood
Probable HGA	1. Clinical disease according to above and 2. presence of permanently high IgG antibody titre of ≥ 1:640 in acute and convalescent sera or 3. at least a four-fold decrease in IgG antibody titre or seroreversion (loss of previously detectable antibody titre) during the investigation period of 12 months.

Adapted from Brouqui 2004 and Bakken 2008. Investigation of blood by microscopy for the presence of bacterial inclusions in granulocytes, i.e. morula, was not performed, based on the previous experience, both by us and other investigators, that morula are very hard to detect in European cases of HGA.

Tick-borne encephalitis (paper III)

Infection with TBE virus was defined as a positive IgM screen in a TBE ELISA, which was confirmed in the specific Rapid Fluorescent Focus Inhibition Test (RFFIT) (Vene et al., 1998). Infection in combination with relevant clinical symptoms was considered as clinical TBE.

Excluded subjects

In total, 70 patients were excluded (Table 9). Nine of the excluded patients in **Papers II** and **IV** were originally included in both studies.

In Paper I no subjects were excluded.

In Paper II, initially 310 serum and CSF samples were obtained from patients in the prospective follow-up study from July 2002 to February 2005 at the Åland Central Hospital. Twenty of these were excluded from the study. Eleven whose CSF/serum sample volumes lacked or were too small, one who had received 2.5 months of antibiotic therapy immediately prior to the study, and three who could not be evaluated because of extensive co-morbidity (one with cardiovascular disease, chronic glomerulonephritis and stroke, one with immunosuppression and recently treated biliary disease, and the third with lymphoma). Since the immune responses work differently in adults and children, five children (5-14 years) were also excluded.

In Paper III, initially 250 patients were enrolled. Forty-four were excluded from the study: twenty-seven who did not come for follow-up blood tests, eight who did not fulfil the inclusion criteria, seven with no follow-up telephone interview, and two who received another diagnosis (one was diagnosed with pneumonia and the other was admitted to a neurological clinic).

In Paper IV the total number of enrolled patients was 156. Fifteen patients were excluded from the study; six due to technical problems, six who could not be evaluated because of missing data or extensive co-morbidity or unclear diagnosis, and three who had received antibiotic therapy immediately prior to inclusion.

Table 9. Excluded subjects in this thesis.

	Paper I	Paper II	Paper III	Paper IV
Enrolled patients, n	23	310	250	156
Excluded patients, n	0	20*	44	15
%	0	6.5	18	9.6

n= number

*= Nine of the excluded patients in Paper II and IV are the same.

Controls

In **Paper I** the healthy controls consisted of 15 healthy individuals who were either staff of the hospital or blood donors. In **Paper III** the 150 healthy controls were blood donors.

Evaluation of data

The medical records and the laboratory interpretation were carefully scrutinized by the author, together with, Pia Forsberg and Ingvar Eliasson (**Paper I**), Pia Forsberg, Dag Nyman (**Paper II**), Ingvar Eliasson (**Paper III**) and Pia Forsberg and Dag Nyman (**Paper IV**).

Methods

Borrelia antibody analyses in serum (Papers I-IV)

In **Paper I** a commercial ELISA-kit (DAKO, Denmark) was used for routine analysis of *B. burgdorferi* IgM and IgG antibodies against *B. burgdorferi* flagellum antigen in serum.

In **Papers II, IV**, conducted on Åland, *Borrelia*-specific antibodies IgM and IgG, were detected by a mixture of the recombinant *Borrelia* antigens p18 (*B. afzelii*), p39 (*B. afzelii*), p41 (*B. burgdorferi s.s.*), p41 internal fragment (*B. garinii*), p100 (*B. afzelii*), OspA (*B. afzelii*) and OspC (*B. afzelii*, *B. garinii*, *B. burgdorferi s.s.*) (RecomWell IgG ELISA, Mikrogen Germany). As a confirmatory test a Western blot was used (Mikrogen RecomBlot immunoblot, Germany). IgM and IgG antibodies against a synthetic *Borrelia* C6 peptide were measured using Immunetics C6Quick ELISA C6 Borrelia kit (Boston MA, USA) (Paper II, IV).

The Clinical Laboratory of Microbiology in Kalmar performed all *Borrelia* antibody tests In **Paper III**. Sera were tested for *B. burgdorferi* IgG and IgM antibodies by a commercial ELISA (Genzyme Virotech GmbH, Germany). Sera were absorbed with RF sorbotech (Genzyme Virotech) before testing for IgM antibodies. Positive or equivocal samples from ELISA were further tested by WB (Genzyme Virotech GmbH, Germany), using an AutoBlot 36 (GeneLabs Diagnostics, USA).

CSF analyses (Papers II, IV)

Pleocytosis was defined as a total leukocyte count ≥5 MNC/µl in the CSF. The occurrence of BBB damage was estimated by calculation of the CSF/serum albumin ratio. A normal age-correlated proportion indicated intact BBB (Reiber, 1994).

The presence of *B. burgdorferi*-specific IgM and IgG antibodies was measured using an IDEIA Lyme neuroborreliosis kit (Lyme NB ELISA kit, K6028, Dako, Denmark), flagellum-ELISA kit. CSF analysed before January 2004 were analysed by Mikrogen RecomWell. Paired patient serum and CSF samples were used to determine the intrathecal *B. burgdorferi* antibody synthesis.

TBEV serology (Paper III)

Sera from the acute phase were tested for anti-TBEV antibodies (IgM and IgG) with an ELISA (Immunozym FSME IgM and IgG, respectively, Progen Biotechnik GmbH, Germany). The evaluation was made according to the manufacturer's instructions. Positive samples were submitted to the Swedish Institute for Infectious Disease Control for confirmation by a rapid fluorescent focus inhibition test (RFFIT) (Vene et al., 1998). The RFFIT test is a neutralization test with high specificity for TBEV and no cross-reactivity with other flaviviruses. The test is

based on the principle that live TBEV is mixed with diluted patient serum samples and the added to tissue culture microplates with BKK-21 S13-cells that are susceptible to TBEV. For visualization of virus foci in infected cells, rabbit anti-TBEV serum is added to each well, followed by conjugate. The plates are then read in a fluorescent microscope, where fluorescent foci can be seen in infected cells. Where neutralizing antibodies are present, no cells are infected and thus fluorescence is absent.

Preparation of cells in blood and CSF (Papers I, II, IV)

In **Papers I, II**, peripheral blood mononuclear cells (PBMC) were separated from heparinized blood using gradient centrifugation on Lymfophrep® (Medinor AB, Lidingö, Sweden), as described previously (Ekerfelt et al., 1997a). In brief, blood was diluted 1:3 in buffer salt solution. Lymphoprep, a polysaccharide solution was applied by syringe beneath the blood. Lymphoprep has the same density as mononuclear cells; therefore these cells will be collected in the interface between the Lymphoprep and the buffer (which also includes plasma) when centrifuged. Other cells will sink and will be found at the bottom of the tube, *i.e*. erythrocytes that will aggregate during centrifugation, and polymorphonuclear cells that will sink due to the high osmolarity. The PBMC were collected and washed with buffer at 4ºC. The cell concentration was adjusted to$1x10^6$ lymphocytes/ml (Paper I).

In **Paper IV**, only CSF was used in the ELISPOT assay. CSF was immediately put on ice and centrifuged. The supernatant was removed and the cells were resuspended. CSF mononuclear cells were diluted in Türks reagent and counted by light microscopy using a Jessen chamber, before centrifugation, followed by resuspension in cell culture medium.

Preparation of *Borrelia* outer surface protein antigen (Papers I, II, IV)

The *Borrelia* antigen used in this thesis, an outer surface protein (Osp) enriched fraction (OF), mainly containing OspA and Osp B, was prepared from *Borrelia garinii* strain Ip90, as previously described (Bergstrom et al., 1991; Magnarelli et al., 1989). The *Borrelia* OF antigen concentration was optimized to be used in a final concentration of 10 μg/ml. This antigen has the ability to discriminate LNB from controls (Forsberg et al., 1995), induce augmented intrathecal IFN- γ secretion (Ekerfelt et al., 1998), demonstrate compartmentalization of IFN-γ response (Ekerfelt et al., 1997a) and to induce strong CSF mononuclear cell response in adults (Widhe et al., 2004) and in children (Widhe et al., 2005).

ELISPOT- enzyme-linked immunospot assay (Papers I, II, IV)

The ELISPOT method was first described by Czerkinsky et al.(Czerkinsky et al., 1988; Czerkinsky et al., 1983; Sedgwick and Holt, 1983) and has been modified by our research group (Ekerfelt et al., 1997a; Forsberg et al., 1995). ELISPOT is

a very sensitive method for detecting low levels of secreted molecules such as cytokines at a single cell level. This assay also detects modest secretion, such as spontaneous from resting, unstimulated cells, or cytokines that are secreted in low concentrations only, e.g. IL-4. Ekerfelt et al. showed that ELISPOT displayed the highest sensitivity and was the only assay that could detect spontaneous secretion of IL-4 when comparing ELISPOT with ELISA and RT-PCR (Ekerfelt et al., 2002). Furthermore, other methods are sometimes affected by problems such as *in vitro* cytokine consumption or cytokines being bound to soluble receptors thereby making the results unreliable. In the ELISPOT assay, the closeness between capture antibodies and cells prevents *in vitro* consumption of cytokines due to cell surface receptor binding. The assay theoretically allows detection of one single cell secreting the cytokine of interest among thousands of cells not secreting the cytokine, thus explaining the high sensitivity of the assay. The principles of the ELISPOT assay are presented in Figure 16.

Briefly, nitrocellulose-bottomed 96-well microtitre plates were coated with anti-human monoclonal capture antibodies against the cytokines of interest. In this thesis, cells secreting IL-4, IL-5, IL-12p70, IL-13, IFN-γ (**Paper I**), perforin and granzyme B (**Paper II**) and IFN-γ (**Paper IV**) were analysed by ELISPOT. Unspecific binding sites were blocked by use of cell culture medium containing bovine serum albumin. Mononuclear cells from blood (**Papers I, II**) and CSF (**Paper IV**) were added to the wells. The cells were stimulated with OF antigen (*Borrelia garinii* Ip90) to yield cytokine secretion.

Wells with unstimulated cells were cultured in parallel, representing the spontaneous secretion. Phytohemagglutinin (PHA) was used as a positive control (**Paper I**) and culture medium without cells was used as a negative control. The cells were incubated for 48h at 37ºC, 5% CO with 95% humidity.

The secreted cytokines were bound to the capture antibodies, thereby causing an imprint of the individual cytokine-secreting cells, here referred to as spots. To get a clear spot of each secreting cell it is important to keep the culture still and undisturbed during the incubation period. The cells were removed by washing. Visualization of the spots was done by enzymatic staining, using a biotinylated secondary cytokine antibody that binds to the cytokine. Streptavidin, conjugated with an enzyme, was then added. When finally adding a substrate for the enzyme a coloured precipitate was formed where the enzyme was present, thus visualizing the imprints as single “spots” (Figure 17).

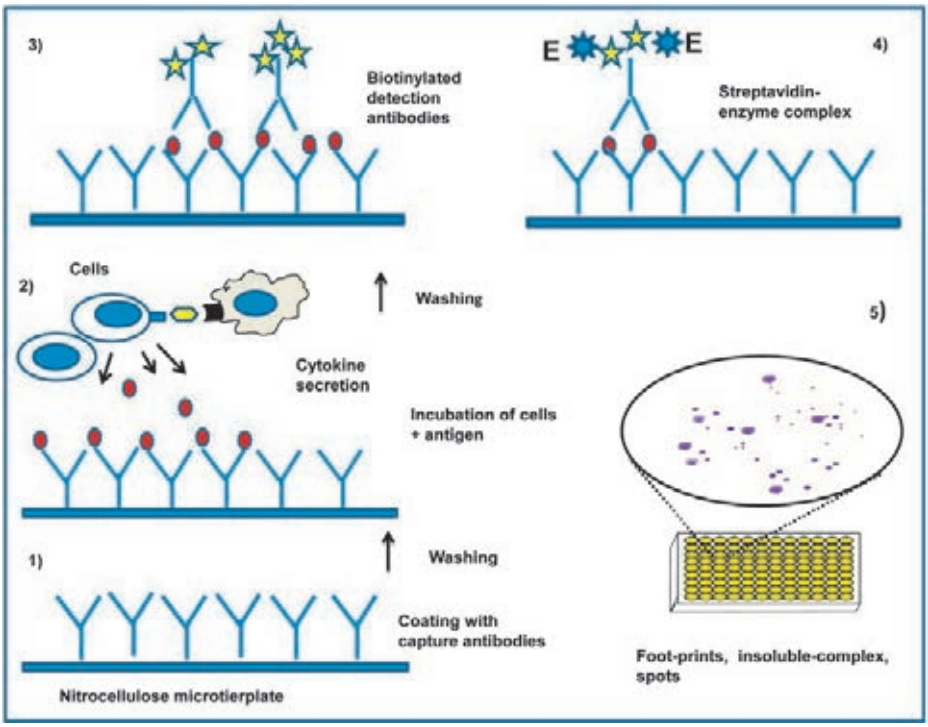

Figure 16. Principles of the ELISPOT assay, 1.Coating with capture antibodies, 2. Incubation of cells together with antigen, 3. Biotinylated detection antibodies are added, 4. Visualization by enzymatic reaction, 5. Insoluble-complex, spots.

Data analysis in the ELISPOT assay

The number of *Borrelia*-induced IFN-γ-secreting cells was calculated by subtracting the number of spots generated by unstimulated cells from the number of OF-stimulated spots. The ELISPOT assay is known to have a rather high intra-assay variation, 23% for IFN-γ and 28% for IL-4. Considering the inter-assay variation this may also be a problem, with 31% for IFN-γ and 38 for IL-4 (Ekerfelt et al., 1997b).

Immunological techniques in general are known to show higher inter- and intra-assay variation compared to common chemical techniques. In addition, assays including culturing of cells are also known to show similar high variation. The high variation for ELISPOT is explained by the measure of the number of cells and because it is usually utilized for analyses of low grade secretion, *i.e.* when few cells secrete the substance of interest.

To compensate for this known variation of the ELISPOT assay, in **Paper I**, the mean value of number of spots in the wells, spontaneous and *Borrelia*-stimulated, were calculated from triplicates.

In **Paper IV** the ELISPOT assay was modified to be feasible in clinical routine laboratories. The counts of spots were adjusted to 5,000 cells/well for all patients by extrapolation. This was done by dividing the number of spots by the number of cells in the well (generating the number of spots/cell) and after that multiplying by 5,000.

The mean of duplicates was calculated when appropriate. CSF cells were assayed in duplicate (two stimulated and two unstimulated wells) when the yield of cells was higher than 25 000 cells and in single (one stimulated and one unstimulated) when the yield was lower than 25 000 cells. In the whole material the number of cells/well varied between 650 cells/well and 115 000 cells/well. Within the LNB-group, the number of cells per well varied from 2000 up to 115 000, median 65 000, and cells from the LNB patients were assayed with 5,000 cells or more per well in 12/14 cases. The number of cells in the non-LNB group varied from 650 up to 35 000, median 5 500. 57/103 were assayed with 5,000 cells or more per well.

The spots were evaluated and counted by a single person with experience of such work (SJ in **Paper I**, AJ in **Paper II**), using an AID ELISPOT reader System (AID, Germany), while in **Paper IV** (MAÅ) a dissection microscope was used. The evaluation and counting in all studies was performed in Linköping and blinded in respect to the diagnosis of the patients.

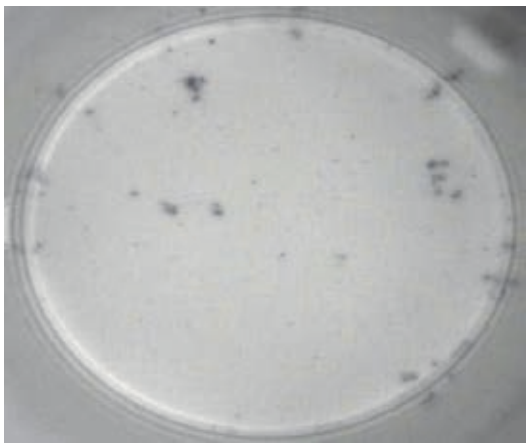

Figure 17. Each spot represents a "footprint" of one cytokine-secreting cell in the ELISPOT assay.

Analytic work on the Åland islands (Paper II, IV)

In **Paper IV,** CSF was immediately put on ice and centrifuged at + 4° C (see under Preparation of cells in blood and CSF) within one hour of lumbar puncture. CSF was frozen and stored at -70° C. In **Paper IV,** the ELISPOT assay was carried out on the Åland islands according to a standardized protocol modified to be feasible for clinical laboratories. The CSF cells were stained by Tűrks reagent,

enabling mononuclear and polymorphonuclear white blood cells to be distinguished and counted in by a light microscopy using a Jessen chamber, before centrifugation. This is not the procedure used in a research laboratory, where cell counting and differentiation of different cell types is done by phase contrast microscopy.

ELISPOT is a time consuming assay and therefore the patients were included only during daytime and during the weekdays, mostly from Monday to Wednesday. The ELISPOT assay was performed and the spots developed on Åland, and the dried microtitre plates were later transported to Linköping where one person evaluated and counted the spots in a dissection microscope. Serum and plasma that were collected in both studies were stored at -70°C and transported to Linköping stored on dry ice (**Paper II**).

Indirect immunofluorescence assay (IFA) (Paper III)

This method was used in **Paper I, III**, for detection of *A. Phagocytophilum.* Antibody testing by an IFA is the most commonly used test to confirm a HGA diagnosis (Aguero-Rosenfeld et al., 2002). IgG antibody titres to *A. phagocytophilum* were determined by IFA using an *A. phagocytophilum* test kit (Focus technologies, Cypress, California, USA). A titre of ≥1:80 was considered as positive. Sera from the acute phase and convalescent sera (6-8 weeks) were investigated. In **Paper III**, sera were also collected six months after enrolment, and for patients with positive *A. phagocytophilum* serology an additional serology was performed 12 months after enrolment (**Paper III**).

The principle for IFA is illustrated in (Figure 18). In short, antigen is fixed onto a glass slide in a well. The Focus test kit utilizes HL-60 cells infected with a HGA strain. Each slide contains 12 wells with inactivated cells infected with *A. phagocytophilum*. First, the patient serum is diluted in PBS. The diluted serum is placed on the slide and incubated. Following incubation, the slide is washed in PBS to remove unbound serum antibodies. After that, fluorescence-labelled antibody to human IgG was added to each well. The slide was incubated allowing the antigen-antibody complexes to react with the fluorescence-labelled anti-human IgG. After incubation, the slide was washed and dried. It was examined by using fluorescence microscopy. A positive reaction appears as apple-green fluorescence of the morulae (Figure 19). A titre of ≥1:80 was considered as positive. Semi-quantitative endpoint titres were obtained by testing serial dilutions of positive specimens. When paired sera were compared they investigated together on the same slide.

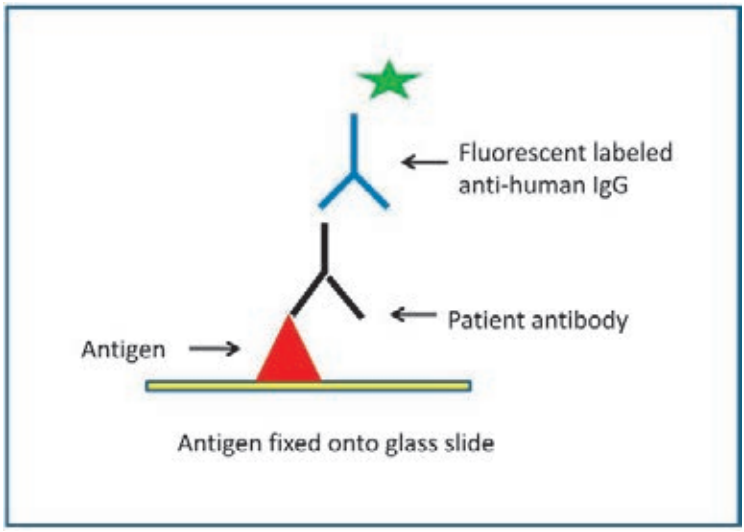

Figure 18. Principles of Indirect immunofluorescence assay.

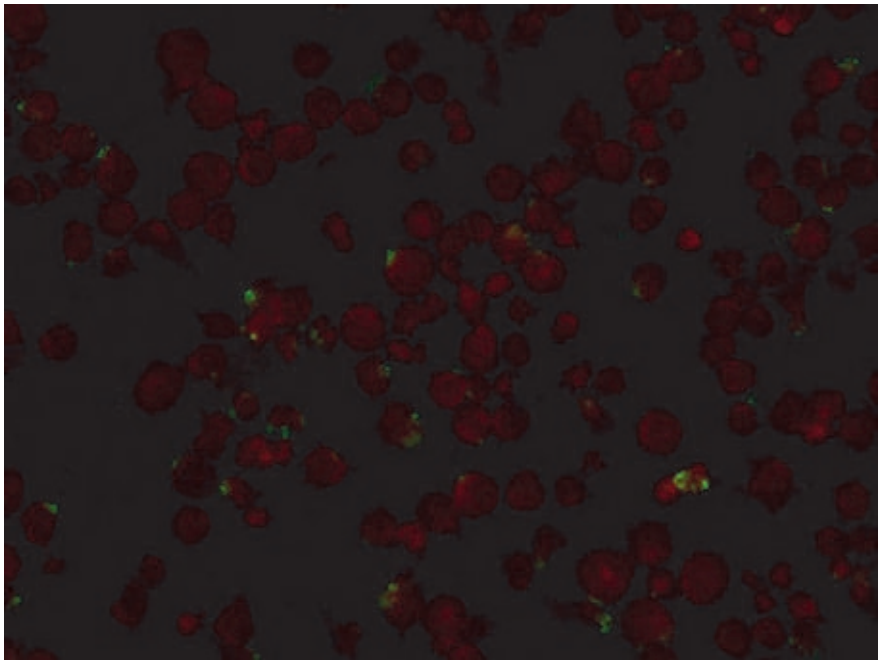

Figure 19. Slide showing the IFA in a fluorescence microscopy. *A. phagocytophilum* infected cells are used as antigens. Apple-green flourescence occurs were antibodies have bound to antigens.(Courtesy by Annika Lindkvist, Ryhov County Hospital, Jönköping)..

Polymerase chain reaction (Paper III)

PCR amplification of *A. phagocytophilum*-specific DNA from acute phase blood can confirm the diagnosis during the early stage of infection (Bakken and Dumler, 2008). No standardised PCR-based methods exist yet and this may yield discrepant results (Brouqui et al., 2004).

In **Paper III,** EDTA blood was obtained from the patients at inclusion. In order to strengthen the results, two different PCR methods were employed. In short, in the first assay, DNA was extracted by QIAamp Tissue Kit using the protocol recommended by the manufacturer (Qiagen Inc, USA). A negative and a positive control sample were included in every run. All samples were stored at –20°C until analysed by PCR. A nested PCR assay targeting the 16S rRNA gene was performed as previously described (Massung et al., 1998). EDTA blood from a clinically ill and *A. phagocytophilum* inclusion-positive cow was used as a positive control, and EDTA blood from a healthy human blood donor was used as a negative control. PCR amplifications were performed in a Perkin Elmer 9600 thermal cycler, using reagents from the Gene Amp PCR kit Amplitaq DNA Polymerase (Perkin Elmer, USA).

In the second PCR assay, *A. phagocytophilum* DNA was extracted in a KingFisher mL automated extractor (Thermo Electron Corporation, USA) by a BioSprint DNA Blood Kit (Qiagen Inc, USA). A negative and a positive control sample were included in every run. All samples were stored at –20°C until analysed by PCR. Amplification for detection of *A. phagocytophilum* was conducted by using MSP2-3F and MSP2-3R directed towards the major surface protein 2 of *A. phagocytophilum* (Massung and Slater, 2003).

The products were visualized by electrophoresis in a 2% agarose gel stained with ethidium bromide. Further details are described in Paper III.

Luminex – multiplex bead array technology

In this thesis the levels of IL-2, IL7, IL-10, IL-12p70, IL-15, IL-17 and GM-CSF were measured in plasma and CSF by multiplex bead array technology. Two different types of custom-made kits (LINCO Research, St Charles, MO, USA) were used. IL-15 and IL-17 were analysed by using standard kits, whereas high sensitivity kits were used for detection of the other cytokines mentioned above. Analyses were performed according to the manufacturer´s recommendation. The principle of Luminex is presented in Figure 20. Theoretically, up to one hundred analytes can be detected in a single sample (Vignali, 2000). Polystyrene beads (microspheres) are dyed with two different fluorescent red colours (red and infrared). Up to 100 different microspheres can be created with a specific colour for each bead making them unique with a special spectral signature. The beads are then coated with e.g, monoclonal capture antibodies against the analyte, e.g. cytokines. The target analyte is then detected by a biotinylated detection antibody.

The detection antibody is labelled with a reporter fluorochrome. Each bead is passing through a flow cytometer one at a time. A red laser classifies the bead, identifying the bead type, while a green laser beam is detecting and quantifying the assay on the bead surface (Leng et al., 2008).

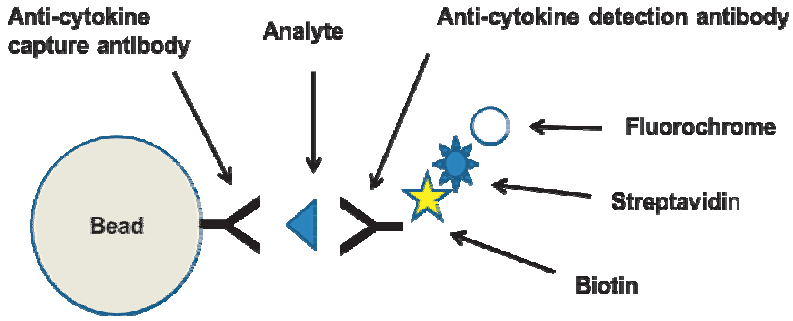

Figure 20. Basic principles of LUMINEX. Beads, dyed with a mixture of red and infrared, are coated with capture antibodies against the target analyte. Biotinylated detection antibodies with a reporter fluorochrome are then added. The beads are then excited by a red and a green laser beam one at a time.

In vitro measurement of functional cytotoxicity

Peripheral blood (50-80 ml) from three individuals previously diagnosed with LNB with persisting symptoms were collected in order to test functional cytotoxicity using an in vitro model. In brief, MNC were separated from peripheral MNC by density gradient centrifugation on Lymphoprep® (described in "Preparation of cells in blood and CSF").

After centrifugation, MNC were removed, washed and resuspended. The cells were counted under a phase contrast microscope and the cell density was adjusted to 1x106 MNC/ml. The suspension was then added to six-well cell culture plates and incubated for 1 h and 15 min at +37°C with 5% CO_2 and 95% humidity. This step allows the monocytes/macrophages to adhere to the bottom of the plate while the lymphocytes stay in solution. Following incubation, the supernatant containing lymphocytes was transferred to Falcon tubes. Additionally, adhered monocytes/macrophages were removed with a cell scraper and transferred to Falcon tubes. Both lymphocytes and monocytes/macrophages were centrifuged. The supernatants were then removed and the cell pellets were resuspended. The lymphocytes were then frozen according to the standard procedure of the lab and stored in liquid nitrogen until use.

Monocytes/macrophages were cultured for seven days at +37°C with 5% CO_2 and 95% humidity. Macrophages were removed by cell scraper. Cell viability was checked by trypan blue exclusion. The same day as the differentiated

macrophages were harvested, frozen autologous lymphocytes were thawed according to the standard procedure of the lab. The *B. garinii* LU59 strain isolated from CSF was used in this study. Spirochetes were thawed and transferred to a Falcon tube containing Barbour-Stoenner-Kelly medium II with 6% rabbit serum. After incubation overnight, the spirochetes were counted under a light microscope using a Bűrker chamber. Macrophages, thawed lymphocytes and live spirochetes were then added to the same well to enable infection of macrophages and subsequent killing of lymphocytes. Optimal assay conditions were investigated using different numbers of lymphocytes, macrophages and spirochetes. 100,000 lymphocytes, 10,000 macrophages and 100,000 spirochetes per well turned out to be optimal. The ELISPOT plates were coated with anti-perforin and anti-granzyme B monoclonal antibodies. The ELISPOT method is described in detail under "Data analysis in the ELISPOT assay". Furthermore, control wells with lymphocytes only, macrophages only, spirochetes only, culture media only and macrophages and spirochetes only were included in all experiments.

Statistics

The statistical analyses in this thesis were in general of non-parametric type, since data were assumed not to be normally distributed. To compare the cytokine secretion between groups, the Kruskal-Wallis test was used as a pre-test and Dunn´s multiple comparison test as a post-hoc test in **Papers I, II**. Differences between proportions (**Paper II**) were tested with Fisher´s exact test and 95% confidence intervals (CI) for proportions were calculated. Student's t-test was used to compare lymphocyte and monocyte counts between single-infected and co-exposed patients (**Paper I**).

In **Paper IV**, The Mann-Whitney U-test was used for comparison of spontaneous and B. burgdorferi induced IFN-γ secretion between the groups. Wilcoxon´s test was used for comparison of spontaneous and *B. burgdorferi* induced IFN-γ secretion within the groups. Logistic regressions analysis was used to adjust for differences in cell count/well between LNB and non-LNB patients and Spearman´s rank correlation test was used for correlation analysis.

Ethical considerations

The research protocols and study design of the investigations reported in this thesis were approved by the following ethics committees: The Local Ethics Committee of the University Hospital, Linköping (**Papers I, II, III**); The Regional Ethics Committee at the Åland Central Hospital, the Åland Islands, Finland (**Papers II, IV**); The Research Ethics Committees of the University of Lund (**Paper III**).

RESULTS AND DISCUSSION

Lyme neuroborreliosis; clinical manifestations, immunopathogenesis and diagnostic aspects (Papers II, IV)

Patient characteristics (Papers II, IV)

Patients with clinically suspected LNB were enrolled in the studies described in **Papers II and IV**, conducted on the Åland islands. This area is known to be hyper-endemic for LB, with an incidence estimated at 1,700 cases/100 000 inhabitants/year in 2011 (Finland´s National institute for health and welfare). All patients underwent a lumbar puncture, and the clinical diagnosis, based on clinical signs, symptoms and laboratory results was determined by physicians experienced with LB.

In Paper II, 290 patients were included, 146 women and 144 men (median age 57 years). Of these, 19 were diagnosed with ongoing LNB (Table 10). Twenty-three patients with other manifestations of *B. burgdorferi*, but without CNS involvement (non-CNS Bb infection), were selected in order to match age and sex in LNB patients. A group of 22 patients with other diagnoses, but no proven ongoing LB (non-Bb controls) were selected and served as controls (Figure 13). The study group in **Paper IV** consisted of 117 patients, 65 women and 52 men (median 58 years, range: 6–87 years) that were used in the calculations for the diagnostic performance of the ELISPOT test. Fourteen patients were diagnosed as having confirmed LNB (median 58.5 years, range 6-87 years) whereas the non-LNB group consisted of 103 patients (median 58 years, range 6-87 years) with heterogeneous diagnoses but no evidence of an ongoing infection with *B. burgdorferi*.

Clinical manifestations of Lyme neuroborreliosis (Paper II)

All of the patients with LNB were followed prospectively by phone interview and/or by clinical examination by a physician (after two weeks, three, six and 12 months). They were treated with antibiotics; two weeks with ceftriaxone 2g x1 i.v., followed by three months with amoxicillin 500 mg x 3 p.o, according to the Ålandic recommendations at that time. All LNB patients were treated with antibiotics before the sample collection was performed.

In **Paper II**, when comparing the confirmed and probable groups statistically, the only significant differences were occurrence of radiculitis (p=0.031) and unspecific pain (p=0.058) (Table 10). Thus, radiculitis was only found in the

group with confirmed LNB, whereas unspecific pain was more common in the probable group. Gender distribution, duration of symptoms, other clinical features and symptoms post-treatment were similar. Four of the LNB patients (one confirmed and three probable) had no pleocytosis at inclusion but fulfilled other criteria, *i.e.*, intrathecal production of IgG antibodies, typical symptoms and response to therapy. The confirmed LNB patient had pleocytosis about one week later and one of the probable patients had pleocytosis when a new lumbar puncture was carried out two months later.

Persisting symptoms

Three (16%) of the 19 patients with LNB had symptoms persisting > six months and even after one year (Table 10). Looking at the cytokine levels in CSF and plasma for these three patients, there were no differences compared with the other LNB patients. Two of them (females, 58, 77 years) presented with radiculitis, one also with bilateral facial palsy, while the third patient (male, 77 years) presented with cognitive disturbances and unspecific pain. Interestingly, Henningsson et al. showed that 13% (14/106) of patients in Jönköping County diagnosed with LNB had persisting symptoms (in this thesis called LNBpspt) for more than six months (Henningsson et al., 2010). Additionally, others have reported persisting symptoms after being treated for LNB (Berglund et al., 2002; Ljostad and Mygland, 2010; Vrethem et al., 2002).

PLDS is a debated issue; however risk factors for developing PLDS following early LNB are identified as, long-lasting symptoms (>6 weeks) before antibiotic treatment, female gender, age >40 years, high levels of *Borrelia*-specific IgG in CSF, high cell count in CSF before treatment, radiculitis and unspecific symptoms such as pain, vertigo and concentration disturbances (Henningsson et al., 2010; Ljostad and Mygland, 2010).

Since advanced age seems to be a risk factor for persisting symptoms one could speculate that the immune system is less effective in eradicating the spirochetes in the elderly. Recent studies indicate that children less commonly develop persisting symptoms and also mobilize an initial strong Th1 response, followed by an IL-4 response, thus switching off the immune system (Widhe et al., 2005). Furthermore, one hypothesis is that a strong early IFN-γ response is needed to eradicate the infection with *B. burgdorferi*. However, if excessive or persistent, this may cause tissue injury, thereby contributing to LNB with persistent symptoms (Kang et al., 1997; Sjowall et al., 2005; Widhe et al., 2004).

Table 10. Patients with Lyme neuroborreliosis In Paper II.

Characteristics of patients diagnosed with LNB (n=19)		Confirmed (n=12)	Probable (n=7)
Age	Median age (range)	68 (51-87)	53 (30-79)
Sex	Male	4	5
Known tick bite	Yes	5	4
Duration of symptoms	0-1 month	7	3
	1-3 months	0	1
	> 3 months	1	3
	> 1 year	1	0
	Not known	3	0
Clinical features*	Radiculitis	8	0
	Peripheral facial palsy	5	1
	Cognitive disturbances	2	1
	Unspecific pain	2	5
	Meningitis	0	0
	Meningoradiculitis	1	1
Laboratory findings	Lymphocytic pleocytosis in CSF ≥5 mononuclear leucocytes µ/L	12	5
	Specific anti-*Borrelia* IgG in CSF	12	3
	Blood-brain-barrier damage	8	4
	Seroconversion or increasing titres of serum anti-*Borrelia* IgG antibodies	12	7
Symptoms post treatment	≤ 2 weeks	4	0
	3 months	2	3
	4 months	2	3
	6 months	1	0
	1 year**	3	0
	Not known	0	1

n= number

CSF= Cerebrospinal fluid

NB= Neuroborreliosis

*= Several patients presented with several clinical features

**=NB with persisting symptoms > 6 months post treatment (NBpspt)

Lyme disease induces a Th1 type response. In humans, increased *Borrelia*-specific secretion of IFN-γ has been reported (Ekerfelt et al., 1997a; Ekerfelt et al., 1998; Forsberg et al., 1995). Thus, an early increased secretion of IFN-γ, followed by a subsequent upregulation of IL-4 is associated with a favourable outcome (Kang et al., 1997; Widhe et al., 2004).

Immune responses in adults with Lyme neuroborreliosis (Paper II)

The pathogenesis of LNB is complex. CNS is considered as an immunologically privileged site and only activated cells pass the BBB (Wilson et al., 2010). Involvement of cytotoxic cells in the immune response against *B. burgdorferi* has been suggested based on *in vitro* studies (Beermann et al., 2000) and activated CD8+ T cells have been shown to accumulate in the CNS during the early phase of LNB (Jacobsen et al., 2003). Additionally, there are indications of a cytolytic phenotype of IFN-γ producing cells from patients with LNB (Ekerfelt et al., 2003) and decreased *B. burgdorferi*-induced upregulation of the IL-12 receptor beta2 on blood CD8+ T cells from patients with LNB with persisting symptoms compared to individuals with asymptomatic *B. burgdorferi* infection (Jarefors et al., 2007).

Cytokines associated with cytotoxicity, IL-2, IL-7, IL-10, IL12p70, IL-15, GM-CSF and the Th17 associated cytokine IL-17 were investigated in CSF and plasma in patients with LNB. In this study all cytokines apart from IL-15 were increased in CSF but not in plasma in patients with LNB compared to patients with non-CNS *Borrelia* infection. IL-2, IL-10 and GM-CSF were also increased in LNB compared to non-*Borrelia* controls (Figure 21).
Cytokines work in complicated networks, exert pleiotropic effects, and the same cytokine can be secreted by different cell types. Thus, there are no cytokines specific to cytotoxic cells *per se*. Aiming to look further into a potential role of cytotoxic cells in LNB, we chose to study a set of cytokines associated with cytotoxic responses.

IL-2 stimulates proliferation of CD8+ T cells after antigen recognition (Figure 12) and promotes growth and the cytolytic function of NK-cells. Additionally, IL-2 is considered as one of the factors required during primary immunization with pathogen to programme the differentiation of fully functional CD8+ T cell memory (Williams and Bevan, 2007). The finding of increased IL-2 in CSF but not in blood in patients with LNB in the current study may, on one hand, mirror a general T-cell activation in the CNS. On the other hand, considering our previous finding of a cytolytic phenotype of IFN-γ-secreting cells in LNB (Ekerfelt et al., 2003) it is also in line with activation of cytotoxic cells in the target organ of NB.

Among the cytokines associated with cytotoxicity, the most dramatic increase was seen in **IL-10.** In patients with LNB, IL-10 was significantly higher compared to patients with non-CNS Bb infection ($p<0.001$) and also compared to the control group ($p=0.001$) (Figure 21). IL-10 exerts pleiotropic effects, and al-

though it is mainly considered to be an immunosuppressive cytokine, it also exerts immunostimulatory effects, i.e. enhancement of antibody production and cytotoxicity (Borish, 1998; Groux et al., 1998; Levings et al., 2002). *In vitro* and *in vivo* experiments show that IL-10 has a growth-promoting activity on activated CD8+ T cells (Groux 1998). Since LNB is a disseminated stage of LB, when effector memory cells are present, the detection of an increase of IL-10 in CSF in the current study may reflect stimulation of cytotoxicity, although we cannot exclude a suppressive role of IL-10.

GM-CSF does not appear to exert direct effects on cytotoxic cells but increases the numbers and cytotoxic activity of effector cells by acting via APC, causing augmentation of antigen presentation and accessory molecules (Murphy, 2012; Shi et al., 2006). The finding of increased levels of GM-CSF in CSF in LNB in the current study also supports the involvement of cytotoxic cells in the target organ in LNB.

Several cytokines are important for maintaining the homeostasis of CD8+ T lymphocytes. **IL-15** is important for the long-term survival and renewal of memory CD8+ T cells. Furthermore, memory CD8+ T cells do not require TCR signals to undergo expansion, but are dependent on IL-15 (Gagnon et al., 2008). In our study, IL15 was not elevated in CSF or plasma. However, we studied patients with an acute LNB, suggesting that the lack of IL-15 indicates that memory CD8+ cells were not involved.

Our study was based on a clinically well characterised group of patients with acute LNB according to case definitions (Table 7). All the patients underwent a lumbar puncture, thus the cytokines were investigated both in CSF and plasma, which is an advantage. However, one limitation is the small number of LNB patients (n= 19), but it is difficult to collect and follow patients over several years. Another consideration is that cytokines are not strictly associated with certain cells or qualities of responses. Therefore, it cannot be fully excluded that our findings of increased CSF levels of six of seven cytokines studied merely reflect a general immune activation in the CNS during LNB.

However, the findings of increased cytokine levels only in CSF, but not in plasma, support the compartmentalization of the immune response to the target organ in LB, in line with previous reports (Ekerfelt et al., 1997a; Ekerfelt et al., 1998; Gross et al., 1998; Grusell et al., 2002; Wang et al., 1995). Some of these studies were focused on *Borrelia*-specific immune responses in CSF (Ekerfelt et al., 1997a; Ekerfelt et al., 1998; Wang et al., 1995), making it likely that the findings in the current study also reflect responses to *B. burgdorferi*.

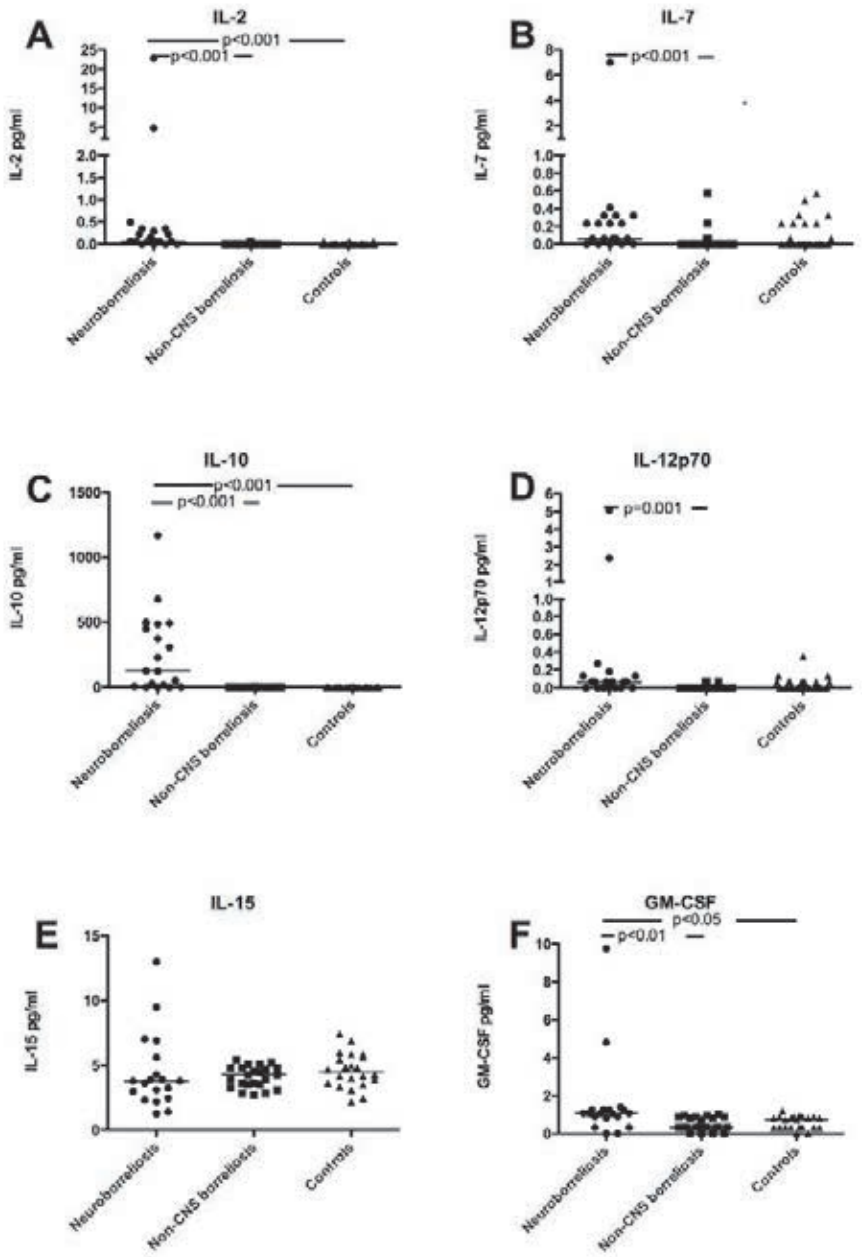

Figure 21. Levels of IL-2, IL-7, IL-10, IL12p70, IL-15 and GM-CSF in cerebrospinal fluid from patients with neuroborreliosis (***n*=19), non-CNS borreliosis (*n*=23) and non-borrelia controls (*n*=22). P-values show statistically significant differences as** analyzed by Dunn´s test, used as a post hoc test when significant differences between the three groups were indicated by Kruskal Wallis test. Median values within the groups are indicated by a line. IL, interleukin; CNS, central nervous system; Controls, non-borrelia controls; GM-CSF, granulocyte-macrophage colony-stimulating factor.

IL-17 in CSF in patients diagnosed with Lyme neuroborreliosis (Paper II)

The Th17 associated cytokine IL-17 was increased in CSF but not in plasma in LNB patients compared to both control groups (Figure 22). No differences were seen in plasma. IL-17 is a rather recently discovered pro-inflammatory cytokine involved in autoimmune diseases, immune-mediated tissue damage and the defence against extracellular bacteria and fungi (Awasthi and Kuchroo, 2009; Di Cesare et al., 2009; Tesmer et al., 2008).

Studies on IL-17 and LB are so far scarce, but it has been suggested that Th17 cells and their associated cytokines are involved in the pathogenesis of LA (Burchill et al., 2003; Codolo et al., 2008; Kotloski et al., 2008). Additionally, Henningson et al. found in a study conducted in the south-east of Sweden, that IL-17 was elevated in 49% in CSF of patients with confirmed LNB. Furthermore, patients with elevated IL-17 in CSF had more pronounced pleocytosis (Henningsson et al., 2011). This is in line with our findings, since the LNB patients had elevated concentrations of IL-17 and the concentration correlated to the level of pleocytosis in CSF R^2= 0.3 (p= 0.022) (unpublished data). Pleocytosis is generally associated with an ongoing inflammatory process in the CNS. However, it is unclear what role of IL-17 in the LNB patients. One possibility is that IL-17 is associated with the inflammation generated during acute LNB.

Interestingly, IL-17, secreted by human Th17 lymphocytes, has the capacity to disrupt the BBB and promote leucocyte recruitment to the CNS (Kebir et al., 2007). The finding of increased IL-17 in CSF in some of the patients with LNB may suggest that a Th17 response is part of the local CNS response. However, the finding of IL-17 alone does not prove a Th-17 response, since IL-17 is also produced by other cells besides T cells (O'Connor et al., 2010).

Th17 cells express the B cell chemoattractant CXCL13, indicating that Th17 cells may actively recruit B cells to the sites of inflammation (Crome et al., 2010). Whether this is happening in LNB patients is unknown, but CXCL 13 is reported to be elevated in LNB patients and is tentatively a marker for LNB (Rupprecht et al., 2005; Tjernberg et al., 2011).

Our findings suggest that IL-17 might have a role in the pathogenesis of LNB. However, further studies are needed to investigate the possible role of Th17 responses in LNB. In conclusion, IL-17 was elevated in CSF in patients with LNB, but the relevance is hitherto unknown.

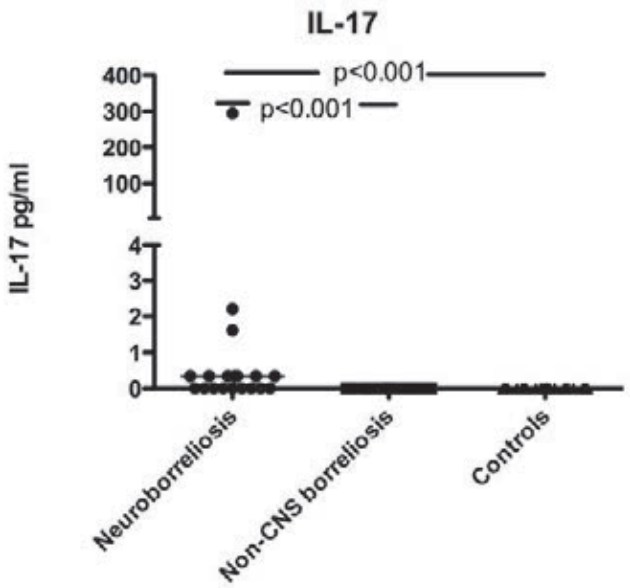

Figure 22. Levels of IL-17 in cerebrospinal fluid from patients with neuroborreliosis (*n*=19), non-CNS borreliosis (*n*=23) and non-Borrelia controls (*n*=22). P-values show statistically significant differences as analyzed by Dunn´s test, used as a post hoc test when significant differences between the three groups were indicated by Kruskal Wallis test. Median values within the groups are indicated by a line. IL, interleukin; CNS, central nervous system; Controls= non-Borrelia controls.

Functional cytotoxicity (Paper II)

In this small pilot experiment, three patients with previous known LNB and with persisting symptoms after treatment were investigated in order to test functional cytotoxicity in vitro. Monocytes/macrophages were cultured and later infected with live spirochetes and exposed to autologous lymphocytes. All three patients seemed to show increased numbers of granzyme B-secreting cells and two of them showed an increase also in perforin-secreting cells, in response to autologous macrophages exposed to live spirochetes (Figure 23). This suggests indirectly, that functional cytotoxicity is part of the immune response in LNB, and further that these responses are not exhausted in LNBpspt, but may play a pathological role. Thus, we cannot show that CTLs have killed macrophages, but at least perforin and granzyme B have been secreted by some cells, most likely CTLs. Perforin is found in granules of cytotoxic cells and is required to deliver granzymes into the cytosol of the target cell. Cytotoxic granulaes also contain proteases, i.e. granzymes (Voskoboinik et al., 2010).

This study has several limitations, one being the few patients studied, another, the lack of controls. It would also have been interesting to investigate the CSF, but since pleocytosis generally is rather moderate in acute LNB and our patients were diagnosed with LNBpspt (i.e. symptoms persisting >6 months), it was not possible to obtain CSF cells in the numbers needed. Therefore, blood MNC were used. The rationale for this was that memory cells continuously enter non-lymphoid organs from the blood stream (Williams and Bevan, 2007). Although memory CD8+ T-cells present in the brain have been reported to equilibrate very slowly with blood-borne memory cells (Klonowski et al., 2004), there is still an exchange between blood and brain which would make it possible to target *B. burgdorferi*-specific effector cells in the blood in LNBpspt.

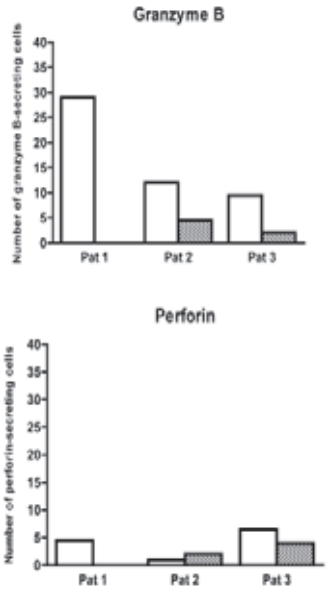

Figure 23 *In vitro* measurment of functional cytotoxicity. Three patients with earlier diagnosed neuroborreliosis and persisting symptoms post treatment were analyzed for perforin- and granzyme B secreting blood lymphocytes in response to *B.burgdorferi* infected autologous macrophages. Open bars represent the number of perforin and granzyme B secreting blood cells in cultures with 100.000 lymphocytes cocultured with 10.000 macrophages infected with B.burgdorferi spirochetes. Filled bars represent represent the numbers of perforin- and granzyme B secreting blood cells in cultures with lymphocytes and uninfected macrophages under the same conditions. The numbers of perforin/granzyme B secreting cells were measured by ELISPOT.

ELISPOT assay in laboratory diagnosis of Lyme neuroborreliosis in clinical practice (Paper IV)

In **Paper IV**, we aimed to investigate the diagnostic performance of an ELISPOT-test. The assay was carried out in a clinical setting on well-characterized LNB material collected on the Åland islands, a highly endemic area for LB.

The numbers of spontaneous and *B. burgdorferi*-induced IFN-γ-secreting cells detected by ELISPOT in LNB and non-LNB are shown in Figure 24. LNB patients showed an increased number of *B. burgdorferi*-induced IFN-γ-secreting cells compared with the non-LNB group (p = 0.01). No difference between the groups was seen regarding spontaneous secretion. When comparing spontaneous secretion with *B. burgdorferi*-induced IFN-γ secretion within the groups, signifi-

cantly lower spontaneous secretion was found within the LNB group ($p = 0.017$), whereas no difference was found within the non-LNB group. Furthermore, the non-LNB group showed a strong correlation between spontaneous and *B. burgdorferi*-induced IFN-γ secretion (r=0.952, p < 0.0001), whereas no correlation was found between spontaneous and *B. burgdorferi*-induced IFN-γ secretion in the LNB group.

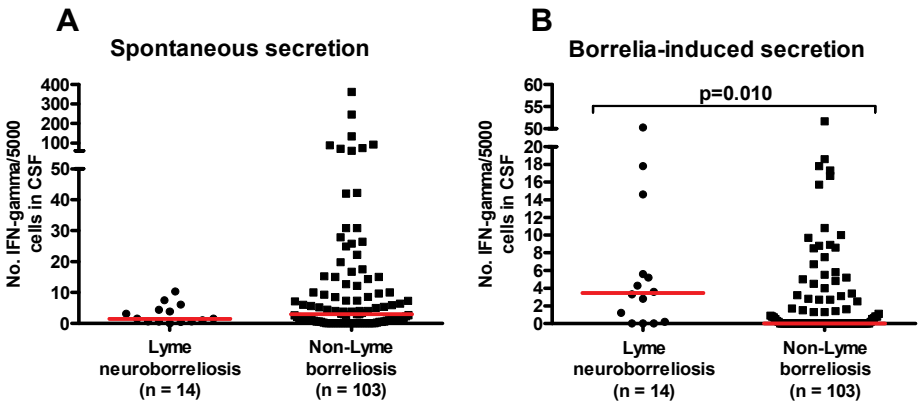

Figure 24. Spontaneous (A) and Borrelia-induced IFN-γ secretion (B) in cerebrospinal fluid from patients with Lyme neuroborreliosis and non-Lyme borreliosis. Medians are shown. P-value represent difference between the groups as compared by Mann-Whitney U-test.

Diagnostic performance of ELISPOT-test

The number of patients showing positive and negative tests for *B. burgdorferi*-induced IFN-γ-secreting cells in LNB and non-LNB at different cut-offs, five and 10 spots, are shown in Table 11. Calculated sensitivity, using the confirmed LNB (n = 14) and the non-LNB group (n=103), yielded a sensitivity of 36% and 21% when five and 10 *B. burgdorferi*-induced IFN-γ-secreting cells were used as cut-offs for positive tests, respectively (Table 11). Our previous studies suggested a stronger diagnostic performance (Ekerfelt et al., 1997a; Forsberg et al., 1995). However, these studies were conducted in areas with lower seroprevalence (5.8%) for LB (Ekerfelt et al., 2001).

The calculated specificity was 82% and 92% when using the cut-offs of five and 10 spots, respectively (Table 11). Surprisingly, 19 of the 103 non-LNB patients, stimulated with outer surface protein (osp) enriched fraction of *Borrelia garinii* Ip90 antigen (OF) were positive in the ELISPOT test but did not have any evidence of an ongoing LB. This finding impairs the specificity of the test considerably.

Table 11. Diagnostic performance of ELISPOT-detected *Borrelia*-induced Interferon-γ secretion in patients with Lyme neuroborreliosis and non-Lyme neuroborreliosis, utilizing different cut-offs.

Diagnostic groups	ELISPOT cut-off ≥5 spots		ELISPOT cut-off ≥10 spots	
	Positive	Negative	Positive	Negative
Lyme neuroborreliosis (n = 14)	5 (36%)	9 (64%)	3 (21%)	11 (79%)
Non-Lyme borreliosis (n = 103)	19 (18%)	84 (82%)	8 (8%)	95 (92%)

Non-LNB patients

The non-LNB patients in this study had no evidence of an ongoing *B. burgdorferi*-infection in the CNS, but some of them were seropositive for *B. burgdorferi* in blood, indicating an earlier infection with *B. burgdorferi*. As mentioned above, 19 of the 103 non-LNB patients were positive in the ELISPOT test (Table 11). This is difficult to explain, since they all had different diagnoses with no single CNS disease that could possibly explain this.

Memory T cells are known to patrol tissues where the infection originally occurred, and they also react to antigens faster than naïve T cells. It is possible that previously generated memory cells circulating in the CSF, acting on some inflammatory trigger, could explain the high number of *B. burgdorferi*-induced IFN-γ-secreting cells in the non-LNB group in the current study, which was performed in this hyper-endemic area for LB. This would also have implications for using the test in low-endemic areas, since cases having a previous LNB may occur also in such areas, decreasing the validity of the test. A clinician working in an endemic area would want a test with a high specificity to help in finding the true negative cases, since the *B. burgdorferi* antibodies reflecting an old infection could persist for years (Hammers-Berggren et al., 1993; Kalish et al., 2001).

Interestingly, when considering spontaneous secretion of IFN-γ, the non-LNB patients seemed to have higher secretion compared to the LNB patients (Figure 24). Investigating the different diagnoses in the non-LNB group reveals a variety of diagnoses, and we were not able to explain this outcome.

Methodological considerations – ELISPOT assay

When using the ELISPOT assay in peripheral blood the cell count in the wells is usually designed to be 100,000 cells/well (**Paper I**). In **Paper IV** we wanted to investigate the diagnostic performance of *Borrelia*-induced IFN-γ-secretion detected by ELISPOT modified to be feasible for use by local clinical laboratories. Thus, we used CSF that normally consists of only a few cells, in the ELISPOT test. Therefore, instructions were developed based on the idea that the CSF should simply be centrifuged, the supernatant removed, and the pellet of cells resuspended in culture medium. A cut-off value for choosing single or duplicate wells for the test should be based on a routine cell count of CSF. This is the rationale for why different numbers of cells were assayed for the different patients, and the

spot counts mathematically adjusted to 5,000 cells/well. Thus, CSF cells were assayed in duplicate (two stimulated and two unstimulated wells) when the yield of the cells was higher than 25,000 cells, and singly (one stimulated and one unstimulated) when the yield was lower than 25,000 cells. The numbers of cells/well for each patient included in **Paper IV** are shown in Tables 13 and 14 and the numbers of patients where the number of cells/well were lower and larger than 5,000, respectively, are summarized in Table 12.

Table 12. Cells/well in the ELISPOT assay, mathematically adjusted to 5000 cells/well, showing the number of patients with numbers of cells/well lower and larger than 5000, respectively.

Cells/well	Patients n	Spots mathematically adjusted by
≤5000	49	Multiplication
≥5000	68	Division

Typically, patients with LNB present with pleocytosis, but exceptions exist. In our study, all of the LNB patients presented with pleocytosis. Within the LNB group, the number of cells per well varied from 2,000 up to 115,000, median 65,000, and cells from the LNB patients were assayed with 5,000 cells or more per well in 12/14 cases (Table 13).

The non-LNB group consisted of patients that came for suspected LNB, but after clinical and laboratory investigations, were diagnosed with other diseases. Nevertheless, 14 of the 103 patients in the non-LNB group had pleocytosis. In the whole quantity of material the number of cells/well varied between 650 cells/well and 115,000 cells/well. Furthermore, the number of cells in the non-LNB group varied from 650 up to 35,000, median 5500. 57/103 patients diagnosed as non-LNB were assayed with 5,000 cells or more per well (Table 14) . One drawback of this study might be that it lacks a healthy control group, but because of ethical reasons this was not possible. On the other hand, this mixture of different diagnoses and patients with suspected LNB represents the reality in everyday life in an endemic setting.

The low number of CSF cells is a limitation of this study and is not optimal for the evaluation of the ELISPOT assay as a diagnostic tool for LNB. This is a known problem since there are usually few inflammatory cells in CSF. However, Asai et al. previously reported that the frequency of spots is not altered by decreasing cell counts, even when there are low numbers of cells per well (Asai et al., 2000). Another shortcoming is that the ELISPOT assay detects cytokines secreted from a single cell, but not the amount of cytokines secreted. Thus, it is not possible to evaluate the secreted amount of a certain cytokine. However, the aim

of this study was to investigate whether ELISPOT is useful as a diagnostic tool in LNB, and that was the rationale for adjusting the spots.

Statistical considerations - ELISPOT assay (Paper IV)

Adjusting the spot counts mathematically to 5,000 cells/well is a methodological compromise in this study. The number of cells/well and the number of spontaneous and *B. burgdorferi* induced IFN-γ-secreting cells before and after mathematical correction are shown for all patients in Tables 13, 14. It cannot be excluded that this strategy affected the result since the relation between the *in vitro* IFN-γ response to *B. burgdorferi* in mononuclear cells and the number of cells per well is not necessarily linear. However, since the non-LNB patients in general had a lower number of cells, and thus would have had less optimal culturing conditions, they would in turn have generated lower numbers of IFN-γ-secreting cells. Thus, the low number of cells in the non-LNB group probably does not explain the weak diagnostic performance of the test, as it was mainly due to the high number of non-LNB patients showing positive tests. Furthermore, Asai et al. showed that low numbers of cells/well still gave adequate numbers of spots. (Asai et al., 2000).

IFN-γ, a Th1 cytokine, is an important mediator of inflammation driven by infection or injury. In addition to its role in the adaptive Th1-response, an innate IFN-γ response exists irrespective of adaptive mechanisms and is an unspecific marker of inflammation, which may have interfered with the results. In the LNB-group, the CSF-leukocyte count, another unspecific marker of inflammation, was elevated in all patients. In addition, the unstimulated IFN- γ secretion was low and a significant stimulation with *B. burgdorferi* antigen was found.

Regression analysis of the uncorrected cell count/well *versus* stimulated and unstimulated IFN-γ production in the diagnostic groups was performed. The LNB group's spontaneous secretion showed a significant association with the cell count with the coefficient of determination R^2 of 0.30, $p = 0.041$ and for the *B. burgdorferi*-induced LNB group R^2 was 0.44 and $p = 0.01$. Thus, there was a difference in IFN-γ production that was associated with the cell count but not entirely explained by it.

The non-LNB group was heterogeneous and a correlation of IFN-γ secretion to the leukocyte count was not found either in *B. burgdorferi*-induced stimulated or unstimulated cells. However, in the subgroup of non-LNB with pleocytosis, a significant correlation for *B. burgdorferi*-induced secretion to the leukocyte count was found, (R^2=0.48, p = 0.006). This may have been an unspecific stimulation by borrelial lipoprotein in the preparation.

Altogether, since the number of cells/well correlated with both the *B. burgdorferi*-stimulated and the unstimulated numbers of IFN-γ-secreting cells in the LNB-group, this suggests a linear relation, justifying extrapolation in the LNB-group. However, since no such correlation was found in the non-LNB group, except for *B. burgdorferi*-induced secretion in the subgroup with pleocytosis, it cannot be excluded that the extrapolation caused the weak diagnostic performance of the test. However, this does not fully explain the weak diagnostic performance of the test, since the non-LNB patients with pleocytosis responded to *B. burgdorferi* stimulation, which as discussed above may have been due to either unspecific stimulation or previously generated *B. burgdorferi*-specific memory cells circulating in the CSF.

The current modified ELISPOT assay showed a weak diagnostic performance as a supplementary test in laboratory diagnosis of patients with clinically suspected LNB. The assay involved several drawbacks, most importantly a low specificity. It also demanded a procedure of extrapolating the results to 5,000 cells/well due to low and varying CSF cell numbers. We therefore concluded that our ELISPOT was not of value as a diagnostic tool in our hyper-endemic area.

One interesting question is if it would be possible to calculate the minimum number of cells/well needed for extrapolating the test.

For future studies it is important to investigate if there is a linear association between cells per well and the number of *B. burgdorferi*-induced IFN-γ-secreting cells and spontaneously IFN-γ-secreting cells. This should preferably be done by titration of numbers of cells per well.

Table 13. Cells/well, spontaneous and *B. burgdorferi* induced secretion before and after mathematically corrected in patients with confirmed Lyme neuroborreliosis.

No	Cells/well	Spontaneous	Stimulated	Net value	Spontaneous corrected*	Stimulated corrected*	Net value Corrected*
1	2 000	3	1	0	7,5	2,5	0,0
2	3 250	1	0	0	1,5	0,0	0,0
3	8 700	0	87,5	87,5	0,0	50,3	50,3
4	18 000	2	14	12	0,6	3,9	3,3
5	23 500	4	9	5,5	0,7	1,9	1,2
6	28 120	9	7	0	1,5	1,2	0,0
7	49 500	31	33	2	3,1	3,3	0,2
8	80 000	61	117,5	57	3,8	7,3	3,6
9	80 640	72	358,5	287	4,4	22,2	17,8
10	83 000	16	259	243	1,0	15,6	14,6
11	97 000	200	300	100	10,3	15,5	5,2
12	100 000	123	233,5	111	6,1	11,7	5,6
13	105 000	10	100	90	0,5	4,8	4,3
14	115 400	16	79,5	64	0,7	3,4	2,8

*=Values recalculated to correspond to 5000 cells/well

Table 14. Cells/well, spontaneous and *B. burgdorferi* induced secretion before and after mathematically corrected in patients with non-Lyme neuroborreliosis.

Cells/well	Spontaneous	Stimulated	Net value	Spontaneous corr	Stim corrected	Net value Corrected
650	0	0	0	0,0	0,0	0,0
1 400	69	42	0	246,4	150,0	0,0
1 620	9	7	0	27,8	21,6	0,0
1 650	29	10	0	87,9	30,3	0,0
1 800	8	6	0	22,2	16,7	0,0
1 815	0	2	2	0,0	5,5	5,5
1 920	2	0	0	5,2	0,0	0,0
2 250	163	166	3	362,2	368,9	6,7
2 500	5	2	0	10,0	4,0	0,0
2 500	2	0	0	4,0	0,0	0,0
2 500	2	4	2	4,0	8,0	4,0
2 600	0	0	0	0,0	0,0	0,0
2 700	4	1	0	7,4	1,9	0,0
2 700	0	0	0	0,0	0,0	0,0
2 700	4	3	0	7,4	5,6	0,0
2 700	40	25	0	74,1	46,3	0,0
2 750	0	0	0	0,0	0,0	0,0
3 000	6	9	3	10,0	15,0	5,0
3 000	10	16	6	16,7	26,7	10,0
3 000	9	19	10	15,0	31,7	16,7
3 025	16	6	0	26,4	9,9	0,0
3 100	0	1	1	0,0	1,6	1,6
3 200	1	0	0	1,6	0,0	0,0
3 250	2	1	0	3,1	1,5	0,0
3 300	8	11	3	12,1	16,7	4,5
3 420	12	5	0	17,5	7,3	0,0
3 500	2	0	0	2,9	0,0	0,0
3 500	6	2	0	8,6	2,9	0,0
3 500	94	65	0	134,3	92,9	0,0

Cells/well	Spontaneous	Stimulated	Net value	Spontaneous corr	Stim corrected	Net value Corrected
3 500	0	0	0	0,0	0,0	0,0
3 500	0	1	1	0,0	1,4	1,4
3 500	0	6	6	0,0	8,6	8,6
3 500	2	13	11	2,9	18,6	15,7
3 600	7	4	0	9,7	5,6	0,0
3 700	0	0	0	0,0	0,0	0,0
3 700	0	0	0	0,0	0,0	0,0
3 720	3	11	8	4,0	14,8	10,8
3 750	2	0	0	2,7	0,0	0,0
3 770	0	0	0	0,0	0,0	0,0
4 000	3	1	0	3,8	1,3	0,0
4 000	1	2	1	1,3	2,5	1,3
4 000	0	1	1	0,0	1,3	1,3
4 000	0	2	2	0,0	2,5	2,5
4 500	54	42	0	60,0	46,7	0,0
4 500	38	54	16	42,2	60,0	17,8
4 770	2	1	0	2,1	1,0	0,0
5 000	42	31	0	42,0	31,0	0,0
5 225	26	80	54	24,9	76,6	51,7
5 300	4	4	0	3,8	3,8	0,0
5 400	10	13	3	9,3	12,0	2,8
5 500	1	0	0	0,9	0,0	0,0
5 500	8	7	0	7,3	6,4	0,0
5 500	0	0	0	0,0	0,0	0,0
5 500	3	4	1	2,7	3,6	0,9
5 500	1	4	3	0,9	3,6	2,7
5 500	7	26	19	6,4	23,6	17,3
6 000	2	0,5	0	1,3	0,4	0,0
6 000	6	7	1	5,0	5,8	0,8
6 000	37	44	7	30,8	36,7	5,8
6 000	18	27	9	15,0	22,5	7,5
6 300	25	29	4	19,8	23,0	3,2
6 300	7	13	6	5,6	10,3	4,8
6 330	8	2	0	5,9	1,6	0,0
6 450	2	6	4	1,6	4,7	3,1
6 500	3	14	11	2,3	10,8	8,5
7 000	10	36	26	7,1	25,7	18,6
7 050	12	24,5	12,5	8,5	17,4	8,9
7 100	2	2	0	1,4	1,4	0,0
7 500	2	4,5	2,5	1,3	3,0	1,7
8 050	23	19,5	0	14,3	12,1	0,0
8 100	2	2	0	1,2	1,2	0,0
8 250	2	1	0	1,2	0,6	0,0
8 250	2	2	0	1,2	1,2	0,0
8 260	3	7	4,5	1,5	4,2	2,7
8 500	0	1	1	0,0	0,6	0,6
8 730	6	15	9	3,4	8,6	5,2
8 750	0	0	0	0,0	0,0	0,0
8 750	1	1,5	0,5	0,6	0,9	0,3
9 000	1	1	0	0,6	0,6	0,0
9 000	3	2	0	1,4	1,1	0,0
9 275	24	13	0	12,7	7,0	0,0
9 300	1	1	0	0,5	0,5	0,0
9 375	133	98	0	70,7	52,3	0,0
9 400	1	2	1,5	0,3	1,1	0,8
9 500	1	3	2	0,5	1,6	1,1
9 750	60	63	3	30,8	32,3	1,5
10 000	2	1	0	1,0	0,5	0,0
10 000	0	0	0	0,0	0,0	0,0

Cells/well	Spontaneous	Stimulated	Net value	Spontaneous corr	Stim corrected	Net value Corrected
10 200	11	9	0	5,4	4,4	0,0
10 340	1	0	0	0,5	0,0	0,0
10 400	0	0,5	0,5	0,0	0,2	0,2
10 880	33	34	1	15,2	15,6	0,5
10 900	13	10	0	6,0	4,6	0,0
11 700	6	5	0	2,6	2,1	0,0
12 000	7	4	0	2,9	1,7	0,0
12 250	11	13	2	4,5	5,3	0,8
17 550	91	121,5	31	25,8	34,6	8,8
18 000	7	9	2,5	1,8	2,5	0,7
20 500	0	2	2	0,0	0,5	0,5
21 250	0	1	1	0,0	0,2	0,2
22 000	2	16,5	15	0,3	3,8	3,4
25 490	469	518,5	49,5	92,0	101,7	9,7
35 000	4	2	0	0,6	0,3	0,0

*=Values recalculated to correspond to 5000 cells/well

Tick-borne co-infections; epidemiology and immunological mechanisms (Papers I, III)

Human granulocytic anaplasmosis (Paper III)

Out of the 206 study-patients, 42 had serological evidence of a concurrent infection with *A. phagocytophilum.* Twenty of these had clinical HGA (Table 18), i.e. serological evidence together with unspecific symptoms or signs (fever, myalgia/arthralgia and/or headache) (Table 20). Eighteen of the HGA cases were confirmed and two probable. Thus, the rate of HGA cases in the total material was 10% (Table 15).

All of the HGA cases apart from four had one or more known tick bites. HGA cases were represented in all age groups (Table 18) . Three counties in the south-east of Sweden participated in the study and 11 of the HGA patients were included in the county of Blekinge, three in Kalmar and six in Östergötland (Table 15)

Table 15. Patients included from three counties in the south-east of Sweden.

County	Included (number)	Serological evidence of current infection	HGA cases
Blekinge	102	25%	11%
Kalmar	38	13%	8%
Östergötland	66	18%	9%
Total	**206**	**20%**	**10%**

Subclinical HGA (Paper III)

As mentioned above, 42 patients had serological evidence of an ongoing infection with *A. phagocytophilum*. Of these, 22 patients that were included due to EM showed serological evidence of concurrent *Anaplasma* infection (Table 20) . They did not develop any other clinical symptoms according to the follow-up interview, and thus were defined as having subclinical *Anaplasma* infection. Eleven of these 22 co-infected patients that had EM and antibodies to *A. phagocytophilum* also showed serological evidence of *B. burgdorferi* infection (Table 20). In conclusion, 20% of the 109 EM patients without other symptoms showed serological evidence of sub-clinical co-infection with *A. phagocytophilum.* Taking all the 174 EM patients into account, including the 65 with symptoms of disseminated infection (unspecific symptoms with or without fever), the rate of co-infection was still similar (21%).

It is not easy to detect patients with a subclinical, ongoing infection without obvious symptoms or signs. In our study we chose to investigate patients diagnosed with EM in order to detect co-infections. It has been argued that there may be cross-reactivity between *Borrelia* and *Anaplasma* serology (Blanco and Oteo, 2002) which could explain positive serology for *A. phagocytophilum* in LB patients. However, Bunnell et al. found no serological cross-reactions in infection of laboratory mice (Bunnell et al., 1999). Furthermore, antibodies may persist for years (Lotric-Furlan et al., 2001), making it difficult to evaluate the status based on only the acute sera. In our study, we used a generally accepted definition for serodiagnosis of *A. phagocytophilum* infection (Table 8). Thus we feel that we can claim that we have clear evidence of subclinical *A. phagocytophilum* infection.

Blood donors previously exposed to A. phagocytophilum and B. burgdorferi (Papers I, III)

In **Paper III**, 50 blood donors from each of the three counties, were screened for *A. phagocytophilum* and *B. burgdorferi*, reflecting seroprevalence in the healthy population (Table 16) . The findings are well in line with previous Swedish studies that show different seroprevalences from different areas (Table 17). The current findings were also comparable to an unpublished study among blood donors from 2001 (Table 16).

Table 16. Blood donors from counties in the south-east of Sweden, seroprevalence of *A. phagocytophilum* and *B. Burgdorferi.*

County, year	Blood donors, n	Seropositivity rate: *A. phagocytophilum*	*B. burgdorferi*
Blekinge			
2003	50	12%	6%
Kalmar			
2001	103	15.5%	
2003	50	14%	8%
Östergötland			
2001	100	14%	
2003	50	12%	10%

Table 17. Seroreactivity of *B. burgdorferi* and *A. phagotophilum* in Sweden, studies in blood donors and population.

	Dumler et al. 1997, Koster, west coast,	**Gustafson et al. 1990, Lisö,**	**Berglund et al. 1993, Aspö, south east**	**Wittesjö et al. 2001, Aspö, south east**	**Ekerfelt et al. 2001, Linköping,**
	Population	**Population**	**Population**	**Population***	**Blood donors**
	n = 185	**n = 346**	**n = 480**	**n = 290**	**n =408**
B. burgdorferi	13.5%	26%	19%	n.d	5.8%
A.phagocytophilum	11.4%	n.d	n.d	28%	n.d

*= The study population was used in Berglund et al´s study 1993.
n.d = not done.

Clinical symptoms and signs of HGA

Headache (16/20) was the most commonly reported symptom, followed by fever (7/20) and myalgia/arthralgia (7/20) among patients diagnosed with HGA. In **Paper III,** interestingly, only seven of the confirmed HGA patients presented with fever (Table 18) These 18 confirmed HGA and two probable cases showed a different clinical spectrum from what is usually reported in the literature. As fever has been an inclusion criterion in most prospective HGA studies so far, patients without fever have probably not been discovered or reported.

Table 18. Features of human granulocytic anaplasmosis (Paper III).

Human granulocytic anaplasmosis		**Confirmed and probable n=20**
Age-groups	5–20 years	1
	21–40 years	3
	41–60 years	11
	≥ 61 years	5
Sex	Male/Female	10/10
Tick-bite	Yes	16
Duration of symptoms before inclusion	0–3 days	8
	4–7 days	5
	8–14 days	0
	15–21 days	3
	≥ 22 days	1
	Symptoms after inclusion	3
Clinical symptoms at inclusion*	Fever	7
	Chills	3
	Headache	16
	Myalgia/ Arthralgia	7
	Neurological**	5
	Gastro-intestinal	2
Antibiotic treatment	Doxycyclin	6
	Phenoxymethylpenicillin	10
	No treatment	4
Symptoms at follow-up	No symptoms	12
	Headache	3
	Myalgia/arthralgia/neurol*/ headache***	5

*= Several patients presented with several clinical features, **= e.g. burning sensation in the arm, vertigo, neck stiffness, nunbness or unspecific neurological symptoms. ***=one or several of these symptoms,

Typically, HGA has been described as an acute febrile illness with fever >39°C, myalgia and malaise (Bakken and Dumler, 2000). In a meta-analysis of clinical manifestations in patients with HGA from North America and Europe, fever was found in 92% of cases (Dumler et al., 2005) . In two studies from North America 85%, respectively 94% of the HGA patients reported fever. (Aguero-Rosenfeld et al., 1996; Weil et al., 2012). Manifestations of HGA are illustrated in Table 19. However, it is not easy to compare the different studies since they are heterogenous and differently designed.

Table 19. Clinical characteristics, signs and symptoms of human granulocytic anaplasmosis reported in different studies from Europe and USA.

Author	HGA n	Fever %	Myalgia %	Arthralgia %	Headache %	Chills/ Sweats %
Bakken et al. 1996, USA (228 included)	41	100	98	27	85	98
Aguero-Rosenfeld et al.1996, USA (18 included)	18	94	78*	78*	61	39
Björsdorff et al. 1999, Sweden (12 included)	12	100	67	NR	100	33
Björsdorff et al. 2002, Sweden (27 included)	4	100	100	50	75	50
Lotric-Furlan et al. 2004, Slovenia (289 included)	13	100	92	69	92	62
Grzeszczuk et al. 2006, Poland (68 included)	2	100	100	100	100	NR
Weil et al. 2011, USA (692 included)	33	85	73	NR	42	NR
Koebel et al. 2012, France (3 included)	3	100	75	75*	100	100

n = number, * = myalgia or arthralgia, NR = not reported

Other European studies reported fever in 100% of the cases (Bjoersdorff et al., 1999a; Bjoersdorff et al., 2002b; Grzeszczuk et al., 2006; Karlsson et al., 2001; Petrovec et al., 1997), and in most cases fever was an inclusion criterion. Interestingly, in a Swedish study with dogs inoculated experimentally with a Swedish *Ehrlichia* isolate, two of the seven dogs did not develop fever. All dogs developed evidence of HGA demonstrated by morulae, seroconversion, and also haematological changes (Egenvall et al., 1998).

Thus, our findings, that HGA cases might have a spectrum of unspecific symptoms but not necessarily fever, might indicate that HGA can be mild and also present without fever, at least in Sweden.

A. phagocytophilum is considered to exert immunosuppressive effects, both in animals and humans (Lepidi et al., 2000; Whist et al., 2003). Even though organ failures and even fatal outcome have been reported (Hardalo et al., 1995), most fatalities following HGA could not be directly attributed to the infection itself but to compromised host defence and the presence of secondary infections (Dumler, 1997). HGA is usually a mild, self-limiting illness and spontaneous recovery of HGA without treatment is also well documented (Bakken and Dumler, 2008). None of the patients in our study were considered to have HGA severe enough to require hospitalization. Furthermore, no known complications were reported.

Another thing to consider is that HGA cases in Europe seem to be milder and to resolve sooner (Brouqui et al., 2004). Morulae and PCR diagnostic positive cases tend to be more often found in patients from North America, as compared to Scandinavian and other European cases (Bjoersdorff et al., 2002b; Blanco and Oteo, 2002). One explanation might be that different clades have evolved in co-evolution with different vectors and mammal reservoirs, thus giving rise to variants with slightly different host preferences and pathogenicity to humans (Massung et al., 2000). This is somewhat analogous to the evolution of *B. burgdorferi*, of which several variants are present in Europe, giving rise to a variety of manifestations, partly connected to sub-species (e.g. *B. afzelii* in skin manifestations and *B. garinii* in LNB), while *B. burgdorferi. s.s* dominates in North America, more profoundly linked to LA (Balmelli and Piffaretti, 1995; Stanek and Strle, 2009).

Table 20. Symptoms of the 206 included study patients, and their relation to *Anaplasma* and *Borrelia* infection. One hundred and nine of the 174 patients with clinical signs of EM had no other recorded clinical symptoms at the time of inclusion. Of these 109 patients, 22 had serological evidence of current *A. phagocytophilum* infection, i.e. subclinical *Anaplasma* infection. In the group with EM and unspecific symptoms, 14 patients had concurrent serological evidence of *A. phagocytophilum* infection, i.e. by definition human granulocytic anaplasmosis (HGA). Six further cases of HGA were identified in the symptomatic group without EM. Out of 174 patients with EM, 74 (43%) had serological evidence of current *Borrelia* infection. Twelve of the patients with unspecific symptoms but without EM also had serological evidence of current *Borrelia* infection. Of these, two patients had concurrent HGA.

Signs/symptoms	Serological status				Total, n
	Anaplasma - Borrelia - n=96	Anaplasma - Borrelia + n=68	Anaplasma + Borrelia – n=24	Anaplasma + Borrelia + n=18	206
Only EM	59	28	11	11	109
EM + unspecific Symptoms	21	30	9	5	65
Unspecific symptoms (no EM)	16	10*	4*	2	32

n = number
Anaplasma = *Anaplasma phagocytophilum*
Anaplasma - = No serological evidence of antibodies
Anaplasma + = Serological evidence of antibodies
Borrelia = *Borrelia burgdorferi*
Borrelia - = No serological evidence of antibodies
Borrelia + = Serological evidence of antibodies
EM = Erythema migrans
Unspecific symptoms= Fever and/or myalgia and/or arthralgia and/or headache

* = one concurrent TBE case included

Laboratory findings and diagnostic aspects

Six patients with confirmed HGA had moderately elevated transaminase levels, while no patient presented with thrombocytopenia at inclusion. Only one had leukopenia and three presented with leucocytosis. Four patients had elevated levels of C-reactive protein.

Other studies have reported that leukopenia, thrombocytopenia, and elevated transaminase levels are found in association with HGA. (Lotric-Furlan et al., 2004; Strle, 2004) However, abnormal laboratory parameters commonly normalize by the end of the second week (Bakken and Dumler, 2008). In addition, usually less than 1% of neutrophils are infected. Thus, pancytopenia cannot be explained by bacterial cytolysis (Bakken and Dumler, 2006). Leukopenia and thrombocytopenia are most likely related to peripheral consumption, sequestration or destruction. Furthermore, most bone marrow examinations reveal hypercellular or normocellular findings (Dumler et al., 1993).

In conclusion, patients with HGA may present with general changes in haematological and chemistry blood tests. These findings provide suggestive clues to the diagnosis, but normal parameters should not discourage the physician from including HGA in the differential diagnosis (Bakken and Dumler, 2008).

Identification of A. phagocytophilum

In this study, examination of morulae in peripheral blood smears from neutrophils was not performed. Previous studies carried out by our group did not reveal any findings of morulae (Bjoersdorff et al., 2002b). Studies in Europe only rarely demonstrate morulae in HGA patients (Hulinska et al., 2009; van Dobbenburgh et al., 1999), while in the US between 25% (Aguero-Rosenfeld et al., 1996) to 68% of the patients present with morulae (Bakken et al., 1996a).

None of the 20 HGA patients were PCR positive, even though they fulfilled the case definition criteria for HGA (Table 8) , based on the European guidelines (Brouqui et al., 2004). The low rate of PCR positivity in HGA has been a general problem in our previous investigations, as well as in other investigations in Europe (Hulinska et al., 2009). On the other hand, in North America the range of PCR positive patients also varies from 69.4% to 75,9% (Bakken et al., 2001).

The PCR technique is of limited value when a patient is presenting with symptoms or signs lasting longer than one week. The sensitivity for PCR is high only during the first week because the bacteraemic phase of the infection rapidly wanes (Bakken and Dumler, 2006; Thomas et al., 2009) (Table 21). Our negative PCR findings are a limitation of the study; however a negative PCR does not exclude HGA, since the IFA:s of the paired acute phase and convalescent phase serum are more sensitive compared to PCR or culture of HGA (Bakken et al., 2002). Furthermore, there is no known correlation between severity of disease and absolute IFA titres (Bakken et al., 2002).

Serologic testing, using an IFA, has been the most common test in diagnosing HGA. In this test, a fourfold titre rise or seroconversion is considered as confirmatory for HGA (Bakken et al., 2002). Testing based on a single serum is not useful, as the test may be negative initially in the acute phase (Walls et al., 1999) or positive due to long-lasting antibodies after a previous infection (Lotric-Furlan et al., 2001).

Still, almost 20 years after the discovery of HGA as a significant clinical infectious disease in humans, the development of state-of-the-art diagnostic tests has not moved very far forward in clinical laboratories. Serology based on indirect IFA, using infected cells as an antigen, is still the most common tool for serodiagnosis, and PCR lacks in clinical sensitivity.

Table 21. Diagnostic tests for laboratory confirmation of human granulocytic anaplasmosis, relative sensitivity. (Adapted from Bakken et al. 2006).

Duration of illness (Days)	Blood smear microscopy	PCR	IFA (serologic test)
0-7	Medium	High	Low
8-14	Low	Low	Medium
15-30		Low	High
31-60			High
>60			High

Erythema migrans and co-infections (Paper III)

EM is the most common feature of LB (Strle and Stanek, 2009). In **Paper III,** 174 patients presented with EM. This is not surprising since EM was one of the inclusion criteria (Figure 14). A total of 186 patients had a current infection with *B. burgdorferi*, i.e., EM or serological evidence of *Borrelia* infection, or both (Table 20). Of the 174 patients with EM, 43% (n=74) also developed *Borrelia*-specific antibodies. This result is in accordance with other studies (Berglund et al., 1995; Bjoersdorff et al., 1999b). In **Paper I** the included patients with EM (n = 23) were screened for *A. phagocytophilum* but not *B. burgdorferi* antibodies, since the study was differently designed, aiming to find patients with concurrent EM and previously exposed to *A. phagocytophilum*.

In **Paper III**, 20 HGA cases were identified. Out of these, seven had fever (Table 18). Among this seven patients, four had EM. Three of the patients with EM and one additional patient with only fever had serological signs of a current

B. burgdorferi infection. Thus, only two of the seven patients that were defined as having HGA with fever did not have any signs of *Borrelia* infection. This means that only these two cases can be considered as classical HGA cases and not co-infected by *B. burgdorferi* (Table 2, **Paper III**).

Both in North America and in Europe about 10% of patients with LB or HGA are reported to have evidence of co-infections with other pathogens (Dumler et al., 2007; Thompson et al., 2001) In Europe, Hulinska et al. investigated in the Czech Republic, the presence of *A. phagocytophilum* and *B. burgdorferi* antibodies in patients with EM, and found that 15% (12/66) of patients were seropositive in IFA IgG to *A. phagocytophilum*. Ten of the patients were PCR-positive (Hulinska et al., 2009). In a Polish study, 68 patients that presented with fever and unspecific symptoms after suspected tick bite were evaluated. Two patients were diagnosed as having confirmed HGA, one with concurrent EM and the other also with a positive TBE serology IgM screen.

Our study indicates that a patient with both EM and unspecific symptoms can easily be interpreted as a case of LB, while he/she may actually constitute a true clinical case of HGA, mixed with EM due to a co-infection with *B. burgdorferi*. On the other hand, a sub-clinical co-infection with *A. phagocytophilum* can be interpreted as clinical HGA, due to symptoms actually caused by concurrent infection with *B. burgdorferi*.

Unspecific symptoms associated with tick-borne infections

In **Paper III,** 32 patients were recruited in the study on the basis of unspecific symptoms or signs (fever, headache, myalgia or arthralgia) in conjunction with a tick bite (Figure 14). Of these, 10 had serological evidence of an infection with *B. burgdorferi*, while four had serological evidence of an *A. phagocytophilum* infection, i.e. clinical HGA. Furthermore, in the group with unspecific symptoms, one patient also had TBE and evidence of *B. burgdorferi* antibodies and another patient had TBE and antibodies to *A. phagocytophilum*, thus co-infections (Table 20).

As mentioned earlier, only seven of twenty patients with suspected HGA presented with fever (Table 18). Fever has been frequently reported in HGA cases (Table 19). Other nonspecific symptoms such as myalgia, arthralgia and headache are also typically associated with HGA, while cough, abdominal pain and rash are more uncommon (Bakken and Dumler, 2008). Unspecific symptoms such as those mentioned above are also associated with other tick-borne infections, e.g. TBE, LB, rickettsioses and babesiosis (Blanco and Oteo, 2002; Parola and Raoult, 2001). In addition, it is known that patients diagnosed with EM (20-51%) may have systemic symptoms such as fatigue, malaise, headache, myalgia and arthralgia (Strle et al., 1999; Strle and Stanek, 2009). However, in the United States these symptoms are more often seen and also associated with more inflammation and signs, suggestive of dissemination of the spirochete. In Europe,

EM is often a less aggressive and more localized infection (Strle et al., 1999). Fever in European patients with EM is an exception, reported in less than 5% of cases (Maraspin et al., 2001; Strle et al., 1999; Strle et al., 2002). Furthermore, it has been reported that unspecific symptoms are common in LB and that they can be presented without a typical EM (Feder et al., 1993).

However, most of our patients were recruited by the inclusion criterion of EM with or without other symptoms, making it difficult to draw any general conclusions concerning the prevalence of unspecific symptoms in LB patients. Nevertheless, it should be noted that there was an obvious age bias in the distribution of fever and unspecific symptoms (Figure 25). Of the total 206 included patients, 80% were above 40 years of age.

Despite the age bias, it is interesting to note that younger persons present more often with unspecific symptoms and fever while older persons more often present with EM with any symptoms. One could speculate that this is due to a stronger immune response in younger persons, but this cannot be concluded from this material.

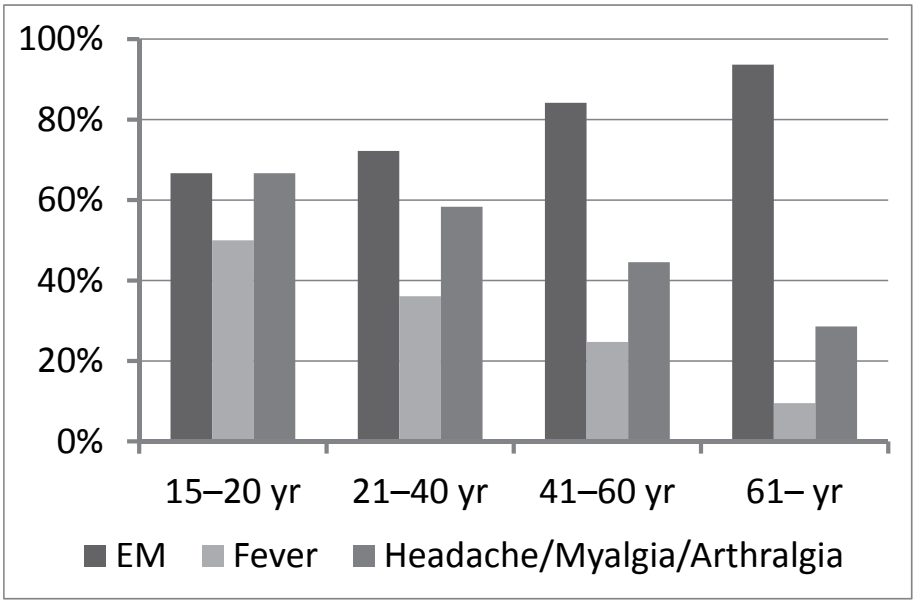

Figure 25. Clinical findings and age distribution of the relative rate of erythema migrans, fever and headache, myalgia and arthralgia in the total material of 206 patients. Age distribution, 15-20 years (n=6), 21-40 years (n=36), 41-60 (n=101), ≥61 years (n=63)

Immunological aspects of HGA (Paper I)

A. phagocytophilum is an intracellular bacterium, partly taking over the control of the first line of defence, the neutrophils. Interestingly *A. phagocytophilum* survive and multiply within the cytoplasmic vacuoles of neutrophils, and while do-

ing so actually delay their apoptosis (Woldehiwet, 2008). The resolution of infection is mediated by a combination of neutralizing antibodies and inflammatory responses of activated macrophages and neutrophils, which are dependent on CD4+ T cells (Brown, 2012). The relatively small amounts of *A. phagocytophilum* in the blood of patients and animals, suggest that the clinical disease is mediated mainly by pro-inflammatory cytokines (Rikihisa, 2011). In humans, IFN-γ and IL-10 have been shown to be elevated in sera from patients with acute infection as compared to sera from convalescent patients or healthy individuals. On the other hand, concentrations of TNF, IL-1β and IL-4 were not elevated (Dumler et al., 2000). Since several cytokines seem to play a significant role in the pathogenesis of HGA, and in the context of the effects of the suggested immune modulation of *A. phagocytophilum* in co-infections (Thomas et al., 2001), we found it interesting to study the peripheral cytokine responses in patients with EM who had previously been exposed to *A. phagocytophilum.*

Patients with erythema migrans with previous exposure to *A. phagocytophilum* (Paper I)

Subjects

In paper I, 23 patients with EM were screened for *A. phagocytophilum*; eight were seropositive for *A. phagocytophilum* (Ap seropositive LB) and 15 were seronegative (Ap seronegative LB). However, they did not display any signs or symptoms of acute HGA. In addition, their *A. phagocytophilum* antibody titres did not change between the two samples drawn. Hence, HGA was considered not to be active. Furthermore, a non-exposed group consisting of 15 individuals, that were seronegative for *A. phagocytophilum* and *B. burgdorferi* were used as a control group. The three diagnostic groups did not differ in regard to sex or age, nor did the single-infected and the double-exposed group differ in lymphocyte or monocyte counts in blood.

Cytokine secretion

When stimulated with *Borrelia* antigen, blood cells from the Ap seropositive patients showed a significantly lower number of Th1-inducing IL-12p70-secreting cells compared to Ap seronegative ($p<0.001$) (Figure 26). The non-exposed healthy controls also displayed a lower number of IL-12p70-secreting cells than the Ap seronegative ($p<0.01$) but no difference was seen between Ap seropositive and non-exposed controls. No significant difference was detected in the spontaneous IL-12p70 secretion between the groups.

Ap seronegative LB patients displayed significantly ($p<0.05$) higher numbers of *Borrelia*-specific IFN-γ-secreting cells than non-exposed healthy controls (Figue 26, b). No difference was seen between Ap seropositive patients and the non-exposed healthy controls. The spontaneous secretion of IFN-γ was higher in

the Ap seronegative group than in the non-exposed healthy control group (p<0.05). No significant difference was seen for IL-4, IL-5 or IL-13 when all groups were compared with a Kruskal-Wallis test, and therefore no further comparisons were carried out.

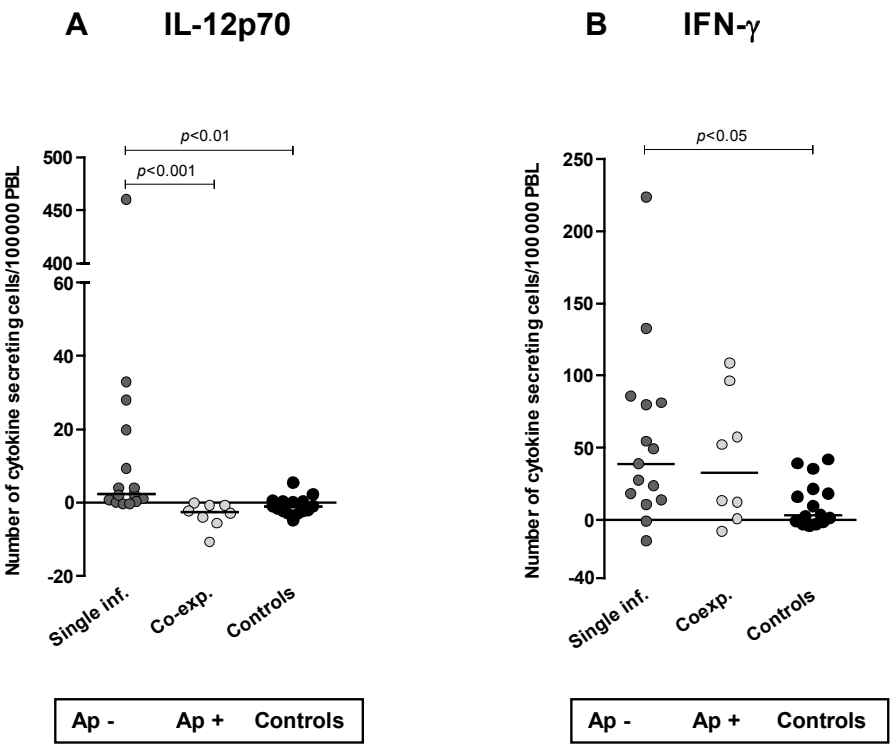

Figure 26. Number of *B. burgdorferi*-specific IL-12p70 (A) and IFN-γ (B) secreting cells/100.000 cells peripheral blood lymphocytes (PBL) from patients with erythema migrans (EM) seropositive or seronegative to *A. phagocytophilum* and healthy controls. P-values show statistical significant differences from comparison with Dunn´s test. The lines mark the median values.
Ap +; EM and *A. phagocytophilum* seropositive (n= 8), Ap -; EM and *A. phagocytophilum* seronegative n=15), Controls; healthy controls, non-exposed (n=15).

Macrophages are one of the main producers of IL-12p70 and have a central role in inducing Th1 responses. They are activated not only by pathogens but also by IFN-γ (Murphy, 2012). In this study, the lack of *Borrelia*-specific IL-12p70 secretion in Ap seropositive LB patients might suggest a reduced Th1 response. However, there were no differences between Ap seropositive LB and Ap seronegative LB in the *Borrelia*-specific IFN-γ production (Figure 26). Speculatively, since the cells from Ap seropositive LB patients did not secrete IL-12p70 when stimulated with *B. burgdorferi* antigen but secreted IFN-γ, this inhibition might

be caused by an inability of macrophages to respond to IFN-γ stimulation. When looking at the non-exposed controls, they responded with a very low or absent IFN-γ and IL-12p70 secretion when stimulated with *Borrelia* antigen. This may be in line with a lack of a memory T-cell response, but further investigations are needed.

Previous studies suggest that the cytokine response in human *A. phagocytophilum* infection is characterized by elevated levels of IFN-γ in the acute phase (Dumler et al., 2000). However, despite a strong IFN-γ response the bacteria survive in neutrophils, Bussmeyer et al. showed impairment of IFN-γ signalling in human neutrophils infected with *A. phagocytophilum*. The secretion of IFN-γ inducible chemokines CXCL10 and CXCL9 was inhibited in infected cells. These chemokines influence the leukocyte trafficking and activation and are important in the protection against intracellular pathogens. Thus, this may result in a reduced recruitment of NK cells and Th1 cells, and thereby a diminished antibacterial response (Bussmeyer et al., 2010).

In our study, none of the Ap positive LB patients had an ongoing HGA, thus this could possibly explain the low secretion of IFN-γ.

One weakness in this study is that the study population is small. Another possible limitation is that *A. phagocytophilum* antibodies can persist for several years (Bakken and Dumler, 2008; Lotric-Furlan et al., 2001), making it difficult to evaluate how long the effects of an infection with *A. phagocytophilum* may affect the immune response. However, studies on sheep suggest that an *A. phagocytophilum* infection might also have prolonged inhibitory effects on immune cells (Gokce and Woldehiwet, 1999; Larsen et al., 1994; Whist et al., 2003). Other studies indicate that *A. phagocytophilum* might remain hidden in different tissues such as the spleen, lung, heart and kidney (Chang et al., 1998; Lepidi et al., 2000).

It is tantalizing to speculate whether it is immune modulation of the infecting bacteria or genetic host factors / immune status that affects the immunological response when exposed to a dual infection. An alternative explanation to immune modulation of the host's immune system could be that certain individuals are more capable of developing a strong Th1 response, and thus are able to eliminate *A. phagocytophilum* and also *B. burgdorferi* more effectively, while others develop a weaker Th1 response and are thereby prone to developing a more prolonged infection with more symptoms and signs, and possibly also with a stronger antibody response.

Co-infections and immunological aspects (Papers I, III)

In **Paper III**, 14 of the 20 HGA cases also presented with an EM. In addition, two of the confirmed HGA cases without EM had serological evidence of a concurrent *B. burgdorferi* infection. None of the patients with HGA required hospitalization and all of them recovered. A more severe illness is associated with high age, anemia, chronic diseases or ongoing immunosuppressive therapy (Bakken et al., 1996a; Dumler and Walker, 2001). Furthermore, there is a direct correlation between patient age and/or comorbid illness and severity (Bakken and Dumler, 2008).

When the first cases of HGA in Sweden were described, three of four of these cases were reported to be co-infected with *B. burgdorferi* (Bjoersdorff et al., 2002b). Thus, as mentioned above, tick-borne co-infections seem to be quite common (Thompson et al., 2001). It has been suggested that co-infections with *A. phagocytophilum* and *B. burgdorferi* contribute to a more severe infection with increased spirochete loads and tissue injury (Thomas et al., 2001). *A. phagocytophilum* affects the granulocytes, and the influence on the host immune system may help a second pathogen to colonize the host. However, much of the pathologic mechanisms in co-infections are not fully understood (Nieto and Foley, 2009).

Co-infected patients appear to report more symptoms as e.g. chills, headache, and arthralgia than patients infected with *B. burgdorferi* alone (Krause et al., 2002). In addition, co-infected patients also report a longer duration of symptoms. Thus, it seems that co-infection with *A. phagocytophilum* and *B. burgdorferi* might result in more severe or persistent symptoms (Swanson et al., 2006), but so far no evidence exists of increased dissemination of the spirochetes among these co-infected patients (Krause et al., 2002).

An experimental study showed that mice co-infected with *B. burgdorferi* and *A. phagocytophilum* had a decreased Th1 response, with diminished levels of IFN-γ, IL-12 and TNF compared to mice single-infected with *B. burgdorferi*. The co-infected mice also developed more severe arthritis and showed an increased pathogenic load of both bacteria (Thomas et al., 2001). In addition, Nyarko et al. showed that *A. phagocytophilum*-infected neutrophils enhanced transmigration of *B. burgdorferi* across human BBB in vitro. Such mechanisms may contribute to increased blood and tissue spirochete loads (Nyarko et al., 2006).

When challenged by *B. burgdorferi* spirochetes the immune defence is shaped towards a strong Th1 response with high levels of IFN-γ (Ekerfelt et al., 1997a; Ekerfelt et al., 1999; Widhe et al., 1998). A weak or delayed Th1 response to *B. burgdorferi* would probably cause a prolonged infection, including a risk for development of persisting symptoms (Widhe et al., 2004). We hypothesized that an infection with *A. phagocytophilum* would induce an impaired Th1 response. Our findings with a reduced number of Il-12p70-secreting cells in pa-

tients with a concurrent EM and previously exposed to *A. phagocytophilum,* could possibly imply a suppression of Th1 responses induced by *A. phagocytophilum*, which may be long-standing even after the infection is resolved.

Our findings are in line with animal studies in which a reduction in lymphocytic proliferation, in response to mitogen stimulation, has been shown for up to six weeks after infection with *A. phagocytophilum* (Gokce and Woldehiwet, 1999; Larsen et al., 1994).

Concluding remarks on infections with A. phagocytophilum

With a prevalence of *A. phagocytophilum*-infected ticks up to 15% (Severinsson et al., 2010) and several reports on infected animals in Sweden (Bjoersdorff et al., 1999c; Engvall and Egenvall, 2002), it is interesting that so few human cases of HGA are discovered. One explanation may be that different strains may have different host preferences and differ in virulence in different hosts. Some strains may develop subclinical infections in humans. Another explanation could be the susceptibility in the host due to differences in immune competence (Rikihisa, 2011; Thomas et al., 2009).

Finally, the unspecific symptoms associated with HGA are not always easy to distinguish from other diseases such as viral infections, flu or other tick-borne infections (Bakken and Dumler, 2008). In addition, patients may not see the physician in the early phase of disease, and PCR as a diagnostic tool is most useful during the first week of illness (Bakken and Dumler, 2006), which could be one explanation for why we did not identify any PCR-positive HGA cases in our study. Furthermore, most HGA cases in Sweden are probably subclinical and therefore never detected.

CONCLUSIONS

Co-infections with several tick-borne infectious agents proved to be common in south-east Sweden. Serological evidence of infections with *B. burgdorferi, A. phagocytophilum* and TBE-virus were detected in patients with EM or unspecific symptoms in association with a tick-bite. HGA is usually described as an acute, febrile illness, often combined with unspecific symptoms i.e., headache, myalgia and/or arthralgia, and also fever. In our study, a number of *A. phagocytophilum* co-infections were discovered in patients with EM. Among these, several cases had no clinical symptoms and no fever, except EM, thus representing sub-clinical co-infections. In addition, some patients with both EM and other unspecific symptoms had co-infections with *A. phagocytophilum*, most likely representing co-infections with clinical manifestations, i.e. HGA. Thus, subclinical or mild HGA is probably a common, unnoticed and self-limiting infection. This may explain the high seroprevalence of *A. phagocytophilum* in the healthy population, represented by blood donors in our investigations.

Patients with serological evidence of previous exposure to *A. phagocytophilum* and currently infected with *B. burgdorferi* had a lower *B. burgdorferi*-induced secretion of IL-12p70 as compared to patients infected with *B.burgdorferi* but seronegative to *A. phagocytophilum*. Since IL-12p70 is important at the onset of the immune response and induces Th1 responses, this finding might mirror a reduced Th1 response, possibly caused by *A. phagocytophilum.*

Patients diagnosed with acute LNB compared to controls, had significantly elevated CSF-levels of the cytokines associated with cytotoxicity, i.e. IL-2, IL-7, IL-10, IL-12p70, GM-CSF and of the Th17 associated IL-17. Only three of the patients with LNB had symptoms lasting longer than six months. Thus it is not possible to draw any conclusions regarding whether cytotoxicity is associated with persisting symptoms in LNB post treatment. However, in a small pilot study, aiming to investigate functional cytotoxicity in patients with persisting symptoms post-treatment in LNB, the results suggest a possible role of cytotoxic cells.

An ELISPOT assay was modified to be feasible for use by clinical laboratories as a supplementary test in laboratory diagnosis of LNB in an endemic setting. However, it showed a weak diagnostic performance, with a sensitivity of only 36% and a specificity of 82%. Thus, this ELISPOT assay was not found to be useful in the laboratory diagnosis of patients with clinically suspected LNB, at least not in hyper- endemic areas.

Suggested advice on clinical management of tick-borne infections

Sweden is endemic for several tick-borne infections *e.g.* Lyme Borreliosis (LB), Human Granulocytic Anaplasmosis (HGA) and Tick-Borne Encephalitis (TBE). All of them can initially cause symptoms and signs such as myalgia, arthralgia, chills, fatigue, headache and fever. Patients who present with unspecific symptoms and signs after exposure to ticks could thus have a tick-borne infection or even a co-infection with several tick-borne agents.

Considering HGA, clinical examination, routine laboratory testing should be performed, but pathologic laboratory findings are not always present. Leukopenia, thrombocytopenia or elevated hepatic transaminases may be elevated, supporting the diagnosis of HGA. If HGA is suspected, empiric antibiotic treatment should start as soon as blood samples are collected, since HGA may develop to a severe infection, especially in immunosuppressed or elderly patients. Diagnosis may be confirmed with serology, but investigation of both the acute phase and convalescent serum must be carried out, which means a diagnostic delay of several weeks. PCR and direct microscopy have shown low sensitivity in most European surveys, but may be added if available.

EM in combination with unspecific symptoms is suggestive of disseminated LB, but could also imply a co-infection with HGA (or TBE). Therefore, this should be considered when choosing antibiotic treatment. The Swedish treatment recommendations for LB are presented in Table 3, page 24 and the treatment for HGA, page 31.

When Lyme neuroborreliosis is suspected, the European guidelines and the Swedish Medical Products Agency (Läkemedelsverket) recommend performing a lumbar puncture for analysis of CSF cell count and differentials and detection of intrathecal antibodies to B. burgdorferi. In order to demonstrate intrathecal antibodies, it is necessary to take both CSF and serum at the same time, as intrathecal production is calculated as a serum to CSF index.

TBE is also known to cause unspecific symptoms in the first phase of infection. In known TBE-endemic areas, TBE may also be considered as a differential diagnosis when a patient is presenting with unspecific symptoms after tick-exposure.

Last, but not least, when looking for information about tick-borne infections on the internet, one should be observant concerning the differences in clinical presentation and diagnostic and therapeutic recommendations that exist between Europe and North America. The clinical manifestations of LB and HGA differ between the continents, and sometimes also the recommended treatment.

Future research

As a continuation of these studies, it would be interesting to investigate more immunological aspects of co-infections with HGA and LB. Many questions remain to be answered. It would also be interesting to try to identify possible constitutional differences in the immune systems and immune responses of patients who develop clinical HGA and disseminated LB symptoms post treatment, as compared to those patients who get sub-clinical or mild infections with rapid eradication of the infectious agents. Are we in fact looking at immunologically distinct sub-populations, with different response patterns? Or have patients who suffer from LB and symptoms post treatment for LB usually had previous exposure to *A. phagocytophilum?* If they have, how does this exposure affect the immune response?

Furthermore, it would be interesting to conduct an epidemiological study on tick-borne infections on the Åland islands, in order to collect more data about the incidence, prevalence, clinical and subclinical manifestations of such infections. To my knowledge, such a surveillance study of *A. phagocytophilum* has never been carried out on the Åland islands. It would also be valuable to collaborate with veterinary medicine to get corresponding high quality epidemiological, clinical and management data regarding tick-borne infections in domestic animals. In this context, babesiosis and infections with *Candidatus Neoehrlichia Mikurrensis* may also be of interest to include.

Another study I would like to get more involved in is the ongoing "STING study", in which Sweden, the Åland islands, and Norway participate. The STING study investigates various epidemiological, microbiological, clinical, diagnostic, immunological and prognostic aspects of tick-borne infections in a Scandinavian context.

Finally, a challenge would be to improve the diagnostic tools of LB, since today there are several limitations in the serology of *B. burgdorferi*, especially in high-endemic settings. The B-cell chemoattractant, CXCL13, is one of the new and promising diagnostic biomarkers of LNB in CSF. It would be interesting to elucidate if other cytokines or chemokines could be used as diagnostic markers in CSF.

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