Clinical and Immunological Aspects of Lyme Borreliosis

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Cover illustration: A B. burgdorferi spirochete (Design: Johanna Sjöwall and Dennis Netzell, Liu-Tryck, Linköping)

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Confidence, like art, never comes from having all the answers; it comes from being open to all the questions

Earl Gray Stevens

Till Christopher, Elias och Lovisa
“A tick family in a rainy forest”, by Elias Sjöwall
CONTENTS

ABSTRACT ......................................................................................................................... 9
SAMMANFATTNING PÅ SVENSKA....................................................................................... 11
ABBREVIATIONS............................................................................................................... 13
ORIGINAL PAPERS........................................................................................................... 15
INTRODUCTION................................................................................................................ 17
Ly me bor reliosis ............................................................................................................. 17
Immunity to infections ................................................................................................... 46
Immune responses in Lyme bor reliosis......................................................................... 52
Immunomodulating effects of antibiotics ....................................................................... 56
INITIATION OF THE STUDY ............................................................................................ 59
AIMS OF THE STUDY ....................................................................................................... 61
MATERIALS AND METHODS.......................................................................................... 63
Subjects (Paper I–IV) ....................................................................................................... 63
Methods (Paper I–IV) ....................................................................................................... 67
Statistics ......................................................................................................................... 79
Ethical considerations ................................................................................................... 79
RESULTS AND DISCUSSION............................................................................................. 81
Interaction between B. burgdorferi and the host (Paper I–II) ........................................... 81
Clinical, immunological and diagnostic aspects of persistent symptoms ............... 95
post-neuroborreliosis (Paper III–IV) ............................................................................ 95
CONCLUDING REMARKS................................................................................................. 105
FUTURE STUDIES........................................................................................................... 107
GENERAL ADVICE ON LYME BORRELIOSIS TO HEALTHCARE PROVIDERS ....... 109
ACKNOWLEDGEMENTS................................................................................................. 111
REFERENCES.................................................................................................................. 115
APPENDIXES................................................................................................................... 135
Paper I–IV ....................................................................................................................... 135
ABSTRACT

Lyne borreliosis (LB) is a tick-borne infection caused by spirochetes of the *Borrelia (B.)* burgdorferi sensu lato complex. The infection is associated with several clinical features, of which erythema migrans (EM) and neuroborreliosis (NB) are the most common in Europe. The prognosis after antibiotic therapy is generally good. However, some patients may have residual symptoms post-treatment. The cause of the delayed convalescence is unclear. There are several factors that may affect the clinical outcome of LB, for example, the early interaction between the host’s immune response and *B. burgdorferi*, the spirochete genotype, antibiotic therapy, as well as the host’s vulnerability.

This thesis aimed to explore the type of early immune response that is generated to *B. burgdorferi* and its importance for the clinical outcome of LB, and to study the condition of persistent symptoms post-NB from clinical, immunological and diagnostic perspectives. In total, 125 adult patients with different clinical features and outcomes of LB and 23 healthy controls were included.

In a prospective follow-up study of EM, we confirmed that the prognosis of EM is good after antibiotic therapy, and that *B. afzelii* is the most common *B. burgdorferi* genotype associated with EM in the Nordic countries. Seven patients (8%) reported persistent symptoms more than six months post-treatment. These patients had also a decreased early expression of inflammatory, Th1-type cytokines in the EM lesions, suggesting an importance of early, local Th1-type immunity to *B. burgdorferi* for a successful clinical outcome of LB. No correlation between clinical characteristics, allergic predisposition, *B. burgdorferi* genotype or serology and the development of symptoms post-treatment was found.

Asymptomatic *B. burgdorferi*-seropositive individuals are interesting from clinical and immunological points of view, since they apparently have encountered *B. burgdorferi* without developing symptoms of LB. In this thesis, asymptomatic individuals were shown to display an enhanced innate inflammatory immune response to live *B. garinii* spirochetes, induced by dendritic cells and whole blood cells, in comparison with patients with a history of subacute NB and healthy controls. Whether this is the optimal immune response to *B. burgdorferi* remains to be determined.

A randomized, placebo-controlled cross-over study showed that three weeks of doxycycline therapy did not significantly improve objective neurological signs, subjective symptoms or quality of life in NB patients with persistent symptoms post-treatment. Nor could any doxycycline-mediated effects on systemic cytokine responses be demonstrated.

Brain magnetic resonance imaging (MRI) findings in NB patients with persistent symptoms post-treatment were shown to be nonspecific and to correlate with age, but not with the duration of symptoms.

In conclusion, this thesis shows that there is an association between the early immune response to *B. burgdorferi* sensu lato and the clinical outcome of LB. The cause of prolonged convalescence post-treatment remains unknown and needs further investigation. However, repeated treatment with doxycycline does not lead to improvement of the persistent symptoms; nor does brain MRI facilitate diagnosis of, or provide an explanation for, the post-treatment symptoms.

Syftet med mitt avhandlingsarbete har varit att undersöka vilket immunssvar som genereras mot *B. burgdorferi* hos människa, dess betydelse för det kliniska utfallen av infektionen samt att studera företeelser av persistera-nde symtom efter antibiotikabehandling ur ett kliniskt, immunologiskt och diagnostiskt perspektiv. Sammanlagt studerades 125 vuxna patienter med olika kliniska manifestationer och utfall av borrelios, samt 23 friska kontroller.

En uppföljande studie av patienter med EM bekräftade att prognosen överlag är god efter antibiotikabehandling och att *B. afzelii* är den vanligaste genotypen som orsakar EM i Norden. 8 procent av patienterna hade dock kvarstående besvär av smarta i rörelseapparaten, känselstörningar eller trötthet mer än ett halvår efter avslutat antibiotikabehandling. Dessa patienter uppvissade ett sänkt uttryck av inflammatoriska signalsubstanser i EM hudbiopsier (vävnad), vilket stödjer hypotesen att ett tidigt inflammatoriskt immunsvar mot *B. burgdorferi* är viktigt för att infektionen skall läka ut utan persisteraende symtom. Inget samband mellan kliniska karakteristika, allergisk benägenhet, *B. burgdorferi* genotyp eller anikroppsbildning och kvarstående besvär kunde ses.

Vissa individer har ett asymptotiskt sjukdomsforlopp vid borrelios, vilket är mycket intressant ur ett kliniskt och immunologiskt perspektiv. Dessa individer visade sig ha ett ökat inflammatoriskt medfört immunsvar mot levande *B. garinii*-bakterier, genererat av dendritiska celler och blodceller, vid jämförelse med patienter med subakut NB och friska kontroller.

I en randomiserad, placebo-kontrollerad läkemedelsprövning kunde vi inte påvisa någon doxycyclin-medierad förbättring av vare sig objektiva neurologiska fynd, subjektiva symtom eller livskvalitet hos patienter med kvarstående besvär efter antibiotikabehandlad NB. Doxycyclin hade heller ingen systemisk inverkan på immunsvaret. Magnetkameraundersökning av hjärnan hos patienter med kvarstående besvär efter antibiotikabehandlad NB visade specifika förändringar som korrelerade med ålder, men inte med symtomvaraktighet.

ABBREVIATIONS

A.  Anaplasma
ACA  acrodermatitis chronica atrophicans
APC  antigen presenting cell
AV  atrioventricular
B.  Borrelia
BALB/c  Lyme borreliosis-resistant mouse strain
BBB  blood brain barrier
BL  borrelial lymphocytoma
CBA  cytometric bead array
CCL/CXCL  chemokine ligand
CCR/CXCR  chemokine receptor
CD  cluster of differentiation
C3H  Lyme borreliosis-susceptible mouse strain
CNS  central nervous system
CRASP  complement regulator-acquiring surface protein
CSF  cerebrospinal fluid
DC  dendritic cell
DNA  deoxyribonucleic acid
EBV  Epstein Barr virus
EM  erythema migrans
ELISA  enzyme-linked immunosorbent assay
ELISPOT  enzyme-linked immunospot assay
FLAIR  fluid-attenuated inversion recovery
GM-CSF  granulocyte macrophage-colony stimulating factor
HGA  human granulocytic anaplasmosis
HIV  human immunodeficiency virus
HLA  human leukocyte antigen
I.  Ixodes
IDSA  Infectious Diseases Society of America
IFN  interferon
Ig  immunoglobulin
IHC  immunohistochemistry
IL  interleukin
IV  intravenous
L  ligand
LA  Lyme arthritis
LB  Lyme borreliosis
LC  Lyme carditis
LD  Lyme disease
LPS  lipopolysaccharide
MCP  monocyte chemotactic protein
MHC  major histocompatibility complex
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>MIP</td>
<td>monocyte inflammatory protein</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NB</td>
<td>neuroborreliosis</td>
</tr>
<tr>
<td>NF</td>
<td>nuclear factor</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OF</td>
<td>outer surface protein-enriched fraction</td>
</tr>
<tr>
<td>Osp</td>
<td>outer surface protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCG</td>
<td>penicillin G (benzylpenicillin)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pcv</td>
<td>phenoxymethylpenicillin</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PLDS</td>
<td>post-Lyme disease syndrome</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPD</td>
<td>purified protein derivative</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>QOL</td>
<td>quality of life</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SF-36</td>
<td>Short Form-36</td>
</tr>
<tr>
<td>spt</td>
<td>symptom</td>
</tr>
<tr>
<td>s.s.</td>
<td>sensu stricto</td>
</tr>
<tr>
<td>SSS</td>
<td>symptom severity score</td>
</tr>
<tr>
<td>TBE</td>
<td>tick-borne encephalitis</td>
</tr>
<tr>
<td>TCM</td>
<td>tissue culture medium</td>
</tr>
<tr>
<td>Tfhs</td>
<td>follicular T helper cell</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>WML</td>
<td>white matter lesion</td>
</tr>
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</table>
The thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I–IV):


INTRODUCTION

Lyme borreliosis
Lyme borreliosis (LB), or Lyme disease (LD), is a multi-organ infectious disease caused by the spirochete *Borrelia (B.) burgdorferi* sensu lato, transmitted to humans by hard *Ixodes* ticks. The infection affects primarily the skin, joints, heart and the nervous system. It is the most common vector-borne disease in the northern hemisphere.

**Historical overview**
In 1883, a German physician (Buchwald) described a patient with a longstanding degenerative skin manifestation. He named the condition "diffuse idiopathic skin atrophy", which is the first known description of what later was characterized as and entitled acrodermatitis chronica atrophicans (ACA). In 1909, a Swedish dermatologist (Afzelius) described the appearance of an erythema migrans (EM), which had developed at the site of a tick bite. The same skin manifestation was renamed erythema chronicum migrans by an Austrian dermatologist (Lipschütz), on the basis of its duration. In 1911, a Swiss pathologist (Burckhardt) described a patient with a solitary cutaneous pseudolymphoma. This skin lesion was later (1943) characterised in detail by a Swedish dermatologist (Bäverstedt) and renamed lymphadenosis benigna cutis. In 1921, Afzelius suggested a relationship between a tick bite and the development of EM. The following year, two French neurologists (Garin and Bujadoux) reported the first case of meningoradiculoneuritis after a tick bite. An association between lymphocytic meningitis and EM was suggested by a Swedish dermatologist (Hellerström) in 1931. Ten years later, the triad of lymphocytic meningitis, cranial nerve palsy and painful radiculitis – “Bannwarth’s syndrome” – was declared; however, without a known association with a tick bite. Later, in the 1950s, both ACA and EM were successfully treated with penicillin.

However, it was not until 1977, when a cluster of American children in Lyme, Connecticut, presented with arthritis preceded by a skin erythema, that the search for the causative pathogen speeded up. This unknown disease was named LD, on the basis of its geographical origin. Finally, in 1982, a previously unrecognized spirochete, now called *B. burgdorferi*, was isolated from *Ixodes (I.)* ticks. Thereafter, the spirochete could soon be detected in patients with LD in the United States (US) and in patients with EM, ACA and Bannwarth’s syndrome in Europe.
The vector (*Ixodes*)

The four main hard tick (*Ixodes*) species transmitting *B. burgdorferi* spirochetes include *I. ricinus* in Europe, *I. pacificus* in western North America, *I. scapularis* in eastern North America and *I. persulcatus* in Asia. Ticks thrive and exist in the leaf litter of the forest ground, where the climate is humid and the risk of desiccation is minimal.19 They are active at air temperatures above 4° C.20 The tick undergoes four stages of development; egg, larvae, nymph and the adult female and male (Figure 1), with each stage lasting approximately one year.21 Blood feeding on the host occurs once in each stage, after which the tick detaches and either develops into the next stage or enters diapause, which is a state characterized by reduced metabolism and delayed development at times when environmental conditions are unsuitable for host seeking or during engorged stages.21 After a blood meal, the mated adult female tick lays eggs and dies. Adult male ticks feed sparingly, if at all.22 The six-legged larvae and the eight-legged nymph primarily feed on smaller hosts, such as rodents and birds, whereas the adult ticks infest larger hosts, especially deer and livestock19 (Figure 2).

**Figure 1.** Different *Ixodes* tick stages: Adult female (A), adult male (B), nymph (C), larvae (D) and mating adult ticks (E) (Courtesy of Pontus Lindblom, Dep. of Medical Microbiology, Faculty of Health Sciences, Linköping University, Sweden).
In unfed ticks the spirochetes colonize the midgut, but then transmigrate during tick feeding through the gut wall and translocate to the tick salivary glands via the haemolymph. The transmission of the spirochetes to humans occurs if the tick’s feeding sites are contaminated with infected salivary secretions or regurgitated midgut contents. The maximum transmission of spirochetes from infected ticks has been experimentally shown to occur 48–72 hours after attachment to the host. Birds are the principal reservoirs of *B. burgdorferi* sensu stricto (s.s.) and *B. garinii*, whereas *B. afzelii* utilizes rodents as the main reservoir hosts. Roe deer are important as principal hosts for adult ticks, although they are incompetent reservoirs for *B. burgdorferi*. Humans should be considered as incidental and dead-
end hosts, unable to maintain a significant spirochetemia, which is necessary for further transmission of the pathogen.

**The *B. burgdorferi* spirochete**

Like other *Borrelia* species, *B. burgdorferi* sensu lato is a spiral-shaped spirochete, measuring 10–30 μm in length and 0.2–0.5 μm in width.\(^9\) It has clinical, morphological and phylogenetic similarities with the spirochete *Treponema pallidum*, the pathogen causing syphilis.\(^{30}\) Ultrastructurally, the cell wall consists of a fragile outer membrane, a protoplasmic cylinder and a cytoplasmic inner membrane around the enclosed cytoplasmic contents. There are 7–11 flagella inside the periplasmic space, giving the spirochete its characteristic shape and motility\(^9\) (Figure 3).

![Figure 3. The structure of the *B. burgdorferi* cell wall (Courtesy of Mona Widhe, Swedish University of Agricultural Sciences, Uppsala, Sweden).](image)

The *B. burgdorferi* spirochete is considered as a gram-negative bacterium. However, its outer membrane is devoid of classic enterobacterium-type lipopolysaccharide (LPS).\(^{31}\) Instead, the outer membrane contains an abundance of outer surface proteins ([Osp]A, OspB, OspC, OspD, OspE and OspF), which are expressed in different sets during transmission from the tick vector to the mammalian host, and these play an essential role in the pathogenicity of LB.\(^{32}\) OspA and OspB are mainly expressed during midgut colonization after a larval feed on an infected host, enabling the spirochetes to adhere to the gut epithelium. During nymphal feeding, the OspA and OspB proteins are downregulated and OspC is expressed on the surface, making it easier for the spirochetes to migrate to the tick salivary glands.\(^{33}\) OspE and OspF are complement-binding virulence factors,\(^{34}\) whereas the specific function of OspD is unknown.\(^{35}\)
INTRODUCTION

As a probable extracellular pathogen, *B. burgdorferi* has had to develop a variety of immune evasion mechanisms in order to protect itself from the host’s innate and adaptive immune responses. For instance, up-regulated OspC on the surface of *B. burgdorferi* binds to a salivary protein, salp15, in the tick salivary glands, thereby protecting *B. burgdorferi* from antibody-mediated killing in the host.\textsuperscript{36} The exact mechanisms of this immune evasion strategy are still unclear. However, the binding of OspC to salp15 could potentially mask the exposure of the highly immunogenic OspC to components of the host immune system.\textsuperscript{10} In addition, Salp15 carries its own host immunosuppressive properties; for example, it inhibits cluster of differentiation (CD)4+ T-cell activation.\textsuperscript{37} Differentially expressed, plasmid-encoded surface lipoproteins on *B. burgdorferi*, i.e. complement regulator-acquiring surface proteins (CRASPs) and Osp E/F related proteins, have been shown to interact with the complement pathway by binding to factor H and/or factor H-like protein-1, thereby inhibiting activation of the complement cascade and subsequent complement-mediated borreliacidal activity. Additionally, *B. burgdorferi* expresses a highly variable lipoprotein, VlsE, on its surface. The genetic sequence encoding for VlsE (*vls* gene system) undergoes random gene conversions, resulting in multiple variations and altered antigenicity of the VlsE protein during the course of infection.\textsuperscript{38} With its invasive nature, *B. burgdorferi* is able to penetrate deep into connective tissue, for example, by binding to type I collagen, a characteristic which may play a role in the long-term survival of the spirochetes (e.g. in ACA).\textsuperscript{39}

*B. burgdorferi* sensu lato comprises 15 genospecies, of which five are considered to be pathogenic to humans; *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. spielmanii* and *B. bavariensis* (*B. garinii* OspA serotype 4).\textsuperscript{40,41} Neither *B. valaisiana* nor *B. lusitaniae* are currently regarded as human pathogenic. However, *B. valaisiana* has been detected by polymerase chain reaction (PCR) in the cerebrospinal fluid of a patient with spastic para-plexis\textsuperscript{42} and *B. lusitaniae* has been isolated from the blood of a patient with vasculitis.\textsuperscript{43} *B. burgdorferi* s.s. is the only known genospecies in North America, whereas all five pathogenic species are found in Europe and Asia. In general, *B. burgdorferi* s.s. is associated with arthritis, *B. garinii* with neuroborreliosis (NB) and *B. afzelii* with ACA, although overlap between species in relation to clinical manifestations occurs, and all may cause EM.\textsuperscript{44,45}

**Epidemiology**

LB is the most common vector-borne illness in North America and is endemic in several regions of both Europe and temperate Asia.\textsuperscript{46,47} In the US, where LB is voluntarily reported to the Center for Disease Control and Prevention (CDC) by national health departments, the reported incidence of LB more than doubled during a 15-year period, with 19,931 cases in 2006. The
INTRODUCTION

incidence was highest among adults aged 40-54 years and children aged 5-14, with a disproportionate increase among young males. EM and arthritis were the most commonly reported symptoms, with a peak incidence of EM in June and July.48

LB is a compulsorily notifiable disease in only a few European countries,49 which do not include Sweden, meaning that reported European incidences are approximate estimations mainly based on positive laboratory antibody test results. The drawbacks of a reporting system based on serologic test results include underreporting of EM (clinical diagnosis), varying methods of analysis and criteria for serological diagnosis and a high seroprevalence for LB in endemic areas, which makes it difficult to distinguish between a past or present infection.50

The highest incidence of LB in Europe has been reported in Slovenia, with an estimated incidence of 206/100 000 inhabitants, and in Austria, with 135 cases/100 000. In southern Europe, the incidence is considerably lower.50 The incidence of LB in southern Sweden, based on a one-year prospective study during 1992-1993, was 69/100 000 inhabitants, of which 77% were EM, with the highest incidence in children aged 5-9 years and adults 60-74 years of age. NB was observed in 16% and arthritis in 7% of the study population. A preceding tick-bite was noticed by 79% of the patients.51 In the Åland Islands, the estimated annual incidence of LB is 1000-2000/100 000 inhabitants,52 and the mean seroprevalence has been reported to be approximately 24% in men and 17% in women, illustrating that LB is hyperendemic in these islands.53

Epidemiological studies indicate that the incidence of LB is increasing both in the US and in several European countries.50,54-56 Several factors certainly contribute to the increase, for example, changes in both human outdoor behaviour (forest activities, visiting summer houses at the coast or in the archipelago, hiking and hunting) and in tick ecology (e.g. increase in host animal populations [e.g. roe deer]),57 greater professional and public awareness of LB and climate change during recent decades with milder winters, extended spring and autumn seasons and warmer, more humid summers, resulting in more surviving ticks and their hosts.58-60

The prime risk factor for acquisition of LB is obviously tick exposure, which is dependent on tick abundance and density, geographical distribution of ticks and the prevalence of ticks infected with B. burgdorferi.61,62 Interestingly, a study in southern Sweden, in a highly endemic area for LB, showed that the risk of tick bite was 4% per 10 hours of outdoor activity and the risk
INTRODUCTION

for acquisition of LB was low, 1/221 (0.5%) tick bites. This low risk may partly be explained by the fact that the population in endemic regions tend to be more observant on tick attachment and take preventive measures, reducing the risk of infection. However, other studies in the US and Europe also indicate that the risk of developing clinical signs of LB after a tick bite is de facto low, even if the tick is infected with *B. burgdorferi*. Gender may also be considered a risk factor for acquisition of LB, since women report more tick bites per time exposed than men and develop symptoms of LB, especially EM, more often than men. In addition, in a study by Jarefors et al., women were shown to be more often re-infected with *B. burgdorferi* than men. All the re-infected women were older than 44 years.

**Prevention**

Effective prevention of LB is mainly based on increased public education and avoidance of tick bites, by using protective and dark clothing in order to reduce attraction of ticks or light-coloured clothing for easy identification of crawling ticks, early detection and removal of ticks and possibly the use of tick repellents containing N,N-diethyl-3-methylbenzamide applied to the skin or clothing, the latter mostly used in the US. However, there is only limited evidence that personal protective measures are effective in reducing the risk of infection.

The role of garlic as an insect repellent is inadequately investigated. In a study by Stjernberg et al., a significant reduction in tick bites was noted in a per protocol analysis in military personnel consuming garlic, compared to those receiving a placebo. Thus, further studies are needed to clarify the potential tick repellent effects of garlic.

Chemical and environmental methods of controlling tick abundance in a certain area are neither defensible from an ecological point of view nor possible to carry out, since both ticks and their hosts are widely distributed throughout the world and new populations are continuously established.

A recombinant OspA vaccine (lymRIX, SmithKline Beecham) was licensed in the US in 1998 for persons 15–70 years of age. It was administered in a three-dose regimen and was shown to be safe and effective in preventing LB. However, it was withdrawn from the commercial market in 2002 due to poor market penetration. Concerns about possible cross-reactions between the recombinant OspA and self-antigens (LFA-1), with increased risk of arthritis, were raised and may to some extent have contributed to the poor sales.
INTRODUCTION

The efficacy of antibiotic prophylaxis following a tick bite, for prevention of Lyme disease, has been a subject of intense debate. A study by Nadelman et al. showed that a single dose of doxycycline (200 mg) given within 72 hours after a tick bite can prevent the development of LB. A meta-analysis of four placebo-controlled clinical trials on the efficacy of antibiotic prophylaxis following a tick bite (I. scapularis) in the US was recently published. The analysis found evidence for use of antibiotic prophylaxis in endemic areas following a tick bite. Pooled data suggested that one case of LB could be prevented for every 50 patients treated with antibiotics. However, in the case of an I. ricinus tick bite in Europe, antibiotic prophylaxis is not justified, since the prevalence of infected ticks is relatively low, and a substantial proportion of infections are subclinical (asymptomatic) and treatment is associated with potential risks as well as significant costs, and might induce antimicrobial resistance. Thus, the general recommendation in Sweden and Europe is to treat with antibiotics only on presentation of appropriate LB-associated symptoms.

Disease stages
Like in syphilis, the clinical features of LB have traditionally been divided into disease stages, with varying names, definitions and included symptoms, for example, stages I–III, early and late disease or early localised, early disseminated and late disseminated infection. In general, the early localised stage (develops days to weeks after a tick bite) includes EM and Borrelia lymphocytoma (BL), whereas multiple EM, early NB and Lyme carditis (LC) are included in the early disseminated stage (weeks to months after a tick bite). Late, disseminated manifestations (months to years after a tick bite) of LB include Lyme arthritis (LA), late neuroborreliosis and ACA (Table 1). Subdivision of LB in stages is becoming increasingly rare in scientific contexts, since it is known that overlap of stages occurs and most patients do not exhibit all stages, or develop symptoms in a chronological order. In fact, seroconversion may occur without development of symptoms.

<table>
<thead>
<tr>
<th>Early, localized stage</th>
<th>Early, disseminated stage</th>
<th>Late, disseminated stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema migrans</td>
<td>Multiple erythema migrans</td>
<td>Lyme arthritis</td>
</tr>
<tr>
<td>Borrelial lymphocytoma</td>
<td>Early neuroborreliosis</td>
<td>Late neuroborreliosis</td>
</tr>
<tr>
<td></td>
<td>Lyme carditis</td>
<td>Acrodermatitis chronica atrophicans</td>
</tr>
</tbody>
</table>
INTRODUCTION

Clinical features, diagnosis and outcome of Lyme borreliosis

The clinical signs and symptoms of LB differ in Europe and North America, depending on the geographical distribution of various *B. burgdorferi* genotypes. In general, EM occurs on both sides of the Atlantic. In North America, *B. burgdorferi* s.s. is the only proven human pathogenic genotype, which explains the more frequently occurring manifestations of arthritis, multiple EM and LC in north American natives. In Europe, where all five genospecies (*B. burgdorferi* s.s., *B. garinii*, *B. bavariensis*, *B. afzelii* and *B. spielmanii*) are present, the clinical picture is more complex. NB is the most common early, disseminated feature of LB in Europe, whereas it is less common in North America, where neither ACA nor BL have been definitively observed.

*Erythema migrans*

EM is the most common clinical feature of LB. It is characterised by an oval or round, gradually expanding erythematous, sometimes bluish-red lesion at the site of the tick bite (Figure 4). It should be at least 5 cm in diameter, to be able to exclude local hypersensitivity reactions caused by the tick bite. The appearance of EM may vary depending on several factors, such as gender, duration of infection and the causative *B. burgdorferi* genotype. In fact, annular forms of EM are more commonly associated with *B. afzelii* infection, whereas EM caused by *B. garinii* are usually non-annular (and homogenous), which is an appearance that occurs more often in women. In addition, lesions caused by both *B. garinii* and *B. burgdorferi* s.s. develop and expand more rapidly and are associated with more general symptoms (e.g. fever, myalgia, arthralgia, malaise) compared to EM caused by *B. afzelii*.

The incubation period from infection to onset of EM is commonly 7–14 days (range 3–30 days). The EM may be accompanied by fatigue, fever, headache, mild neck stiffness, arthralgia and myalgia. Multiple EM may also occur, indicating hematogenous dissemination of the spirochetes. The EM lesions may heal spontaneously, often within weeks to months, but may persist for longer.
The diagnosis of EM is clinical. Serologic testing is insensitive in the acute phase (the first 2–3 weeks of infection), false positive reactions may occur and antibody responses may be affected by early antibiotic treatment. In endemic regions, background seropositivity may be high, which may further complicate assessment of a positive serologic test. For a secure microbiological diagnosis of EM, *B. burgdorferi* should be detected in skin lesions with PCR or by culture. Findings on histopathological examination of EM biopsies include a diffuse mononuclear superficial and perivascular infiltrate, mainly composed of lymphocytes, histiocytes and plasma cells. In very early lesions, an additional, but small number of neutrophils and eosinophils may be present.

The clinical outcome of EM after appropriate antibiotic treatment is good. A minority of EM-patients suffer from subjective, nonspecific symptoms for varying periods post-treatment, including musculoskeletal pain, neurocognitive impairment, headache and/or fatigue. The reason for the persistent symptoms is not known (this issue is further discussed in the Introduction, “The post-Lyme disease syndrome”). However, in a study by Cerar et al., nonspecific symptoms after treatment of EM did not differ significantly in comparison to patients without LB.

**Borrelial lymphocytoma**

BL (or lymphadenosis benigna cutis) occurs almost exclusively in Europe, and is associated with *B. afzelii* and *B. garinii* infection. It is characterised by a painless, bluish-red nodule or plaque, mainly found on the ear lobe,
INTRODUCTION

nipple or scrotum in children. It is rare in adults. A concomitant EM is often observed (Figure 5). *B. burgdorferi* serology is usually positive at the time of presentation and spirochetes may be found by PCR or occasionally in culture from a skin biopsy. The nodule is histopathologically characterised by a typical dense lymphocytic infiltrate of plasma cells in the dermis and/or subcutis.

![Borrelial lymphocytoma on an ear lobe in a child (left) and on the mamilla (adult), with a surrounding erythema migrans (right) (Courtesy of Barbro Hedin-Skogman [left], Children’s Medical Clinic, Dalarna, Sweden and Katarina Ornstein [right], the Local Health Care Clinic, Hässleholm, Sweden).](image)

**Figure 5.** Borrelial lymphocytoma on an ear lobe in a child (left) and on the mamilla (adult), with a surrounding erythema migrans (right) (Courtesy of Barbro Hedin-Skogman [left], Children’s Medical Clinic, Dalarna, Sweden and Katarina Ornstein [right], the Local Health Care Clinic, Hässleholm, Sweden).

In a prospective study by Maraspin *et al.* on BL in adults, the median time to complete disappearance of the skin lesion was 28 days after initiation of antibiotic treatment and the outcome after one year follow-up was favourable.

**Lyme carditis**

LC is a rare clinical feature of LB. The manifestation is more frequently seen in the US than in Europe, with estimates that approximately 4–10% of untreated patients with LB in the US develop carditis. The estimated incidence in Europe is much lower, 0.3–4.0%. LC is characterised by various atrioventricular (AV) blocks in the heart, due to conductance disturbances. The disease course is commonly benign and the symptoms usually resolve within 3–6 weeks, even without antibiotic treatment. However, intensive cardiac surveillance may be necessary in severe cases. Cardiac manifestations are commonly observed in conjunction with an EM or in association with neurological symptoms or arthritis. *B. burgdorferi* antibodies are commonly present in serum at the time of symptom onset and they support, together with the typical clinical findings, the diagnosis. In addition to anti-
INTRODUCTION

biotic therapy, corticosteroids and salicylates are recommended if the conduction disturbances do not improve within 24 to 48 hours after initiation of treatment. However, there is no evidence that this adjunctive therapy speeds recovery of the disease. Complications are rare and may include a permanent AV block with possible need for pacemaker, development of chronic dilated cardiomyopathy, and congestive heart failure. In very rare instances, a complete heart block may cause a fatal outcome of LB.

**Neuroborreliosis**

Neurological symptoms usually occur 1–12 (mean 4–6) weeks after the infecting tick bite and NB in Europe mainly occurs from July to December. NB is typically an acute illness, with more than 95% of cases classified as early NB, – defined as a condition in which the neurological symptoms last <6 months. Less than 5% have a symptom duration exceeding six months at diagnosis (late NB) (Table 2).

![Table 2. Classification of neuroborreliosis. Adapted from Mygland et al., 2010](image-url)

<table>
<thead>
<tr>
<th>Early NB</th>
<th>Neurological symptoms with a duration &lt;6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manifestations confined to the PNS (cranial nerves, spinal roots or peripheral nerves)</td>
<td></td>
</tr>
<tr>
<td>CNS manifestations (meningitis, myelitis, encephalitis)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Late NB</th>
<th>Neurological symptoms with a duration &gt;6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNS manifestations (radiculopathy, polyneuropathy)</td>
<td></td>
</tr>
<tr>
<td>CNS manifestations (progressive encephalomyelitis, cerebral vasculitis)</td>
<td></td>
</tr>
</tbody>
</table>

NB, neuroborreliosis; PNS, peripheral nervous system; CNS, central nervous system

The most common peripheral nervous system (PNS) manifestations of early NB in Europe are a painful radiculitis and facial nerve palsy (Figure 6). Although affecting PNS, the pathological events of radiculitis and facial nerve palsy most often occur intrathecally, since they are often accompanied by pleocytosis. Plexus neuritis and mononeuritis multiplex occur in 5–10% of NB patients. The most common CNS manifestation of early NB is mononuclear (lymphocytic) meningitis, which together with radicular pain (worst at night) and facial nerve palsy is called Bannwarth’s syndrome.
INTRODUCTION

ache alone is not a prominent symptom of early NB and seldom occurs without radicular pain or paresis.\textsuperscript{121} Other CNS manifestations, such as myelitis and encephalitis, are rare in early NB. Case reports of patients with confusion, cerebellar ataxia, hemiparesis, acute stroke-like symptoms and cerebral vasculitis have been described.\textsuperscript{121,123}

Figure 6. Peripheral, right sided facial nerve palsy (Courtesy of Nucleus Medical Media, Inc., GA, USA).

Late PNS manifestations of NB include mononeuropathy, radiculopathy and polyneuropathy, whereas progressive encephalomyelitis and cerebral vasculitis, although rare, are included in late CNS manifestations.\textsuperscript{121,124}

For a correct and reliable diagnosis of NB (both early and late), neurological signs and symptoms must be associated with inflammatory cerebrospinal fluid (CSF) parameters, including a mononuclear pleocytosis and the presence of intrathecally produced immunoglobulin (Ig) M and/or IgG antibodies against \textit{B. burgdorferi},\textsuperscript{49,121} assessed \textit{e.g.} by calculation of the serum/CSF antibody index.\textsuperscript{125} However, pleocytosis may be absent in late NB, and specific \textit{B. burgdorferi} antibodies may be lacking initially, but are present in almost all patients 6–8 weeks after the onset of symptoms.\textsuperscript{125} Further supporting CSF findings include elevated albumin ratio (indicating blood-brain barrier [BBB] injury) and the presence of oligoclonal bands (indicating ongoing inflammation in the CSF), although these signs, as well as pleocytosis, are not specific for NB.\textsuperscript{49} Various kinds of radiological findings, diagnosed by brain magnetic resonance imaging (MRI), occur in patients with different stages of NB, but are mostly nonspecific. Focal lesions in the white matter of the brain, as well as nerve-root and meningeal enhancement have been described.\textsuperscript{126}
INTRODUCTION

*B. burgdorferi* may be isolated from the CSF by culture, but the method is very time-consuming and has a low sensitivity. However, it may be of help in individual cases, especially in patients with immune deficiencies or if *B. burgdorferi* antibodies are absent in the CSF. PCR of CSF samples has approximately the same sensitivity as culture, *i.e.* 10–30% (median) in early NB. Interestingly, clinical as well as CSF findings have been shown to differ in NB caused by *B. garinii*, the most common pathogen associated with NB in Europe, and *B. afzelii*. Lymphocytic pleocytosis, more pronounced general and neurological symptoms, and the typical Bannwarth’s syndrome more often occur in *B. garinii* infections, whereas the clinical features in NB caused by *B. afzelii* are less specific and more difficult to diagnose.128

Most patients with NB improve with antibiotic therapy. Various post-treatment outcomes of NB have been reported, with persistent symptoms of diverse duration and character, such as fatigue, cognitive impairment, headache, arthralgia, myalgia or discomfort of persistent neuropathy. However, few studies have compared the persistent symptoms with a control group of patients without LB. Persistent symptoms with objective findings, mainly by means of neurological deficits, do occur but are not as common as subjective complaints. A thorough clinical and laboratory evaluation of patients with remaining complaints is very important, in order to exclude possible treatment failure, differential diagnoses or the presence of a new illness unrelated to NB. This issue is further discussed in the Introduction, “The post-Lyme disease syndrome”.

**Acrodermatitis chronica atrophicans**

ACA, which is present only in Europe and Asia and is primarily associated with *B. afzelii* infection, is classified as a chronic and long-lasting, usually progressive manifestation of LB. Characteristics of the lesion are a bluish red discoloration of the skin, with accompanying skin atrophy on extensor surfaces of the extremities (hands, feet) (Figure 7). Involvement of peripheral nerves, adjacent to the skin lesion, is common, usually as axonal polyneuropathy and radiculitis/neuralgia, with mild sensory disturbances (dysesthesia, paresthesia). ACA is seldom preceded by other objective, visible signs of LB and it is commonly observed in the elderly, predominantly in females. Virtually all patients with ACA are *B. burgdorferi* seropositive.
The diagnosis is based on the clinical appearance, the presence of *B. burgdorferi* antibodies (IgG) in serum and a typical histopathological picture, consisting of telangiectases in combination with a dense lymphocytic infiltrate of plasma cells, involving the whole or part of the dermis and sometimes also the subcutaneous fat, with or without atrophy. Spirochetes may be found, even after long disease duration, in cultures of ACA skin biopsies.

The clinical outcome of ACA after antibiotic treatment is variable. A study by Kindstrand *et al.* showed a gradual improvement of both cutaneous lesions and symptoms of neuropathy after antibiotic treatment, but virtually no effect on signs of peripheral nerve deficits (especially numbness), assessed by neurophysiological tests.

**Lyme arthritis**

LA is by far the most studied feature of late, disseminated LB. It manifests as asymmetrical mono- or oligoarticular arthritis, with intermittent or chronic swelling of mainly large joints, typically the knees. In untreated patients, the severity of the illness ranges from subjective mild joint pain, to intermittent attacks of joint swelling, to chronic synovitis persisting for months to years. LA occurs more commonly in North America than in Europe, and this is explained by the fact that *B. burgdorferi s.s.*, the subtype associated with arthritis, is the only subtype present in North America. Detailed data on prevalence of LA in Europe are lacking, and the fact that arthralgia and arthritis are often grouped together, complicates the comparison of results from different studies.
INTRODUCTION

The diagnosis of LA is based on the medical history, clinical features, exclusion of other causes of arthritis and the presence of B. burgdorferi IgG antibodies in serum. PCR-detection of borrelial deoxyribonucleic acid (DNA) in the synovial fluid is a sensitive, complementary method for correct diagnosis. Isolation of spirochetes from synovial fluid by culture is rarely successful.96

Not all patients with LA respond well to antibiotic treatment. Interestingly, it has been shown that patients with persistent joint swelling post-treatment have distinct immunogenetic markers, including the presence of the human leukocyte antigen (HLA)-DR4 or -DR2 allele and antibody reactivity to spirochetal Osp.148,149 Arthritis may persist in a small percentage of patients post-treatment, despite evident eradication of the spirochetes. In the treatment-resistant cases, it has been postulated that a T cell epitope of OspA may cross-react with a human protein (LFA-1), leading to an autoimmune response, which maintains the joint inflammation.150 This form of arthritis is termed “antibiotic-refractory LA”,151 a type of synovitis which persists despite 60 days of treatment with antibiotics (of which 30 days involve intravenous [IV] antibiotic therapy), in conjunction with negative B. burgdorferi PCR analysis of synovial fluid specimens.76 The recommended treatment of this condition is with non-steroidal anti-inflammatory drugs and hydroxychloroquine, and if the arthritis persists 3–6 months, arthroscopic synovectomy may be considered.152 Hitherto there has not been enough evidence to suggest treatment with other disease-modifying anti-rheumatic drugs, such as methotrexate or infliximab.152

Other manifestations of Lyme borreliosis

Rare manifestations of LB may occur with disseminated disease. Iritis, chorioiditis, optic neuritis, retinal detachment, retinal vasculitis, myositis and osteomyelitis have been described in sporadic cases.153

Asymptomatic infection

Epidemiological studies of populations in LB endemic regions have shown that B. burgdorferi-seropositivity, without evidence of clinical infection, is not uncommon.92,154,155 However, an asymptomatic infection is probably more common in Europe than in North America,156 since infection with B. burgdorferi s.s. is associated with more signs and symptoms of infection at the time of disease onset.155 The underlying mechanisms of subclinical infection are mostly unknown, but infection with non-invasive strains of B. burgdorferi have been proposed as one explanation.157
INTRODUCTION

Co-infections

Besides transmitting *B. burgdorferi*, *Ixodes* ticks may be co-infected with the tick-borne encephalitis (TBE) virus, *Anaplasma (A.) phagocytophilum*; the intracellular bacteria causing human granulocytic anaplasmosis (HGA) and intraerythrocytic protozoans of the genus *Babesia*, which cause babesiosis.  

The clinical characteristics of HGA include self-limiting, influenza-like symptoms (also called "tick-associated fever" in Sweden), but the infection may range from a subclinical seroconversion to a severe illness with a fatal outcome, mainly seen in immunosuppressed individuals. Studies on the prevalence of HGA infection in Southeast Sweden have indicated that *A. phagocytophilum* occurs as a common co-infection with *B. burgdorferi*, with a seroconversion rate as high as 11% during a single tick season. The seroprevalence rates in other European countries range from zero or very low, up to 28%. 

TBE occurs only in Europe and Asia, and in Sweden approximately 200 cases of illness are identified per year. TBE is characterized by a typical biphasic course of illness in roughly 75% of infected individuals. The first stage usually presents with fever, myalgia, fatigue and headache, lasting for 2–7 days. This stage is followed by an afebrile and asymptomatic period lasting for 2–10 days. The second febrile phase includes symptoms of meningitis in approximately 50% of the patients, meningoencephalitis in 40% and meningoencephalomyelitis in 10%. Specific treatment of TBE is missing, why the treatment is directed at symptom-relieving measures and neurologic rehabilitation. Active vaccination is the most effective method for preventing TBE; the modern vaccines have been shown to be both safe and effective.  

Babesiosis, which is a common cause of co-infection with *B. burgdorferi* in North America, is a disease ranging from a silent infection to a fulminant, malaria-like illness. As with HGA, most patients experience a viral-infection like illness with fever, chills, myalgia, arthralgia, vomiting and fatigue with physical findings of hepato-splenomegaly and jaundice. The fulminant illness mainly affects immunocompromised patients (lack of spleen, malignancy, human immunodeficiency virus [HIV]). Both babesiosis and HGA are treated with antimicrobials. 

The presence of co-infections should be taken into consideration when diagnosing and treating patients with atypical or unusually severe clinical features of LB.
INTRODUCTION

**Methods for detection of *B. burgdorferi* infection**
Both direct (microscopy, culture and PCR) and indirect (*B. burgdorferi* antibody analyses) laboratory methods are used for detection of *B. burgdorferi* infection.

**Microscopy**
Spirochetal structures were detected by silver staining in chronic skin lesions long before it was known that *B. burgdorferi* was the causative pathogen of LB. Silver staining methods have also been used for detection of spirochetes in EM skin lesions, BL, synovial tissue as well as in the CSF of patients with Bannwarth’s syndrome. However, this method is limited by the low density of spirochetes usually found in clinical specimens, particularly in extracutaneous manifestations, and is thus currently used in laboratories mainly to assess spirochete growth in culture aliquots.

**Culture**
*B. burgdorferi* can be recovered, – by culture in different modifications of the original Kelly medium (Barbour-Stoenner-Kelly II [BSK II], BSK-H and Kelly medium Preac-Mursic) at 30–34°C under microaerophilic conditions, from various tissues and body fluids, including biopsy specimens of EM, BL and ACA, as well as from CSF, synovial fluid, cardiac tissue and blood. Isolation of spirochetes by culture is the best diagnostic evidence of LB. However, the method has several limitations, including low sensitivity, and it is time-consuming (generation time of *B. burgdorferi* is 7–20 h) and expensive, and thus not suitable for use in routine clinical practice. In addition, the method has the highest sensitivity with regard to EM (prior to antibiotic treatment), although microbiological confirmation is seldom needed since EM is mainly a clinical diagnosis.

**The polymerase chain reaction**
The PCR is currently the most sensitive technique for detection of *B. burgdorferi* in clinical specimens, especially in tissue sections (EM, BL and ACA) and in synovial fluid. Various methods for extraction of nucleic acids from clinical samples are in use, although they vary in analytic sensitivity. The plasmid-encoded *ospA*, which occurs in multiple copies in each *B. burgdorferi* genome, is the most commonly used target for amplification. The PCR methodology may be used for confirmation of the clinical diagnosis of LB, molecular species identification and/or typing, and for detection of co-infecting pathogens. The overall diagnostic sensitivity of PCR is approximately the same as that of culture, with the exception of PCR of synovial fluid and synovial tissue, which significantly surpass culture with regard to sensitivity.
INTRODUCTION

PCR has hitherto not been used in routine clinical practice, due to the low sensitivity of the method in the CSF and blood (narrow time interval for spirochete dissemination). The PCR method may be hampered by false-positive results due to accidental contamination of samples with a small quantity of target DNA,\textsuperscript{167} and negative results do not exclude infection with \textit{B. burgdorferi} (i.e. low sensitivity of PCR, spirochetes are already eradicated or have disseminated).\textsuperscript{168} Interestingly, a novel PCR method (Light Upon eXtension real-time PCR) has been evaluated in ticks that have bitten humans, and this has proved to be more sensitive than a corresponding TaqMan assay.\textsuperscript{89} However, the diagnostic performance of this method in clinical specimens remains to be evaluated.

\textbf{Serological assays}

Analysis of \textit{B. burgdorferi} antibodies in serum is the most common method of diagnosing LB. The general recommendation in both Europe\textsuperscript{127} and in the US\textsuperscript{171} is to apply a two-tier testing approach for antibody analysis. This procedure was introduced at the end of the 90s, in order to increase the specificity of \textit{B. burgdorferi} antibody testing.\textsuperscript{167} A positive result in a sensitive conventional enzyme-linked immunosorbent assay (ELISA), detecting both IgM and IgG antibodies against \textit{B. burgdorferi} should according to European recommendations be confirmed by a more specific Western immunoblot.\textsuperscript{127} A number of different assays with various antigens are currently available, but the most commonly used are: antigen mixtures of the \textit{B. burgdorferi} whole-cell sonicate, purified flagellar components, recombinant antigens (OspC, the BBK32 protein, p39, the DbpA protein, p41) and the synthetic C6 peptide derived from the VLsE sequence.\textsuperscript{166,172}

The advantages of ELISA include the ease of testing, the quantifying of antibody levels and the automated procedure. The Western blot is based on the use of antigens separated by molecular size and detection of antibody reactivity to these specific antigens. However, the method involves technical difficulties, subjective interpretation and is time-consuming. Since second generation ELISA assays, based on recombinant antigens, show much higher sensitivity and specificity compared to the former ones, it has recently been discussed whether the two-step approach should be modified or even replaced by newer diagnostic approaches.\textsuperscript{89,167,173}

Development of sensitive serological assays is more complex in Europe, due to the heterogeneity of the different \textit{B. burgdorferi} strains. Moreover, a number of commercial serological assays are currently available, without a European or Swedish standardization.\textsuperscript{174,175} Other dilemmas include the cross-reactivity of \textit{B. burgdorferi} IgM antibodies with antibodies against
INTRODUCTION

other microorganisms, for example, *Treponema pallidum*, cytomegalovirus, Epstein-Barr virus (EBV), or with the rheumatoid factor. In addition, both *B. burgdorferi* IgG and IgM antibodies sometimes persist, independent of treatment response, for months to several years post-treatment, which complicates differentiation between a current and past infection. To date, no single antigen has proved superior to two-tier testing with regard to sensitivity and specificity. However, the C6 peptide has been shown to be highly immunogenic and broadly cross-reactive among all *B. burgdorferi* genospecies. Furthermore, promising results on assays using the C6 peptide have recently been reported, both when testing European and American patients with different manifestations of LB. However, the assays differ in many ways, which complicates comparison of the results. Based on the current knowledge of the pros and cons of serological testing, the Swedish Medical Product Agency has defined criteria for analysis of *B. burgdorferi* antibodies in serum (Table 3).

Table 3. Swedish recommendations for analysis of *B. burgdorferi* antibodies in serum.

<table>
<thead>
<tr>
<th>Serologic analysis is indicated in</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>NB – concomitant analysis of <em>B. burgdorferi</em> antibodies in the CSF and serum. Most patients are antibody positive in serum eight weeks after onset of symptoms</td>
<td></td>
</tr>
<tr>
<td><strong>Lyme arthritis</strong> – almost always accompanied by <em>B. burgdorferi</em> specific antibodies in serum</td>
<td></td>
</tr>
<tr>
<td><strong>ACA</strong> – almost always accompanied by <em>B. burgdorferi</em> antibodies in serum</td>
<td></td>
</tr>
<tr>
<td><strong>Lyme carditis</strong> – <em>B. burgdorferi</em> antibodies in serum comparable with those in NB</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serologic analysis is NOT indicated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>After a tick-bite or tick-exposure, without symptoms or clinical signs compatible with LB</td>
<td></td>
</tr>
<tr>
<td>Erythema migrans – a clinical diagnosis</td>
<td></td>
</tr>
<tr>
<td>Unspecific, subjective symptoms, for example, fatigue and malaise</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment control</strong> – antibodies may persist for months to several years</td>
<td></td>
</tr>
</tbody>
</table>

ACA, acrodermatitis chronica atrophicans; NB, neuroborreliosis; LB, Lyme borreliosis; CSF, cerebrospinal fluid; *B. borrelia*
INTRODUCTION

Antibody analyses in the cerebrospinal fluid

Determination of the CSF-serum antibody index is essential in and required for the diagnosis of NB,\textsuperscript{121,127} since interruption of the BBB often occurs in NB, which enables transudation of serum antibodies into the CSF. A positive CSF antibody index confirms past or present \textit{B. burgdorferi} infection in the CNS.\textsuperscript{173} Several methods are available for analysis of intrathecal antibody production, such as capture immunoassays, and CSF-serum indices determined by ELISA and Western blot. Various recombinant antigens are used in the assays, for example, the internal fragment of flagellin, DbpA, BBK32, OspC and the synthetic C6 peptide. However, there are currently no standardized CSF-antibody tests in either Europe or in North America.\textsuperscript{167} The still most commonly used method in Sweden is the commercial flagellum-based ELISA (personal communication with Urban Forsum, Linköping, Sweden), although newer methods are about to be introduced in routine diagnostics.

\textit{B. burgdorferi}-specific antibody production (IgM and IgG) in CSF begins within two weeks after onset of neurological symptoms. \textit{B. burgdorferi}-specific IgG antibodies are present in virtually all patients with NB by six weeks after disease onset\textsuperscript{125,185} and may persist for a long time,\textsuperscript{186} whereas intrathecal \textit{B. burgdorferi}-specific IgM antibodies usually disappear within 3–6 months post-treatment.\textsuperscript{125}

Additional methods

Various complementary methods, mainly used in research contexts, have been evaluated for diagnostic purposes in regard to LB. Recently, the B cell chemokine CXCL13 has aroused great interest as a sensitive and specific diagnostic biomarker in the CSF in early NB.\textsuperscript{187} Recent studies indicate that CSF CXCL13 is linked to disease duration in NB and could be a marker of disease activity and treatment response.\textsuperscript{188,189} Other assays include T cell proliferation assays, detection of \textit{B. burgdorferi} antigens in body fluids,\textsuperscript{127} the enzyme-linked immunospot (ELISpot) assay (described in detail in “Methods”),\textsuperscript{190} assessment of \textit{B. burgdorferi} cyst formation and analysis of CD57+/CD3- lymphocyte subpopulations in blood.\textsuperscript{121} However, currently none of these additional methods are recommended as diagnostic tests, due to the cumbersome nature of some of the assays, the uncertain clinical significance and concerns about specificity and standardization.

Treatment

The treatment recommendations for LB differ worldwide.\textsuperscript{191} Several randomized treatment trials have compared different treatment regimens for different manifestations of LB. Doxycycline, amoxicillin, phenoxy-

\textsuperscript{37}
INTRODUCTION

Ylpenicillin (PcV) and cefuroxime axetil have been shown to be equivalent alternatives for treatment of EM in adults.111,113,192-195 Contrariwise, various effects regarding macrolides (especially azithromycin) have been described.193,196-199 IV ceftriaxone, cefotaxime and penicillin G (PcG), as well as oral doxycycline and amoxicillin are recommended for treatment of LA.152,200,201 However, randomized treatment trials of LC and ACA are lacking. Common antibiotic regimes for treatment of LC include amoxicillin, doxycycline and ceftriaxone.120,202 Clinical improvement of chronic ACA skin lesions have been described with PcG, cefuroxime, ceftriaxone and doxycycline.142,203

According to the evidence-based guidelines of the European Federation of Neurological Societies, adult patients with early NB, with PNS manifestations, should be treated with a single 14-day course of either doxycycline (200 mg daily) or ceftriaxone (2 g daily IV); both regimens have been shown to be equally effective.121,204,205 In an evidence-based review of Halperin et al., PcG, ceftriaxone and cefotaxime and both IV and oral doxycycline were proven to be effective for the treatment of nervous system LB.129 Both Mygland et al.121 and Halperin et al.129 recommend that patients with NB with CNS manifestations or who have experience treatment failure with oral doxycycline should be treated with IV ceftriaxone. In 2009, the Swedish Medical Product Agency published evidence-based guidelines for treatment of LB (Table 4).

*B. burgdorferi* has been shown to have high *in vitro* susceptibility to azithromycin and ceftriaxone, whereas the *in vitro* activity of doxycycline, amoxicillin and PcG exhibit greater variations.206,207 However, the published susceptibility results vary considerably in terms of different test conditions and in the criteria for determination of antibiotic-induced killing of spirochetes and growth inhibition *in vitro*. In addition, the *in vitro* activity of many antimicrobial agents do not always correlate with the clinical experience, which is exemplified by the merely moderate activity of β-lactam antibiotics against *B. burgdorferi* *in vitro*, whereas they have good effect *in vivo*. Factors, such as interactions of the BSK medium with antimicrobials *in vitro*, different temperatures conditions *in vivo* and *in vitro*, and immunological factors during infection *in vivo*, may influence the results.206

Corticosteroids (a tapering course) are usually recommended in Bell’s palsy, a peripheral palsy of unknown aetiology affecting the facial nerve.208 However, the use of corticosteroids in early NB or in facial nerve paralysis with known or highly suspected Lyme aetiology is not recommended, due to lack of evidence.121,129,191
INTRODUCTION

Table 4. Swedish recommendations for treatment of Lyme borreliosis in adults.95

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Antibiotic</th>
<th>Dosage</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solitary EM</td>
<td>PcV</td>
<td>1 g x 3</td>
<td>10 days</td>
</tr>
<tr>
<td>- during pregnancy</td>
<td>PcV</td>
<td>2 g x 3</td>
<td>10 days</td>
</tr>
<tr>
<td>- penicillin allergy</td>
<td>Doxycycline</td>
<td>100 mg x 2</td>
<td>10 days</td>
</tr>
<tr>
<td></td>
<td>Azithromycin</td>
<td>500 mg x 1 day 1,</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>250 mg x 1 day 2-5</td>
<td>5 days</td>
</tr>
<tr>
<td>EM with fever or multiple EM</td>
<td>Doxycycline</td>
<td>100 mg x 2</td>
<td>10 days</td>
</tr>
<tr>
<td>- during pregnancy</td>
<td>Ceftriaxone</td>
<td>2 g x 1 IV</td>
<td>10 days</td>
</tr>
<tr>
<td>Borreliac lymphocytoma</td>
<td>Doxycycline</td>
<td>100 mg x 2</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>PcV</td>
<td>1 g x 3</td>
<td>14 days</td>
</tr>
<tr>
<td>Neuroborreliosis</td>
<td>Doxycycline</td>
<td>200 mg x 1</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>2 g x 1 IV</td>
<td>14 days</td>
</tr>
<tr>
<td>Lyme carditis</td>
<td>Doxycycline</td>
<td>100 mg x 2</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>2 g x 1 IV</td>
<td>14 days</td>
</tr>
<tr>
<td>ACA</td>
<td>Doxycycline</td>
<td>100 mg x 2</td>
<td>21 days</td>
</tr>
<tr>
<td></td>
<td>PcV</td>
<td>2 g x 3</td>
<td>21 days</td>
</tr>
<tr>
<td>Lyme arthritis</td>
<td>Doxycycline</td>
<td>100 mg x 2</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>2 g x 1 IV</td>
<td>14 days</td>
</tr>
</tbody>
</table>

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

The post-Lyme disease syndrome

Remaining objective clinical manifestations are uncommon after adequate treatment of LB.76,209 However, in some adult patients, subjective musculoskeletal (myalgia, arthralgia) and neurocognitive symptoms (fatigue, headache, malaise, memory impairment and cognitive difficulties) persist after antibiotic therapy, without signs of residual or new objective manifestations of the infection.76 This phenomenon has been termed “post-Lyme disease syndrome” (PLDS), and is characterised by continuous or relapsing nonspecific, subjective symptoms lasting more than six months after treatment of LB.49,121 However, strictly standardized case definitions for this condition are lacking, which complicates the comparison of results of different studies.76 The frequency of remaining subjective complaints is partly dependent on the follow-up time point post-treatment, since a gradual decrease in symptoms has been observed.113,136,138 The post-treatment symp-
INTRODUCTION

toms occur in the general population as well,\textsuperscript{210-212} and have been shown to occur as frequently in controls without LR.\textsuperscript{213-216} Children appear to be less likely to develop PLD symptoms (\textit{i.e.} duration <6 months) or PLDS.\textsuperscript{215,217}

A large controlled treatment trial of patients with PLDS showed that a majority of the study subjects reported both fatigue and cognitive dysfunction at baseline, but had normal neuropsychological test scores, including objective measures of attention and memory,\textsuperscript{218} which was consistent with results from a study by Shadick et al.\textsuperscript{219} Additional or prolonged antibiotic treatment of persistent symptoms has not been proven to be more beneficial than placebo,\textsuperscript{218,220-222} but contrariwise involves a substantial risk for various adverse events, such as septicaemia spreading from IV catheters, fungal infections and anaphylactic reactions.\textsuperscript{76}

During the last decade, the PLDS has attracted considerable public attention and has been a frequently debated topic in both scientific and public contexts. Patient support associations have been established worldwide and the phenomenon has also engaged legal authorities and insurance companies, mainly in North America.\textsuperscript{223} In an attempt to clarify the definition of PLDS, the Infectious Diseases Society of America (IDSA) has proposed strict criteria for this syndrome (Table 5).

Risk factors for development of PLDS following early NB have been identified and include: age >40 years, long-lasting symptoms (>6 weeks) prior to antibiotic treatment, high levels of \textit{B. burgdorferi} specific IgG antibodies and a high pre-treatment cell count in the CSF, as well as NB with concomitant radiculitis and female gender.\textsuperscript{56,131}

Despite extensive research, the pathogenic mechanisms and causes of PLDS are still mainly unknown. Besides post-infectious sequelae, there may be other causes of persistent symptoms post-treatment (discussed below in “Possible causes of PLD symptoms”). Differential diagnoses, such as fibromyalgia, chronic fatigue syndrome, autoimmune diseases and degenerative musculoskeletal, psychiatric and neurological disorders should be considered and excluded before the diagnosis of PLDS is made.\textsuperscript{76,224-226}
INTRODUCTION

Table 5. Proposed definition of the post-Lyme disease syndrome.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>An adult or child with a documented objective episode of early or late LB according to European or North American case definitions</td>
</tr>
<tr>
<td>After treatment of the episode of LB, with recommended treatment regimens, there is resolution or stabilization of the objective manifestations of LB</td>
</tr>
<tr>
<td>Onset of any of the following subjective symptoms within six months of the diagnosis of LB and persistence of continuous or relapsing symptoms for at least a six-month period after completion of antibiotic treatment: fatigue, widespread musculoskeletal pain, complaints of cognitive difficulties</td>
</tr>
<tr>
<td>Subjective symptoms are of such severity that, when present, they result in substantial reduction in previous levels of occupational, educational, social, or personal activities</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive culture or PCR results, using reliable methods, with evidence of current B. burgdorferi infection</td>
</tr>
<tr>
<td>An active, untreated, well-documented co-infection</td>
</tr>
<tr>
<td>The presence of objective abnormalities, found on physical examination or on neuropsychological testing, that may explain the symptoms</td>
</tr>
<tr>
<td>Fibromyalgia or chronic fatigue syndrome before the onset of LB</td>
</tr>
<tr>
<td>A prolonged history of undiagnosed or unexplained somatic complaints, for example musculoskeletal pain or fatigue, before the onset of LB</td>
</tr>
<tr>
<td>A diagnosis of an underlying disease or condition that might explain the symptoms</td>
</tr>
<tr>
<td>Laboratory or imaging abnormalities indicating an undiagnosed process distinct from post-Lyme disease syndrome</td>
</tr>
</tbody>
</table>

Adapted from Wormser et al., 2006. Stanek et al., 2011. Wormser et al., 2006.

LB, Lyme borreliosis; PCR, polymerase chain reaction; B., Borrelia.

Other terms, such as “Post-treatment chronic Lyme disease” and “Chronic Lyme disease” are often used interchangeably with PLDS in scientific literature. However, the term “chronic” should be avoided in this context. Instead, “chronic”, or preferably “late LB”, should merely be used in the sense of an untreated, late manifestation of LB, i.e. ACA, arthritis and encephalomyelitis. Patients with nonspecific subjective symptoms, diagnosed with “chronic LD”, have been classified by Feder et al. in 4 categories, il-
INTRODUCTION

Illustrating the great heterogeneity of this group of patients (Figure 8). Category 4 is defined as PLDS, and is the patient category that will henceforth be discussed.

<table>
<thead>
<tr>
<th>Category 1</th>
<th>Category 2</th>
<th>Category 3</th>
<th>Category 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms of unknown cause, with no evidence of <em>B. burgdorferi</em> infection</td>
<td>A well-defined illness unrelated to <em>B. burgdorferi</em> infection</td>
<td>Symptoms of unknown cause, with positive <em>B. burgdorferi</em> serology, without history of objective clinical findings consistent with LB</td>
<td>Post-Lyme disease syndrome</td>
</tr>
</tbody>
</table>

*Figure 8. The 4 categories of illness, included in the concept of “chronic Lyme disease”.*

*Adapted from Feder et al., 2007*

LB, Lyme borreliosis; *B., Borrelia*

**Possible causes of PLD symptoms**

The mechanisms underlying PLD symptoms (duration <6 months) and PLDS (duration >6 months) remain insufficiently understood, but are likely to be multifactorial. Based on information from numerous publications and reviews on this issue, a number of possible or hypothetical causes and mechanisms may be involved in the development of PLD symptoms and PLDS (Figure 9). These are discussed below.
Figure 9. Possible causes and mechanisms of post-Lyme disease symptoms and the post-Lyme disease syndrome.

PLDS, post-Lyme disease syndrome; HGA, Human granulocytic anaplasmosis

At present there is fairly convincing evidence that PLD symptoms are not caused by a persistent infection with *B. burgdorferi*. The studies that have found support for persistence of *B. burgdorferi* infection in different body fluids and tissues detected by unconventional methods, have been questioned by expert groups in the field of LB and have not been reproduced or confirmed by independent investigators. In 2006, the IDSA defined evidence-based arguments for PLDS not being caused by a persistent *B. burgdorferi* infection: 1) the lack of antibiotic-resistance in the *B. burgdorferi* genus; 2) the lack of correlation of persistent symptoms with laboratory evidence of inflammation or with development of objective, physical signs of LB; 3) lack of a precedent for such a phenomenon in other spirochetal infections, such as syphilis; 4) the concentration of *B. burgdorferi* antibodies usually decline or disappear despite persisting symptoms; 5) LB lacks characteristics of other infections that justify longer courses of antibiotic treatment (intracellular pathogens, infections in an immunodeficient host, infections involving a biofilm or foreign material) and finally; 6) patients with PLDS do not respond to a further course of IV antibiotics. In addition, the “cystic”
INTRODUCTION

forms of *B. burgdorferi*, which have been seen to occur in unfavourable conditions for *B. burgdorferi*,
[226] have not been shown to have any clinical relevance.76

There is limited evidence of a significant role of co-infections with for example, *A. phagocytophilum or Babesia microti* in the development of PLD symptoms and studies in humans addressing this issue are few.209,218,229

Autoimmune mechanisms have been suggested to contribute to the pathogenesis of antibiotic treatment-resistant LA (mentioned in the Introduction, “Lyme arthritis”)150 but whether such mechanisms are involved in PLDS is not clear. However, antibodies against OspA, which are associated with treatment-resistant LA, have also been found in the CSF of patients with early NB.230 A study by Klemper et al. found no association between any HLA class II allele or genotype and PLD symptoms.231 Hypothetically, damage to nervous tissue in NB, either due to direct effects of the *B. burgdorferi* spirochetes or due to the inflammatory immune response generated to the spirochetes, could cause liberation of self-antigens, which were previously unavailable to host immune surveillance, leading to activation of self-reactive immune cells and further tissue damage.232 Interestingly, anti-*B. burgdorferi* flagellin-antibodies, cross-reactive to neural mouse tissue, have been found in patients with PLDS. The importance of these findings is not known.233

Debris of dead *B. burgdorferi* spirochetes, with triggering antigens, may hypothetically maintain a low-grade inflammatory response at the site of the initial infection, with ongoing cytokine stimulation and subsequent tissue damage.234,235 Indeed, pro-inflammatory cytokines, such as Interleukin (IL)-6, tumour necrosis factor (TNF) and IL-1β are known to cause for example, fatigue, malaise, pain and neurocognitive impairment.234 However, analysis of spontaneous cytokine levels in CSF in patients with PLDS has not been performed. Additionally, an improperly regulated initial immune response to *B. burgdorferi*, with weak pro-inflammatory cytokine secretion, may lead to a persisting, local or systemic, low-grade inflammatory response due to insufficient activation of important downregulatory immune mechanisms following eradication of the *B. burgdorferi* spirochetes (further discussed in the Introduction, “T helper cell responses in Lyme borreliosis”).

Recovery from disease and recovery from illness are not always equated. Many factors, including personal characteristics, previous experiences in life and social circumstances may affect the recovery from disease and illness.236 Previous studies of various infectious diseases (brucellosis, influenza) have
INTRODUCTION

suggested that delayed convalescence with persistent subjective symptoms of various kinds may be related to the emotional state of the patient before onset of the infection.\textsuperscript{237} Furthermore, in patients with PLDS, an association between stressful life events prior to \textit{B. burgdorferi} infection, prior treatment with psychotropic medication and persistent symptoms post-treatment have been described.\textsuperscript{238} Thus, other factors, such as psychiatric co-morbidity and psychological factors may contribute to the illness of PLDS. In a study by Hasset \textit{et al}, depression, a generalized anxiety disorder and other psychological factors were shown to be overrepresented in patients with well-characterized PLDS,\textsuperscript{239} whereas a previous study by Elkins \textit{et al},\textsuperscript{240} could not find such distinguishing psychiatric disorders in PLDS patients. Thus, the cause and effect of psychiatric co-morbidity in the pathogenesis of PLDS remains unknown.

\textbf{Post-infectious symptoms}, such as chronic fatigue, musculoskeletal pain and neurocognitive difficulties have been shown to occur, besides in PLDS, after several infections caused by, for example, EBV, Parvovirus B19, Ross River virus, \textit{Coxiella burnetii} and \textit{Brucella} species.\textsuperscript{241-244} \textbf{Intercurrent illnesses}, for example, fibromyalgia, may occur during or after \textit{B. burgdorferi} infection.\textsuperscript{226} Antibiotic treatment does not resolve the symptoms.\textsuperscript{225}

Remaining objective sequelae after adequate treatment of NB mainly include persisting cranial nerve palsies (first and foremost facial nerve palsy),\textsuperscript{245,246} whereas skin atrophy and objective neurological and neurophysiological findings of peripheral nerve deficit are common findings that persist after treatment of ACA.\textsuperscript{142} The sequelae may lead to various degrees of persistent subjective discomfort, which may complicate assessment of the patient post-treatment. However, to date no specific structural lesions in the CNS have been found with MRI technique in patients with PLDS.\textsuperscript{247}

At last, it is possible that the persistent symptoms merely represent a \textit{natural, expected course} of response to the \textit{B. burgdorferi} infection. This is illustrated by the fact that a progressive decrease in symptoms is commonly seen over time.\textsuperscript{113,204} As concluded by Wormser \textit{et al}, the post-treatment symptoms tend to be more related to the aches and pains of daily living (which also occur in the general population), rather than to LB or other obvious reasons.\textsuperscript{76}
INTRODUCTION

Immunity to infections

The immune system is the body’s defence mechanism against invading pathogens, tumours and foreign substances. It consists of a variety of effector cells and molecules with different features, which need to be carefully regulated and coordinated in order to rapidly and efficiently eradicate non-self-material and at the same time restrict self-inflicted (tissue) damage due to inflammation. To protect the host effectively against non-self, the immune system must fulfil four main tasks. The first is immunological recognition of non-self, which is provided by a delicate interplay between cells of both the innate and adaptive immune system. The second task is to activate immune effector functions, such as complement, cytokines, antibodies and effector cells, which cooperate in order to destroy and eliminate the invader. The harmful effects of the powerful immune activation must be counterbalanced by immune regulation, which is the third task. Finally, to be efficient in the long run, the immune system needs to be capable of generating immunological memory to previously exposed agents.²⁴⁸

Innate recognition of pathogens

Cells of the innate immune system (monocytes/macrophages, dendritic cells [DCs], natural killer [NK] T cells, γδ T cells, granulocytes and mast cells) are the first line of defence against pathogens and constitute a natural barrier against the environment.²⁴⁸ Innate cells lack the specificity of the adaptive immunity, but can, however, discriminate between self and non-self, e.g. by means of pattern recognition receptors (PRRs), which bind conserved, repetitive pathogen-associated molecular patterns (PAMPs) of a given microbial class. The PRRs include the collectin family of proteins (e.g. mannose-binding lectin and surfactant proteins), macrophage mannose receptor, the fMet-Leu-Phe-receptor, scavenger receptors as well as NOD-like and Toll-like receptors (TLRs).²⁴⁸⁻²⁵⁰ To date, ten different TLRs have been identified in humans, which recognize different PAMPs and act in various combinations (homo- or heterodimers).²⁵¹ Peptidoglycan and bacterial lipoproteins are recognized by TLR2; double-stranded ribonucleic acid (RNA) by TLR3; LPS and heat-shock proteins by TLR4; flagellin by TLR5; and CpG motifs of bacterial DNA by TLR9. TLR1 and 6 form heterodimers with TLR2. The agonists and function of TLR10 are still unknown.²⁵² The ligation of the different PAMPs with their corresponding TLRs on antigen-presenting cells (APCs) and other innate cells activates MyD88-dependent intracellular signalling pathways, which culminate in activation of nuclear factor (NF)-κB and the induction of a variety of stereotyped responses, such as inflammation. However, in recent years it has become evident that individual TLRs can also induce MyD88-independent immune responses, which are tailored to a spe-
cific microbial infection. Thus, these receptors are involved in both innate and the forming of adaptive immune responses.\textsuperscript{253}

**Maturation of dendritic cells**

DCs are professional APCs with a unique ability to induce primary immune responses by phagocytising, processing and presenting antigens to naïve T cells. There are different subsets of DCs, the two main subsets being plasmacytoid and myeloid DCs.\textsuperscript{254} Myeloid DC originate from proliferating CD34\textsuperscript{+} hematopoietic progenitor cells in the bone marrow and from non-proliferating DC precursors in peripheral blood.\textsuperscript{255} These circulating precursors (CD14\textsuperscript{+} monocytes, CD11c\textsuperscript{+} and CD11c\textsuperscript{-} precursor DCs) enter peripheral tissues and lymphoid organs, where they differentiate into immature DCs under the influence of different stimuli, such as IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF).\textsuperscript{254-256} Immature DCs are very efficient in antigen capture, using either macropinocytosis, receptor-mediated endocytosis via lectin receptors or Fc-receptors, or by engulfment of apoptotic bodies.\textsuperscript{255} Following antigen uptake, immature DCs undergo a developmental program called DC maturation, which includes up-regulation of major histocompatibility complex (MHC) class I and II and co-stimulatory molecules (CD80/86 and CD40) as well as CD1a and CD83 on their surface.\textsuperscript{257,258} Following antigen processing, DCs migrate to lymphoid organs, where they can prime naïve T cells, which need to recognize both the antigen bound to MHC molecules and co-stimulatory molecules on DCs in order to respond and to be able to establish immunological memory. The DC-T cell interaction activates the adaptive immune system.\textsuperscript{251} IL-12p70 is the main cytokine produced by mature DCs. Characteristics of immature and mature DCs are shown in Figure 10. Due to the heterogeneity of different subsets of DCs, there is no single cell-surface antigen that identifies all DCs.\textsuperscript{259}
Figure 10. Characteristics of immature and mature dendritic cells (DCs). Different stimuli can initiate and inhibit DC maturation. Adapted from Banchereau et al., 2000. CCR, chemokine receptor; CD, cluster of differentiation; MHC, major histocompatibility complex; IL, interleukin, LPS, lipopolysaccharide; TNF, tumour necrosis factor; GM-CSF, granulocyte macrophage-colony stimulating factor; L, ligand.

Adaptive immunity

Adaptive immunity is mediated by T and B cells, who contribute to the defence against non-self by antigen-specific effector cells, antigen-specific antibodies and immunological memory. The activation and polarisation of naive CD4+ T helper (Th) cells is conveyed through a tight cross-talk with pathogen-primed DCs and requires three stimulatory signals: 1) ligation of the T cell receptor with peptides presented on MHC class II molecules on the surface of DCs; 2) interaction between the co-stimulatory molecules CD80/86 and CD28 (on T cells), which allows Th cells to develop into cytokine-secreting effector cells, and finally; 3) the polarising signal, including ligation of CD40 by CD40 ligand (L) and the secretion of Th cell-polarising molecules (e.g. IL-12p70 [Th1] and the chemokine ligand (CCL)2 [Th2]) by DCs. The Th1-cell polarising factors include in particular IL-12p70 and interferon-γ (IFN-γ), whereas IL-4 is particularly considered a Th2-polarising factor. On the other hand, peptides (intracellular bacterial and viral) presented on MHC class I molecules on DCs polarize T cells to develop into CD8+ cytotoxic T cells. B cells also act as APCs, by presenting antigens on MHC class II molecules. Interaction of CD40 on antigen-primed B cells with CD40L on polarized T cells promotes B cell maturation, proliferation and differentiation into antibody-producing plasma cells.
T helper cell subsets

CD4+ T cells are generally divided into Th1, Th2, Th17, follicular helper T cells (Thfs) and regulatory T cells (Treg). In short, Th1 cells produce IFN-γ, TNF and TNF-β (lymphotoxin-α). They stimulate innate and T cell immune responses and promote cell-mediated immunity by cellular cytolytic activity. Th1 cells stimulate macrophages, CD8+ T cells and NK cells and are important in immune responses to intracellular pathogens. However, they also induce synthesis of opsonising and complement activating antibodies, in particular IgG1 and IgG3 antibodies by B cells. Uncontrolled, excessive Th1 responses may cause tissue damage, chronic inflammatory diseases and autoimmunity. Th2 cells are characterized by production of IL-4, IL-5, IL-9 and IL-13. Th2 cells are involved in humoral responses affecting mast cells and eosinophil granulocytes and constitute our protection against extracellular pathogens (in particular parasites). Th2 cells stimulate B cells to synthesize IgG4 and IgE. Aberrant Th2 reactions are involved in allergy. Th17 have a role in the defence against some extracellular bacteria and fungi and may play an important role in emergence of autoimmunity. The main cytokines secreted by Th17 cells are IL-17, IL-21 and IL-22. Thfs, although not fully established yet as a unique lineage, express high levels of CXCR5, IL-10 and IL-21 and are involved in long-lived, protective antibody responses that are generated in germinal centres in various lymphoid organs. Treg cells, on the other hand, are a group of heterogeneous, immunosuppressive cells, which maintain self-tolerance and immune homeostasis. They have several ways of acting and mainly produce IL-10 and transforming growth factor (TGF)-β.

Cytokines

Cytokines are small proteins produced by various cell types in response to activating stimuli and are capable of inducing immune responses through ligation with their specific receptors. They exert their effects in autocrine, paracrine or endocrine manners and are involved in growth, differentiation, activation and inhibition of immune responses. Their effect is, however, highly dependent on the cellular source, target and its responsiveness to the stimulus and not least, the specific phase of the immune response during which they are produced. Several cytokines have pleiotropic effects, whereof certain have both pro-inflammatory and anti-inflammatory potential (i.e. pleiotropic effects), such as IL-10 and TGF-β, although these cytokines in most circumstances are regarded as anti-inflammatory. Chemokines are a family of chemoattractant proteins, which induce chemotaxis (cell trafficking). Members of this family are mainly divided into two groups, depending on their chemical structure: CC and CXC chemokines (ligands), which act on different sets of receptors (CC receptors [CCR] and CXC receptors [CXCR]). The studied cytokines and chemokines in Paper I-
INTRODUCTION

IV are described in detail in Table 6. The references in Table 6 are as follows: Murphy et al., 2008,248 Borish and Steinke, 2003,263 Commins et al., 2010,265 Borish, 1998,264 Levy, 2009266 and Trinchieri, 2003.267
## INTRODUCTION

Table 6. Characteristics of the cytokines and chemokines included in the thesis.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Main producer cells</th>
<th>Actions</th>
<th>Paper</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>Macrophages, T cells</td>
<td>Stimulates growth and differentiation of DC, neutrophils and macrophages</td>
<td>III</td>
<td>Murphy et al., 2008; Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Th1, Tc and NK cells, macrophages</td>
<td>Stimulates macrophage activation and killing by NK cells and neutrophils, inhibits IL-4-mediated effects</td>
<td>I-III</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Macrophages, epithelial cells</td>
<td>Activates T cells and macrophages, pyrexia</td>
<td>II-III</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IL-2</td>
<td>Th2 cells, mast cells</td>
<td>Stimulates T cell proliferation</td>
<td>III</td>
<td>Murphy et al., 2008; Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 cells, mast cells</td>
<td>Activates B cells, IgE switch, stimulates Th2 and suppresses Th1 differentiation</td>
<td>I-III</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IL-5</td>
<td>Th2 cells, mast cells</td>
<td>Promotes eosinophil growth and differentiation</td>
<td>III</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IL-6</td>
<td>Macrophages, T cells, endothelial cells</td>
<td>Stimulates acute phase protein production, pyrexia, B and T cell growth and differentiation, inhibits IL-1 and TNF synthesis</td>
<td>I-III</td>
<td>Borish, 1998; Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IL-10</td>
<td>Monocytes, Th1, Th2, Treg and Tc cells, B cells, mast cells, phagocytic cells</td>
<td>Inhibits Th1 and Th2 cells, macrophages, NK cells, stimulates B cell proliferation and differentiation</td>
<td>I-III</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>IL-12</td>
<td>DC, macrophages, neutrophils</td>
<td>Proliferation of NK cells, B cells, NKT cells and Th1 and Tc cells, promotes Th1 differentiation</td>
<td>I-III</td>
<td>Trinchieri, 2003</td>
</tr>
<tr>
<td>TNF</td>
<td>Macrophages, neutrophils, NK cells, T cells</td>
<td>Promotes inflammation, endothelial activation</td>
<td>II-III</td>
<td>Murphy et al., 2008; Commins et al., 2010</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Chondrocytes, monocytes, fibroblasts, platelets, T cells</td>
<td>Inhibits activation and proliferation of B and T cells, phagocytic cells and NK cells, chemotactic for macrophages, stimulates IgA switch in B cells</td>
<td>III</td>
<td>Borish and Steinke, 2003</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Producer cells</th>
<th>Actions</th>
<th>Paper</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 (CCL8)</td>
<td>Phagocytic cells, endothelial cells, T cells, eosinophils, neutrophils, hepatocytes, keratinocytes</td>
<td>Chemotactic for neutrophils, stimulates neutrophil degranulation, the respiratory burst and adherence to endothelial cells, promotes T cell differentiation</td>
<td>II-III</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>MCP-1 (CCL2)</td>
<td>Monocytes, macrophages, DC</td>
<td>Stimulates chemotaxis of monocytes, T cells, DC, NK cells, inhibits IL-12 production in APC and promotes Th2 differentiation</td>
<td>I</td>
<td>Borish and Steinke, 2003; Murphy et al., 2008</td>
</tr>
<tr>
<td>MIP-1α (CCL3)</td>
<td>Macrophages</td>
<td>Stimulates chemotaxis of T cells, monocytes, DC, NK cells, eosinophils, B cells and promotes Th1 differentiation and stimulates IL-12 production in APC</td>
<td>I</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>MIP-1β (CCL4)</td>
<td>Macrophages</td>
<td>Stimulates chemotaxis of T cells, monocytes, DC, NK cells, eosinophils, B cells and promotes Th1 differentiation and stimulates IL-12 production in APC</td>
<td>I</td>
<td>Borish and Steinke, 2003</td>
</tr>
<tr>
<td>RANTES (CCL5)</td>
<td>Macrophages, NK cells, epithelial cells, eosinophils, endothelial cells, keratinocytes, T cells</td>
<td>Chemotactic for macrophages, NK cells, eosinophils, basophils, DC and promotes Th1 differentiation</td>
<td>I</td>
<td>Levy, 2009; Murphy et al., 2008</td>
</tr>
<tr>
<td>Eotaxin (CCL11)</td>
<td>Endothelial cells, epithelial cells, fibroblasts, NK cells, macrophages</td>
<td>Eosinophil and basophil chemotactic, promotes Th2 differentiation</td>
<td>I</td>
<td>Borish and Steinke, 2003; Murphy et al., 2008</td>
</tr>
</tbody>
</table>
Immune responses in Lyme borreliosis

*B. burgdorferi* is a complex, highly adaptable extracellular pathogen, which expresses immunogenic and pro-inflammatory lipoproteins on its surface. The multifaceted clinical features of LB are mainly caused by a vigorous inflammatory host response in the infected tissues, rather than by direct tissue damage from the spirochete itself. Both innate and adaptive immune responses are involved in the defence against the pathogen (Figure 11).

![Diagram](image-url)

**Figure 11.** An illustration of the immune response elicited to *B. burgdorferi* after inoculation of the spirochetes in the skin during tick feeding. Green arrows indicate defence, red arrows inhibition and blue arrows synthesis of immune mediators. Immunological details are provided in different parts of the Introduction. IL, interleukin; CXCL, chemokine ligand; TNF, tumour necrosis factor; DC, dendritic cell; IFN, interferon; APC, antigen presenting cell.
INTRODUCTION

**Activation of innate cells by B. burgdorferi**

The skin constitutes the first barrier to *B. burgdorferi* infection. LB begins with dermal inoculation of spirochetes into the host during tick feeding. To overcome the host’s innate defence mechanisms, the spirochetes take advantage of immunomodulatory substances present in the tick saliva, which are injected into the skin during tick feeding. These pharmacologically active substances have been shown, for example, to impair homeostasis, host immunity and wound healing and facilitate the transmission of pathogens to the host. Early during infection, a powerful inflammatory response to the invading spirochetes and their immunogenic lipoproteins is established via activation of innate cells, for example, DCs, macrophages and neutrophils. DCs have been shown to rapidly engulf live *B. burgdorferi* spirochetes mainly using coiling phagocytosis, with localisation of spirochetal fragments in the cytosol and inside phagolysosomes, and upon activation they secrete pro-inflammatory cytokines, such as IL-12p70 and IL-8. *B. burgdorferi* antigens are presented on MHC class II on DCs and effectively activate T cells. Neutrophils are activated by *B. burgdorferi* spirochetes and lipoproteins to produce an oxidative burst (release of reactive oxygen species), and the spirochetes are phagocytised, for example, by tube phagocytosis in a complement-dependent manner. Macrophages are likewise highly effective in phagocytising *B. burgdorferi* spirochetes using tube phagocytosis and spirochetal lipoproteins stimulate macrophages to secrete pro-inflammatory cytokines, for example, IL-1β, IL-6, TNF and IL-12. However, anti-inflammatory cytokine responses to *B. burgdorferi* have also been observed. Spirochetes and their lipoproteins have been shown to elicit IL-10 secretion in murine and human mononuclear cells. Apoptosis has also been shown to occur in human monocytes following lipoprotein stimulation or phagocytosis of *B. burgdorferi* spirochetes, illustrating possible means of limiting the inflammatory response. The innate recognition of *B. burgdorferi* lipoproteins and the subsequent inflammatory response is mainly mediated by CD14 and TLR2, in heterodimer combinations with either TLR1 (TLR1/2 binds triacylated lipoproteins) or TLR6 (TLR2/6 bind diacylated lipoproteins). The TLR-mediated immune activation has been shown to be especially important for *B. burgdorferi* killing, but not for overall spirochete-induced inflammatory responses, since a deficiency in the TLR signalling pathway have been shown to result in increased spirochete burden, without impact on the inflammatory response.

**Adaptive responses to *B. burgdorferi***

The adaptive immune response, which comprises antibodies and effector cells, has the mandate to control the *B. burgdorferi* infection and give rise to immunological memory.
INTRODUCTION

Antibodies
The activation of B cells and the production of antibodies during B. burgdorferi infection are important for reducing the spirochete burden, for disease modulation and for providing protective immunity.\(^{284}\) Polyclonal activation of B cells by B. burgdorferi lipoproteins is mediated by TLR2. T cell-independent antibody production in marginal zone B cells (natural IgM prior to isotype switch), early during infection, has been experimentally shown to be sufficient to prevent B. burgdorferi infection and to reduce pathogen burden.\(^{285}\) Following activation by effector T cells, B cells start producing B. burgdorferi-specific IgM and IgG antibodies.\(^{18}\) The antibodies are directed against an increasingly diverse array of antigens as the infection progresses, which illustrates the large antigenic repertoire of differentially expressed surface antigens during the course of infection.\(^{286}\) The earliest antibody responses develop to flagellin (FlaB) and p66, followed by OspC, VlsE, decorin-binding protein A (DbpA) and additional antigens,\(^{286}\) whereas antibodies against OspA and OspB develop later during the course of infection.\(^{18}\) Further aspects of LB serology are discussed in the Introduction, in “Serological assays”.

T helper cell responses in Lyme borreliosis
The immune response to B. burgdorferi is mainly characterised by a pro-inflammatory and Th1-type response, as illustrated by several human studies (discussed below). The early immune response to B. burgdorferi in patients with EM is dominated by such a pro-inflammatory, IFN-\(\gamma\) response.\(^{287}\)\(^{289}\) B. burgdorferi-specific IFN-\(\gamma\) secreting cells have also been demonstrated in peripheral blood in patients with various clinical features of LB,\(^{290}\) both in peripheral blood and CSF in patients with neurological manifestations of the infection,\(^{290,291,292}\) and in synovial fluid in patients with LA,\(^{293,294}\) whereas B. burgdorferi-specific IL-4 responses overall are insignificant. Oksi et al. showed similar results with increased B. burgdorferi-induced IFN-\(\gamma\) production and decreased spontaneous IL-4 secretion in peripheral blood mononuclear cells (PBMC) in patients with musculoskeletal and neurologic manifestations of LB.\(^{295}\) Interestingly, in a recent study by Nordberg et al., the Th17-like cytokine IL-17 was shown to be increased in CSF in patients with early NB, suggesting a B. burgdorferi-elicited, local Th17-response in the CNS.\(^{296}\) The importance of Th17-responses in NB need, however, to be further investigated.

B. burgdorferi-induced cytokine patterns and the type of immune response (Th1/Th2) generated in response to the spirochetes have also been shown to correlate with the outcome of LB. An initial pro-inflammatory response to B. burgdorferi both in peripheral blood and CSF,\(^{297}\) followed by downregulat-
INTRODUCTION

ing, anti-inflammatory cytokine responses, seems to be associated with successful resolution of the infection in humans. Lack of such anti-inflammatory responses has been indicated in patients with persisting symptoms after treatment of NB and in patients with ACA.\textsuperscript{297-299} However, asymptomatic \textit{B. burgdorferi}-seropositive patients, in whom the spirochetes are assumed to be successfully eradicated without development of clinical signs and symptoms of LB, display similar pro- and anti-inflammatory cytokine responses to \textit{B. burgdorferi} as patients with objective manifestations of LB.\textsuperscript{300} Correlations between early cytokine patterns in \textit{B. burgdorferi}-infected (EM) skin and the clinical outcome post-treatment of EM have not previously been studied.

The importance of a correct Th1/Th2 balance during the disease course has also been studied in animal models of LB, which have been instrumental in the interpretations of Th1/Th2 data in humans. Keane-Myers and Nickell\textsuperscript{301} showed that LB-susceptible (C3H) mice responded to \textit{B. burgdorferi} (analysed five weeks post-infection) with higher levels of IL-2 and IFN-\(\gamma\) and lower levels of IL-4. Moreover, they developed more severe arthritis and had a higher spirochete load compared to LB-resistant, mainly IL-4 responding (BALB/c) mice. The importance of IL-4 for the clinical outcome was illustrated by the fact that its neutralization led to increased joint swelling and higher spirochete load in both mouse strains, whereas neutralization of IFN-\(\gamma\) reduced both joint swelling and the spirochete burden.\textsuperscript{301} However, the authors did not analyse cytokine responses during the course of infection. This was done later by another group,\textsuperscript{302} who showed that BALB/c mice responded to \textit{B. burgdorferi} infection with an early IFN-\(\gamma\) response, followed by a subsequent up-regulation of IL-4 secretion. These mice had a complete resolution of arthritis as compared to C3H mice, which had a lower initial IFN-\(\gamma\) response, lacked a subsequent IL-4 response, and developed persistent arthritis. Furthermore, depletion of IL-12p70 resulted in attenuation of arthritis in C3H mice, but at the expense of an increased spirochete burden, suggesting an important role of IFN-\(\gamma\) in the eradication of \textit{B. burgdorferi}.\textsuperscript{302,303} Experimental Th2-deviation (mercury and progesterone) of \textit{B. burgdorferi}-infected mice resulted, likewise, in milder symptoms and signs of arthritis,\textsuperscript{304} but delayed eradication of the spirochetes.\textsuperscript{305} In addition, the anti-inflammatory cytokine IL-10 has been shown to be co-regulated with IFN-\(\gamma\) in LB-resistant mouse strains and consequently to contribute to the resolution of experimental LA.\textsuperscript{306}

Thus, a strong, initial pro-inflammatory response to \textit{B. burgdorferi} seems to be important for eradication of the spirochetes and is partly responsible for the development of symptoms and signs of infection. However, the pro-inflammatory response needs to be followed by downregulatory, anti-
INTRODUCTION

inflammatory cytokine responses at a proper moment, in order to tune down the inflammation and minimize subsequent tissue damage. This down-regulation of inflammation, by secretion of, for example, IL-4, IL-10 and TGF-β, appears as a feedback mechanism provided that the initial immune response is strong enough. This reasoning, initially based on studies in mice, has formed the basis for the working hypothesis of Paper I and II in this thesis.

Immunomodulating effects of antibiotics

In addition to their antibacterial effects, many antimicrobials affect the immune system. In a review by van Vlem et al., the main effects of antibiotics (in vitro, ex vivo and in vivo) on immune responses were divided into immuno-enhancing (imipenem, cefodizime and clindamycin) and immuno-depressing (erythromycin, roxithromycin, cefotaxime, tetracycline, rifampicin, gentamicin, telcoplanin and ampicillin), depending on their effect on, for example, phagocytosis, chemotaxis, lymphocyte proliferation, as well as on cytokine and antibody production. However, several antibiotics (e.g. most β-lactams) have no immunomodulating impact (neutral effects) at all. Since immunomodulating effects of doxycycline were studied in Paper III, a review of its impact on the immune system will follow below.

Tetracyclines

One of the most well studied groups of antimicrobials, from an immunological point of view, is that of the tetracyclines. They were discovered in 1948 as natural fermentation products of a soil bacterium, Streptomyces aureofaciens. They are a family of broad-spectrum antibiotics, of which the two most commonly used are doxycycline and minocycline. All tetracyclines are bacteriostatic and exert their antimicrobial effect by binding to the bacterial ribosome, and thereby inhibiting protein synthesis. Various in vitro effects of tetracyclines in general, and doxycycline in particular, have been described, which are presented in Figure 12.

The immunomodulating effects of tetracyclines have long been used clinically in the treatment of, for example, inflammatory diseases, such as rheumatoid arthritis and rosacea. Through their anti-apoptotic activity, tetracyclines have also been shown in animal models to operate in a neuroprotective manner in, for example, traumatic brain injuries and Parkinson’s and Huntington’s disease, by inhibiting caspase activity, inducible nitric oxide (NO) synthase and the pro-inflammatory cytokines TNF and IL-1β, and in addition, reducing activation and proliferation of microglia. Tetracyclines have also been evaluated in clinical trials of multiple sclerosis (MS).
INTRODUCTION

cutaneous sarcoidosis,\textsuperscript{315} abdominal aortic aneurysm,\textsuperscript{316} and in patients with acute ischemic stroke\textsuperscript{317} and myocardial infarction.\textsuperscript{318} However, the dosage and duration of treatment vary in the studies. Interestingly, in an experimental simian model of HIV CNS disease, minocycline reduced the severity of encephalitis, suppressed viral load in the brain and decreased the expression of CNS inflammatory markers.\textsuperscript{319} Doxycycline and minocycline have also been experimentally shown, in a dose-dependent manner, to reduce the production of TNF, IL-6 and IL-8 in human monocyctic cells lines and rhesus monkey brain astrocytes infected with live and sonicated \textit{B. burgdorferi} spirochetes,\textsuperscript{320} suggesting a dual therapeutic effect of tetracyclines in LB.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure12.png}
\caption{The main immunomodulating effects of tetracyclines \textit{in vitro}. Figure based on the following reviews.\textsuperscript{309,321,322} (+) indicates stimulation, (-) inhibition.}
\end{figure}

MMP, matrix metalloproteinase; NO, nitric oxide; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor
INITIATION OF THE STUDY

In 1999, after finishing the sixth semester at medical school at the Faculty of Health Sciences in Linköping, I wanted to try something new and challenging! Research was an unfamiliar topic to me, but seemed interesting. My husband (and study colleague) had heard about the Biomedical Research School in Linköping, a one-year research course resulting in a Master’s degree in medicine, and he persuaded me to complete the application form. Fortunately, I was accepted! At the beginning of that year, I got to know Christina Ekerfelt, Pia Forsberg and Jan Ernerudh, three enthusiastic and skilful researchers, who introduced me to the “Borrelia research group”. Already during the preclinical part of medical school, I became fascinated by the delicate and well regulated interplay between host immune cells and invading pathogens. Thus, in my Master’s thesis, I studied the innate interaction between *B. burgdorferi* spirochetes and DCs from patients with different clinical outcomes of LB. I also had a fantastic opportunity to visit Marie Larson and co-workers at Ralph Steinman’s lab at the Rockefeller University, Manhattan, New York, during which I, among other things, learned the method of DC culture.

After taking my medical school examination, I registered as a PhD-student and continued full-time with my research project, which came to deal with immune responses to *B. burgdorferi* in relation to clinical outcome and a clinical and immunological understanding of the PLDS. During these two years, I learned the basics of Good Laboratory Practice and devoted myself to feed cells, developing new methods, the ELISPOT- and other cytokine assays, applications and planning of new research projects and establishing co-operation with other skilful research groups. I have managed this knowledge and experience with care and it has been of great benefit to me in my work in the medical profession! After a two-year internship and completion of the half-time seminar (and two years of parental leave), I was employed as a resident physician at the Clinic of Infectious Diseases in Linköping, during which time I completed the clinical parts of my research project. During my PhD-studies, the research group has become another family to me and it has expanded to include many talented researchers engaged in interesting projects, which I hope to be involved in later on.

"Only action brings ideas to life"

Dan Millman
AIMS OF THE STUDY

The work of this thesis aimed to explore the type of early immune response generated to *B. burgdorferi* and its importance for the clinical outcome of LB in adult patients. Another aim was to increase knowledge about patients with persistent symptoms after treatment of NB from clinical, immunological and diagnostic perspectives. The specific aim of each paper was to:

I. Determine whether early cytokine expression in the skin and in peripheral blood in patients with EM is associated with the clinical outcome of *B. burgdorferi* infection

II. Explore whether differences in innate immune responses, generated by DCs and whole blood cells infected with live *B. garinii* spirochetes, might contribute to the clinical outcome of LB

III. Examine whether: 1) persistent symptoms and quality of life after treatment of NB improve with doxycycline treatment; 2) doxycycline has an influence on systemic cytokine responses and; 3) improvement of persistent symptoms could be due to immunomodulating effects of doxycycline

IV. Evaluate, using brain MRIs, the presence of specific lesions in the nervous system in patients with persistent symptoms after treatment of NB and to correlate MRI findings with the duration of symptoms
MATERIALS AND METHODS

Subjects (Paper I–IV)
Altogether, 148 subjects were included in the thesis (125 patients and 23 controls).

Patients (Paper I–IV)
In total, 125 adult patients (67 females and 58 males) were included in the four papers, of which some were included in more than one paper (Figure 13). The patients were divided into different groups according to clinical outcome (as defined in “Definition of clinical outcome”): subacute NB (n=7, Paper II), patients with persistent symptoms >3<6 months after treatment of NB (n=2, Paper IV), patients with persistent symptoms >6 months after treatment of NB (n=35, Paper II–IV), ACA (n=1, Paper II), and B. burgdorferi-seropositive, asymptomatic subjects (n=7, Paper II). In addition, 88 patients with EM participated in Paper I (Table 7).

Table 7. The diagnosis of the patients included in Paper I-IV.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Paper I Follow-up study of EM (n=88)</th>
<th>Paper II Immune responses in LB (n=21)</th>
<th>Paper III Cross-over study with DOX (n=15)</th>
<th>Paper IV Brain MRI findings in post-treatment NB (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subacute NB</td>
<td>-</td>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NB persistent symptoms &gt;3&lt;6 mos</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>NB persistent symptoms &gt;6 mos</td>
<td>-</td>
<td>6</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>ACA</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
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<td>EM</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Controls</td>
<td>-</td>
<td>7</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>28</td>
<td>15</td>
<td>32</td>
</tr>
</tbody>
</table>

EM, erythema migrans; LB, Lyme borreliosis; DOX, doxycycline; MRI, magnetic resonance imaging; NB, neuroborreliosis; ACA, acrodermatitis chronica atrophicans.
MATERIALS AND METHODS

Three patients participated in three of the studies (Paper II–IV). Additionally, six patients were included both in Paper II and IV and three in Paper III and IV (Figure 13). Patients in Papers II, III and IV were recruited from Östergötland and those in Paper I from the Åland Islands, Finland. Patients in Papers II, III and IV had a history of LB with different disease outcomes after treatment and had previously been well examined and diagnosed by a physician at the Clinic of Infectious Diseases in Linköping. Patients in Paper I were examined either by the co-authors (MN and DN) or by physicians at the Åland Central Hospital who were very experienced in diagnosing LB. In addition, asymptomatic subjects with presence of *B. burgdorferi*-specific IgG antibodies in serum (found by screening of blood donors), without a history of clinical signs of or receiving any previous treatment for LB were included in Paper II. All patients with objective manifestations of LB (i.e. not asymptomatic) were treated with at least one course of antibiotics according to local treatment recommendations, either prior to (Paper II–IV) or at inclusion (Paper I) in the studies.

![Diagram](image_url)

**Figure 13.** Subjects included in the thesis (n=148).

Certain clarifications are needed concerning the patients included in paper IV. All included patients had a history of well-characterized NB, according to European definition criteria, and persistent symptoms of various character and dignity >3 (n=2) or >6 (n=14) months after treatment of NB (Table 8). Most of the patients had current symptoms at the time of the MRI-
examination. They were referred to in Paper IV as “chronic NB” in accordance with the former nomenclature (clarified under “Definition of clinical outcome”). They had all received at least one course of recommended antibiotic treatment of NB at time of diagnosis and at the routine clinical follow-up (Table 8). A re-lumbar puncture at least once post-treatment showed no signs of treatment failure (all patients lacked pleocytosis post-treatment). In one of the patients (no. 2), lumbar puncture was performed >1 year after disease onset and this patient thus lacked CSF pleocytosis, but had evidence of a positive intrathecal anti-B. burgdorferi IgG antibody index (Table 8). Patient no. 14 lacked both pleocytosis and intrathecal B. burgdorferi antibodies. This patient had a history of ACA with subsequent development of neurological symptoms suspected to be due to NB (i.e. radiculitis, headache and vertigo). The neurological symptoms had been present >18 months (lumbar puncture failed twice) and the patient had received two courses of doxycycline treatment at the time of lumbar puncture. The CSF-analysis revealed a BBB damage with an elevated albumin ratio, an increased CSF total IgM index and the presence of oligoclonal bands, indicating an inflammatory process within the CNS with intrathecal antibody production.223

Table 8. Clinical characteristics of the patients included in Paper IV.

<table>
<thead>
<tr>
<th>No</th>
<th>Age (years)</th>
<th>Sex</th>
<th>CSF B. IgG antibody index</th>
<th>CSF B. IgM antibody index</th>
<th>Presence of CSF mononuclear pleocytosis#</th>
<th>Symptom duration (years) at MRI</th>
<th>Antibiotic treatment at diagnosis and follow-up</th>
<th>Re-lumbar puncture post-treatment</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>54</td>
<td>f</td>
<td>pos</td>
<td>neg</td>
<td>yes</td>
<td>5.9</td>
<td>DOX, CTRX</td>
<td>No pleocytosis, B. IgG, O-bands</td>
</tr>
<tr>
<td>2</td>
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<td>neg</td>
<td>no</td>
<td>4.7</td>
<td>DOX, CTRX</td>
<td>No pleocytosis, B. IgG, O-bands</td>
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<td>pos</td>
<td>neg</td>
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</tr>
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<td>6</td>
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<td>pos</td>
<td>neg</td>
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<tr>
<td>8</td>
<td>83</td>
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<tr>
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<td>No pleocytosis, B. IgG, O-bands</td>
</tr>
<tr>
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<td>pos</td>
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<td>DOX</td>
<td>No pleocytosis, B. IgG</td>
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<tr>
<td>12</td>
<td>70</td>
<td>m</td>
<td>pos</td>
<td>neg</td>
<td>yes</td>
<td>0.9</td>
<td>DOX</td>
<td>No pleocytosis, B. IgG</td>
</tr>
<tr>
<td>13</td>
<td>69</td>
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<td>DOX, CTRX</td>
<td>No pleocytosis, B. IgG</td>
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<tr>
<td>14</td>
<td>77</td>
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<td>neg</td>
<td>neg</td>
<td>no</td>
<td>1.6</td>
<td>DOX</td>
<td>No pleocytosis, O-bands, elevated albumin ratio, pos total IgM index</td>
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<tr>
<td>15</td>
<td>47</td>
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<td>pos</td>
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<td>pos</td>
<td>yes</td>
<td>0.3</td>
<td>DOX</td>
<td>No pleocytosis, B. IgG</td>
</tr>
</tbody>
</table>

#Antibody index: (OD-CSF/OD-serum) x (OD-CSF − OD-serum), according to Hansen and Lebech, 1991.125

#Mononuclear pleocytosis was defined as ≥5 x 10⁶ mononuclear cells/L in the CSF.

f, female; m, male; MRI, magnetic resonance imaging; pos, positive; neg, negative; DOX, doxycycline; CTRX, ceftriaxone; B., Borrelia; Ig, immunoglobulin; O-bands, oligoclonal bands; CSF, cerebrospinal fluid; OD, optical density
MATERIALS AND METHODS

Controls (Paper II, IV)
Altogether, 23 controls (11 females, 12 males) were included in the thesis. In Paper II, the controls consisted of seven individuals, who lacked *B. burgdorferi* antibodies in serum by serologic analysis prior to study inclusion. They were all staff at the University Hospital, Linköping. In Paper IV, the controls consisted of 16 healthy individuals, who were matched for gender and age (approximately within 10 years) and who were recruited from orthopaedic outpatients and staff at the Clinic of Radiology, University Hospital, Linköping. No history of LB or other neurological diseases was reported by the controls and none of them had received treatment for LB.

Excluded subjects (Paper I, IV)
In all, 25 subjects were excluded from the Papers; 21 patients from Paper I and 1 patient and three controls from Paper IV. In Paper I, 18 patients were excluded due to missing data from the 6-month follow-up and three due to presence of symptoms >3 weeks before inclusion in the study. In Paper IV, one patient and the matched control were excluded after MRI examination, since the patient’s symptoms were caused by another diagnosis than NB. This matched pair was not replaced. In addition, one control in Paper IV was excluded due to claustrophobia (MRI examination was initiated, but discontinued) and another one due to an incidental finding of a meningioma in the MRI examination. These two controls were replaced by other matched, healthy volunteers.

Diagnostic criteria (Paper I–IV)
The diagnosis of the included patients is presented in Table 7. Diagnostic criteria of EM were based on clinical findings of a circular, expandible skin rash, >5 cm in diameter. A preceding tick-bite and EM-associated symptoms, such as fever, myalgia, arthralgia and fatigue, supported the diagnosis. ACA was diagnosed on the basis of a typical clinical appearance, the presence of *B. burgdorferi*-specific IgG antibodies in serum and a characteristic histopathological picture. The criteria for definite NB were the presence of symptoms associated with NB, presence of *B. burgdorferi*-specific IgM and/or IgG antibodies and a mononuclear pleocytosis in the CSF, according to European guidelines. Mononuclear pleocytosis was defined as the presence of \( \geq 5 \times 10^6 \) mononuclear cells/L in the CSF. However, pleocytosis was not present in all patients, on whom CSF analysis was performed after long symptom duration or after initiation of antibiotic treatment (Paper II–IV). In Papers II and III, all patients with NB had *B. burgdorferi*-specific antibodies in CSF. However, one patient included in Papers II (with ACA diagnosis) and IV (no. 14, Table 8) lacked *B. burgdorferi*-specific antibodies in CSF.
MATERIALS AND METHODS

Definition of clinical outcome (Paper II–IV)
The asymptomatic patients in Paper II had the following characteristics: presence of B. burgdorferi-specific IgG antibodies in serum (found by screening of healthy blood donors), no history of or treatment for LB, and an IFN-γ response detected with the ELISPOT assay, as previously described. The clinical outcome of NB was considered in relation to the duration of symptoms post-treatment, and accordingly classified as: subacute, if the symptom duration was <6 months post-treatment and the symptoms had disappeared at study inclusion (Paper II), or chronic, if symptoms persisted >3 months (Paper IV) or preferably >6 months (Paper II–IV) post-treatment, and were present at study inclusion. All patients with persistent symptoms after treatment of NB had been re-examined by a physician at the Clinic of Infectious Diseases in Linköping, according to the clinical routine, and a lumbar puncture had been performed without showing any residual pleocytosis. The term “chronic” was previously used in the scientific literature to describe patients with various sequelae after treatment of LB and was thus easily confused with an untreated, late B. burgdorferi infection. The definition was therefore re-evaluated in 2007, in order to clearly distinguish post-treatment symptoms and disabilities from a late (chronic), untreated B. burgdorferi infection. The term was renamed “NB with persistent symptoms post-treatment” and was defined as symptoms persisting >6 months after adequate antibiotic treatment of NB. The time limit of six months was chosen in order to have a margin for resolution of symptoms in patients with a subacute disease course and to obtain a clinically well characterized group of patients. In paper III, all patients had a symptom duration exceeding six months.

Methods (Paper I–IV)
I have had the privilege to learn several research methods while working with this thesis. Thus, I have been responsible for or involved in most of the methods, with the exception of the antibody assays in serum and CSF, the cytometric bead array (CBA), the PCR and the brain MRIs.

Antibody assays in serum (Paper I–IV)
A commercial ELISA-kit (Dako, Denmark) was used for routine analysis of B. burgdorferi-specific IgM and IgG antibodies against the B. burgdorferi flagellum antigen in serum (Paper II–IV). Additional antibody-analyses included measurements of B. burgdorferi-specific IgM and IgG antibodies against a mixture of the recombinant B. burgdorferi 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, garinii, B. burgdorferi s.s.) (RecomWell ELISA, Microgen, Germany), followed by Western blot (RecomBlot,
MATERIALS AND METHODS

Mikrogen, Germany) (Paper I). IgM and IgG antibodies against a synthetic *B. burgdorferi* C6-peptide were measured using a QuickELISA C6 kit (Immunetics, USA) (Paper I).

**CSF analyses (Paper II–IV)**
In patients with NB, pleocytosis was defined as a total leukocyte count ≥5 x 10⁶ cells/L in the CSF, of which a majority consisted of mononuclear cells. The presence of *B. burgdorferi*-specific IgM and IgG antibodies in the CSF was analysed with the flagellum-ELISA kit (Dako, Denmark). A CSF antibody index >0.3 was required for intrathecal *B. burgdorferi*-antibody synthesis and for the diagnosis of NB. Additional routine analyses of CSF included (not consistently analysed in all patients): albumin ratio, lactate, total IgM and IgG antibody synthesis index and the presence of oligoclonal bands.

**Preparation of cells (Paper II–III)**
Heparinized peripheral blood was used for preparation of the different cell types included in Papers II and III.

**Peripheral blood mononuclear cells**
Heparinized peripheral blood from the patients was separated by density gradient centrifugation on Lymphoprep®, as previously described. The mononuclear cells were removed from the buffy coat layer, washed twice and re-suspended in culture medium before being counted in a Bürker-chamber using a phase contrast microscope.

**Culture and stimulation of dendritic cells**
The method for culture of DCs was originally developed by Bender *et al.*, and modified in co-operation with Marie Larsson at the Rockefeller University, NY, USA. The mononuclear cells were re-suspended in 5% pooled, heat-inactivated human serum, and cultured undisturbed in 6-well culture plates, 1 x 10⁶ cells/ml (5 ml/well), for one hour at 37°C, 5% CO₂ with 95% humidity. Adherent cells (*i.e.* monocytes) were carefully scraped off and washed, whereas the non-adherent (*i.e.* T, B and NK cells) cells were discarded. Further purification of the mononuclear cells was performed using the Monocyte-negative isolation kit® (Dynal Biotech, NO), according to the manufacturer’s instructions. The isolated monocytes, which were untouched during the isolation procedure due to blocking of Fc-receptors, were re-suspended in culture medium, supplemented with IL-4 (52 μg/ml) and GM-CSF (50 ng/ml). The cells were cultured in a strictly sterile milieu in 24-well cultureplates at 37°C, 5% CO₂ with 95% humidity for seven days, with exchange of fresh medium and cytokines every second or third day. The culture medium was left devoid of antibiotics, since previous tests indicated a better survival
MATERIALS AND METHODS

of both DCs and spirochetes in antibiotic-free medium (data not shown) and to avoid potential immunomodulating effects of the antimicrobial agent on the cells. The typical morphological changes seen during differentiation were visualized with an inverted microscope (Nicon TMS, Japan), on every occasion of medium exchange.

On day seven of culture, DCs were counted, assessed for viability by trypan blue staining and re-suspended in 1% plasma to a concentration of 2 x 10^5 cells/ml. Live B. garinii (low passage-strain lp90) spirochetes were activated in a 34°C C-water bath and washed with PBS, before being stimulated with 3% rabbit serum. DCs were stimulated with live spirochetes (in a ratio of 1:10) in human plasma. The optimal ratio of DCs to spirochetes (1:5, 1:10, 1:20), as well as the DC-generated cytokine response to live and ultrasonicated spirochetes, and also to an Osp-enriched fraction (OF) of B. garinii (strain lp90) (as described by Bergström et al.\textsuperscript{327}) was previously evaluated (data not shown), showing that live spirochetes, in line with previous studies,\textsuperscript{281,328} were the most potent stimulators of cytokine production in DCs, and also imitated the \textit{in vivo} infection best.

\textbf{Stimulation of whole blood cells}

For studying the interaction between innate immune cells and live \textit{B. burgdorferi} spirochetes, an experiment described by Nau \textit{et al.}\textsuperscript{329} was repeated with some modifications. Heparinized peripheral blood was washed twice with sterile PBS. Live \textit{B. garinii} (lp90) spirochetes were activated in a 34°C C-water bath, followed by washing with PBS and stimulation with 3% rabbit serum. The live spirochetes were added (2 x 10^7/well) to the whole blood samples, re-suspended to a double volume with heat-inactivated human serum, and cultured undisturbed for 24 hours in 6-well (2 ml/well) culture plates at 37°C, 5% CO\textsubscript{2} with 95% humidity. Un-stimulated whole blood and whole blood stimulated with heat-inactivated human serum containing 3% rabbit serum were used as controls. After 24 hours, the blood samples were centrifuged and the supernatants were immediately collected and frozen at −70°C.

\textbf{Stimulation of peripheral blood mononuclear cells}

PBMC, re-suspended in tissue culture medium (TCM) (as described by Forsberg \textit{et al.}\textsuperscript{190}) to a concentration of 1 x 10^6 cells/ml, were incubated for 48 h at 37°C, 5% CO\textsubscript{2} with 95% humidity, with 1 ml of either OF, LPS (\textit{E. coli}), or phytohemagglutinin (PHA); all diluted with TCM. PBMC incubated with merely TCM were used for analysis of spontaneous cytokine secretion. After incubation, the cell suspensions were centrifuged and the supernatants collected and stored at −70°C.
The enzyme-linked immunospot assay (Paper II–III)
The ELISPOT assay, which is based on an ELISA technique, is a highly sensitive method for visualization of cytokine secretion on a single cell level. Each spot, which develops in the assay, represents a single cytokine-secreting cell. Thus, the ELISPOT assay provides both qualitative (type of immune response) and quantitative (number of secreting cells) information on cytokine responses. The assay was originally described by Czerkinsky et al.\textsuperscript{330} and has been modified by our research group\textsuperscript{196,291} for detection of cytokine-secreting cells in blood and CSF. The principle of the ELISPOT assay is illustrated in Figure 14. In short, nitrocellulose-bottomed culture plates were coated with monoclonal mouse anti-human antibodies directed to IL-4, IL-10, IL-12p70, IFN-γ and TNF (Paper II) or IL-4, IL-12p70, IFN-γ and TGF-β (Paper III). Cell suspensions, either DCs (20 000 cells/well, Paper II) or PBMC (100 000 cells/well, Paper III) were added to the wells in triplicate and stimulated with antigens; live \textit{B. garinii} (Ip90 strain) spirochetes (Paper II) or OF (Paper III). Purified protein derivative (PPD), LPS (Paper II) and an influenza-vaccine (Paper III) were used as reference antigens for recall responses and PHA was used as a positive control (Paper III). Culture medium without cells was used as a negative control. The cells were cultured undisturbed for 48 h at 37° C, 5% CO\textsubscript{2} with 95% humidity.

Development of spots was conducted with matched biotin-conjugated monoclonal antibodies, which were directed against the primary antibodies, and alkaline phosphatase-conjugated streptavidin. The final step included addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, diluted in alkaline phosphatase buffer. The spots were evaluated and counted by a single person (JS in Paper II and MAÅ in Paper III), blinded with respect to the patient’s diagnosis, using the AID EliSpot Reader System (AID, Germany). The same settings were used for all patients throughout each study. The median of triplicates was used for non-parametric statistical calculations. In order to determine the antigen-specific cytokine secretion, the number of spots in the un-stimulated wells (spontaneous secretion) was subtracted from the number in the antigen-stimulated wells. The OF-specific IFN-γ secretion was considered positive for values >15 spots/100 000 cells and >10 spots/100 000 cells concerning IL-4, based on previous results (Paper III)\textsuperscript{331}.
Figure 14. The principle of the ELISPOT assay (Courtesy of Mona Widhe, Swedish University of Agricultural Sciences, Uppsala, Sweden).

Flow cytometry (Paper II)
The principles of flow cytometry and fluorescence-activated cell sorting enable detection, separation and counting of different cell types on the basis of their characteristic cell-surface proteins. The cells of interest are incubated with monoclonal antibodies (directed to a specific protein on the surface of or within the cells) labelled with fluorescent dyes. When the labelled cells pass through a laser beam, the laser light is scattered and the dye molecules will become excited and emit light of certain wave lengths depending on the dye. The light is detected by sensitive photomultiplier tubes, giving information about the size (forward scatter) and granularity (side scatter) of the cells as well as the fluorescence emissions, i.e. reflecting the expression of cell-surface proteins on each cell. In paper II, the expression of characteristic
cell surface markers on un-stimulated, semi-mature DCs (cultured for 7 days) was analysed. Random and blinded (the patient’s diagnosis was unknown) samples of DCs were labelled with directly conjugated monoclonal antibodies directed to CD1a, CD80, HLA-DR, CD11c, CD3, CD19, CD14 (BD Biosciences, USA), CD86, CD83 (Caltag Laboratories, USA) and with their corresponding isotype controls (BD). A minimum of $1 \times 10^4$ cells were counted. The CellQuest Pro software (BD) was used for the analysis.

**Cytometric bead array (Paper II)**
The CBA (BD, USA) is a flow cytometer application, which enables quantification of multiple cytokines simultaneously in a single, small volume sample. Each bead has the same colour, but unique fluorescence intensity, so that beads can be mixed and run simultaneously in a single tube. The beads are coupled to an antibody, and directed against a specific cytokine. The secondary labelled antibody binds to the cytokine, thereby eliciting fluorescence with intensity proportional to the amount of cytokines. The fluorescence intensity is detected by the flow cytometer. The cytokine concentrations are determined using the CBA analysis software. In Paper II, the CBA was used for simultaneous detection of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF (Human Inflammation Kit; BD, USA) in whole blood cell supernatants, performed according to the manufacturer’s instructions and analysed by FACSCalibur (BD). The samples, analysed for the presence of IL-6 and IL-8, were stored undisturbed at +6° C overnight (12 h) and diluted 1:10 before analysis. The other samples were analysed undiluted.

**Luminex (Paper I–III)**
The Luminex Multiplex assay is based on the same principle as CBA, and is illustrated in Figure 15. However, the Luminex beads are labelled with mixtures of two different fluorochromes, which enables simultaneous detection of different cytokines by beads of different colours. The Luminex assay is suitable for the detection of, for example, cytokines, antibodies and nucleic acids in a solution, depending on how the beads are pre-treated. In Paper I, serum samples obtained at inclusion and plasma samples from the 3- and 6-month follow-ups were analysed for the presence of IL-4, IL-10, IL-12p70 and IFN-γ with the Milliplex™ MAP High sensitivity Human cytokine kit (Millipore Corporation, USA), according to the manufacturer’s instructions. In Paper II, the *B. garinii*-stimulated whole blood cell supernatants were analysed for the presence of regulated upon activation, normal T cell expressed and secreted (RANTES or CCL5), monocyte chemotactic protein-1 (MCP-1 or CCL2), monocyte inflammatory protein-1α (MIP-1α or CCL3) and MIP-1β (CCL4) and eotaxin (CCL11) (Human Chemokine 5-plex, Biosource International Inc, USA), according to the manufacturer’s instructions. In Pa-
per III, the presence of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, IFN-γ and TNF in serum was determined with a Human Ultrasensitive Cytokine 10-plex kit (Invitrogen, USA), according to the manufacturer’s instructions. All cytokines were analysed in undiluted sera. The antigen-stimulated PBMC-supernatants were analysed for the presence of IL-6 and IL-8 (single bead kits, Invitrogen, USA) and IL-1β, IL-4, IL-10, IFN-γ, GM-CSF and TNF (6-plex kit, Invitrogen, USA), both according to the manufacturer’s instructions. The samples used for analysis of IL-6 and IL-8 were diluted 1:100 and the other cytokines were analysed in undiluted samples. For all assays, the Luminex™ 100 System (Luminex Corporation, USA) was used for analysis of MFI and the StarStation software (Applied Cytometry Systems, UK) for data acquisition and analysis. Detailed descriptions are given in Paper I–III.

Figure 15. The principle of the Luminex cytokine detection assay.

Skin biopsy (Paper I)
The punch biopsy technique is illustrated in Figure 16. A 4 mm skin punch biopsy was taken under local anaesthesia from the outer edge of the red EM zone and from healthy skin at an opposite body site (control). Each biopsy was split into two pieces, immediately snap-frozen in a mixture of isopen-
tane and liquid CO₂ and stored at -70° C. Serial cryostat sections (6 μm, Leitz CM3050 cryostat, Leica Microsystems, Sweden) were later obtained. Two to six sections were placed in each well of a three-well slide, which had been pre-coated with 0.1% poly-L-lysine. The slides were air-dried and kept at –70° C until stained.

Figure 16. Illustration of the skin punch biopsy technique and the three skin layers (© Reed-Elsevier, USA). Image from: http://www.answers.com/topic/skin-biopsy.

Immunohistochemistry (Paper I)

Immunohistochemistry (IHC) is a method which can be used for detection of cytokine expression in tissues. Specific antibodies, labelled with a fluorescent dye or enzyme, bind to their antigens (in this study, cytokines) in the tissue. The fluorochrome-stained cells are visualized with a fluorescent microscope, which exposes the cells to light and excites the fluorescent dye. The excited dye emits light at a characteristic wavelength, which is visible through a selective filter. In brief, the slides were fixed in an acetone bath immediately after being picked from −70° C, and then air dried. Saponin-phosphate buffered saline ( [PBS], Sigma-Aldrich, Sweden and Medicago AB, Sweden) was used for permeabilisation and as a washing and dilution buffer throughout the assay. Two blocking steps, first with a streptavidin/avidin blocking kit and secondly with 5% normal serum (a mixture containing goat, mouse and rat serum) followed. Antibodies were initially titrated for maximal fluorescence in sections of inflamed tonsil, and validation of the staining method was done with isotype antibodies and PBS as negative controls. The slides were incubated in a humidified chamber for 60 min at room tem-
MATERIALS AND METHODS

Temperature (RT) with primary antibodies directed to IFN-γ, IL-12p70, IL-4 and IL-10 and with their corresponding isotype antibodies. After washing, the slides were incubated for another 60 min at RT with secondary Cy3- or FITC-conjugated antibodies. After additional washing, a drop of SlowFade®Antifade mounting solution was added to each well, cover glasses were mounted and the slides were stored at +4°C (for a maximum of 48 h) until being analysed. The slides were blinded and all sections were analysed according to a protocol by two of the authors (JS and LF), using a Nikon Eclipse E600 (and Nikon EZ-C1 software version 3.30, Nikon Instruments Europe, the Netherlands) at excitation wavelengths of 546 (Cy3) and 488 (FITC) nm. Cytokine expression was estimated on a scale from 0 to ++++; where +, ++ and +++ corresponded to 1–10, 10–20 and >20 cytokine expressing (positive) cells/section, respectively. These estimations were then given numeric values (0=0, +=1, ++=2 and +++=3). Thus, a semi-quantitative and comparable estimation of the cytokine expression in the skin specimens was obtained.

The polymerase chain reaction (Paper I)
The PCR is a molecular and biochemical method, used for production of large amounts of a certain DNA sequence. The PCR-method used in Paper I was developed by Comstedt et al. and is described in detail elsewhere. In short, DNA was extracted using the Puregene DNA isolation protocol (Genta Systems, USA) and stored at −20°C. DNA extracts were analysed for the B. burgdorferi complex by a quantitative PCR assay with probes and primers specific for the 16S ribosomal RNA gene. Serially diluted B. burgdorferi 31 and B. hermsii HS1 DNA were used as standards. B. burgdorferi species were identified by direct sequencing of the amplicons generated from the /rrs/(16S)-/rrl/(23S) intergenic spacer or 16S gene PCR. When necessary, nested modification of these assays was used to increase amplification success. Further details are given in Paper I.

Other assays in peripheral blood (Paper I, III)
Allergic propensity was examined by measuring total and allergen-specific IgE antibodies in plasma (ImmunoCAP™, Phadia AB, Sweden), according to the manufacturer’s instructions. Specific IgE antibodies directed to common food (egg white, cow milk, fish, wheat, peanut and soy bean) and inhaled allergens (timothy grass, birch and mugwort, animal dander [cat, horse and dog], house dust mite Dermatophagoides pteronyssinus and spores of the mould Cladosporium herbarum) were measured with the Phadiatop Combi® assay (Phadia AB). These screening tests indicate if an individual is sensitized to a specific allergen. Routine blood samples included the sedimenta-
tion rate, the C-reactive protein, haemoglobin, white blood cell count, liver transaminases, creatinine and plasma protein electrophoresis (Paper III).

**Magnetic resonance imaging (Paper IV)**

MRI, nuclear magnetic resonance imaging or magnetic resonance tomography, is an imaging technique used in radiology to visualize detailed structures inside the body. It provides a good contrast between different soft tissues, making it especially useful in imaging of, for example, the brain, muscles and connective tissue. In contrast to computed tomography scans or traditional X-rays, MRI does not use any ionizing radiation. Instead, it applies a powerful magnetic field to align the magnetization of the hydrogen nuclei in the body and then uses radio frequency fields to systematically alter the alignment of these nuclei. This makes the nuclei produce a signal (alternating current) detectable by a scanner. This information is recorded and used to construct an image of the scanned body area. In Paper IV, a 1.5 Tesla scanner (Signa Horizon Echospeed, General Electric, USA) with a standard head coil was used for the brain MRI scans. A standardized dose of gadolinium contrast medium of 0.2 ml/kg body weight was administered IV for the last two sequences. All examinations were reviewed by a blinded neuroradiologist (LD) and by one of the authors (AA), according to a standardized protocol including ten sequences. All types of MRI findings (increased/decreased signal or contrast enhancement) in the white or gray matter, cranial nerves, CSF or the leptomeninges were screened for in each sequence and measured, if possible. Extracranial findings (e.g. findings in the eyes, sinuses or skull) were also noted in the protocol. White matter lesions (WML) were divided into periventricular, central and subcortical or noted as being located in the basal ganglia. Subependymal lesions (periventricular capping) were separated from other WML and graded as 0 (none), 1 (slight), 2 (moderate) or 3 (severe), based on the thickness (mm) of the MRI findings and in accordance with a previous study by de Leeuw et al.\textsuperscript{334}

**The follow-up questionnaire (Paper I)**

At inclusion, patients with a newly discovered EM completed a questionnaire with information about: age, gender, general health status, other diseases that may have an influence on the immune system (e.g. rheumatoid arthritis, systemic lupus erythematosus, other collagenosis, HLA-B27 positivity or malignancy), treatment with immunosuppressive drugs or antibiotics at inclusion or within two months prior to inclusion, date of tick bite and whether other symptoms associated with the EM were present and their location. Besides this information, the size, duration, number and location of the EM were noted by the physician. The follow-ups at 3, 6, 12 and 24 months after inclusion were conducted by a research nurse at the time of blood sampling, or by phone, and were based on the same questionnaire

76
MATERIALS AND METHODS

with special emphasis on potential persistent symptoms post-treatment. If symptoms reported at the six-month follow-up were present >3 weeks prior to inclusion, the patient was excluded from the final data analyses.

Neurological examination (Paper III)
The neurological examinations were carried out according to a protocol, by one of the two investigators (JS or PF), before start of and at the end of doxycycline and placebo treatment, respectively. The examination protocol contained the following parts: evaluation of neck stiffness, the finger-nose test, Romberg's test, Grasset's sign, walking on the heels and toes, assessment of eye movements, evaluation of nystagmus, cranial nerve function (especially the facial nerve), sensory examination, deep tendon reflexes, the Babinski plantar response, muscle strength in the extremities, and fundoscopy. The outcome of the different examinations was defined as deviant, if one or more of the examinations were deviant, otherwise it was defined as normal. The difference between the outcome of the examination after and before treatment with doxycycline and placebo was calculated and used for comparison.

The SF-36 health survey (Paper III)
The perceived quality of life (QOL) was assessed according to the standardised Swedish version of the Short Form (SF)-36 health survey (IQOLA SF-36 Standard Swedish Version 1.0) before the start and after completion of treatment with doxycycline and placebo, respectively. The SF-36 includes eight subscales that measure physical functioning, physical limitations on usual role-related activities, bodily pain, general health perceptions, vitality, social functioning, emotional limitations on usual role-related activities, and mental health. The subscales constitute the basis for calculation of the summary scores of the mental (MCS) and physical (PCS) components of the SF-36 survey (IQOLA SF-36 v.1, the HRQL-group, Section of healthcare research, University of Gothenburg and Sahlgrenska University Hospital, Gothenburg, Sweden) (Figure 17). The scores range from 0 (worst) to 100 (best). The difference between the summary scores after and before the start of treatment was used for statistical comparison between doxycycline and placebo treatments.
The symptom severity score (Paper III)

At the first visit to the doctor, the patient’s symptoms were defined and assessed by the patient and the severity level written in a form according to a 10-graded symptom severity score (SSS): 0=no symptoms, 1–2=mild, 3–4=mild-moderate, 5–6=moderate, 7–8=severe, and 9–10=unbearable symptoms (Table 9). The symptoms were assessed at the same time every day during the three-week course of treatment with doxycycline and placebo, respectively. The difference between the SSS after and before the start of treatment was calculated for the symptoms with the two highest SSS (symptom 1 and 2, Table 1, Paper III) and used for statistical comparison.

Table 9. The symptom severity score (in Swedish) used in Paper III

<table>
<thead>
<tr>
<th>Week</th>
<th>Symptom</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
<th>Score 4</th>
<th>Score 5</th>
<th>Score 6</th>
<th>Score 7</th>
<th>Score 8</th>
<th>Score 9</th>
<th>Score 10</th>
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<tr>
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</tr>
</tbody>
</table>

Note: The above table is a simplified representation of the actual data shown in the image.
MATERIALS AND METHODS

Statistics
The statistical methods used in the thesis were in general of non-parametric type, since data (especially immune response data) were assumed not to be normally distributed and outliers were present in several of the analyses. Logistic regression was used for comparison of multiple clinical parameters between two unrelated groups (Paper I). Fisher’s exact test (for nominal data, two groups) and Mann Whitney U test (two groups) were used for comparison between unrelated groups and Wilcoxon signed rank test and McNemar test were used for paired data within and between two groups, respectively. Kruskal-Wallis test was used as a pre-test for comparison between multiple unrelated groups, and in case of showing a trend to significance ($p \leq 0.1$) or a true significance ($p \leq 0.05$), was supplemented by Mann Whitney U test as a post-hoc. A manual Dunn-Bonferroni correction was performed to correct for multiple comparisons. Correlations were calculated using the Spearman’s rank correlation test. P-value $\leq 0.05$ (Papers I–IV) or p-value $\leq 0.0125$ (i.e., $p \leq 0.05/4$) after manual correction for multiple variables (Paper I), was considered significant. SPSS 11.5 (Paper II) or PASW Statistics 18 for Windows (Paper I, III), and John Macintosh Project version 7.0.1 (SAS Inc., USA) (Paper IV) were used for the statistical analyses. The GraphPad Prism versions 4.0 (Paper II) and 5.02 (Paper I and III) for Windows were used for graph design.

Ethical considerations
Ethical approval was obtained from the Regional Ethics Committee at the Åland Central Hospital, the Åland Islands, Finland (Paper I), the Local Ethics Committee of the University Hospital, Linköping (Paper II) and the Regional Ethical Review Board at Linköping University (Paper III and IV). Informed, written consent was obtained from all patients and controls.
RESULTS AND DISCUSSION

Interaction between *B. burgdorferi* and the host (Paper I–II)

**Clinical outcome of erythema migrans (Paper I)**

Of the 109 prospectively included patients with EM, 18 were excluded after completion of the study due to incomplete follow-up and three due to the presence of symptoms >3 weeks prior to inclusion. Seven (8%) of the remaining 88 patients reported persistent symptoms at the six-month follow-up. The symptoms were: arthralgia in the knee (2/7), elbow (2/7), wrist (1/7) and ankle (1/7), fatigue (1/7), back pain (1/7) and hypoesthesia in the skin close to the previous site of EM (1/7). Five of the patients had remaining symptoms at the 12-month follow-up and three still had symptoms 24 months post-treatment (arthralgia and hypoesthesia). None of the patients developed signs of treatment failure and none of them received any additional antibiotic treatment for the persistent symptoms. Thus, it is evident that the clinical outcome of antibiotic-treated EM in this study was good, as has been demonstrated in several previous studies.\textsuperscript{108,110,111} The cause of the post-treatment symptoms and whether they can be associated with the *B. burgdorferi* infection has not been clarified in this study and thus, needs to be further investigated. However, musculoskeletal symptoms and fatigue are common symptoms in the general population as well,\textsuperscript{210,221} and have also been shown to occur after other acute illnesses, such as EBV infection\textsuperscript{212} and influenza\textsuperscript{237}. Furthermore, the symptoms tended to disappear gradually over time, similarly to that seen in other studies.\textsuperscript{113,114} A control group of people without LB, living on the Åland Islands, would have been optimal for comparison, but this was difficult to implement during a two-year period, because of the endemic occurrence of *B. burgdorferi* infection among the population.\textsuperscript{53}

**Patient characteristics**

In general, the clinical baseline characteristics of the EM patients (Table 10) from the Åland Islands are well in line with previous Swedish studies.\textsuperscript{51,60,179,337} Of the total of 88 patients, 55 were women and 33 were men, and the median age was 57 years. The median duration of EM at inclusion was six days. One patient had noticed the EM 215 days prior to inclusion, and this is commented on in the discussion part in Paper I. Three patients had multiple EM, whereof two had fever and fatigue at inclusion, but all three were asymptomatic at the six-month follow-up. They received treat-
RESULTS AND DISCUSSION

ment with doxycycline (n=2) and amoxicillin (n=1). The low number of patients with multiple EM observed in this study was expected and consistent with European data. Fifty-eight (66%) patients reported a prior tick-bite. Irrespective of clinical outcome, the most commonly reported symptoms at baseline were fatigue, myalgia, arthralgia, headache and malaise. The monthly distribution of EM among the study participants ranged from June to December, with most patients being included in July (Figure 18). A majority of the EM were located in the lower extremity or on the trunk, with the knee creases (fossa poplitea) being the most common site, which is in line with findings by Berghlund et al.51 and Bennet et al.70 All patients, except one, completed the antibiotic treatment without disruption. The patient, who was the exception, discontinued treatment after seven days due to a suspected allergic reaction, but she was asymptomatic at all follow-up time points. Analysis of B. burgdorferi antibody kinetics was not the primary purpose of the study, partly due to the high background seropositivity among the inhabitants of the Åland Islands,53 the low sensitivity of serologic assays in general in early, localized LB and because EM is merely a clinical diagnosis,49 and thus was not consistently analysed at inclusion.
Table 10. Characteristics of patients with erythema migrans included in Paper I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with symptoms at 6 months (A)</th>
<th>Patients with no symptoms at 6 months (B)</th>
<th>Statistical comparison (A vs B)</th>
<th>In total (A+B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>81</td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>Sex f/m</td>
<td>5/2</td>
<td>50/31</td>
<td>NS</td>
<td>55/33</td>
</tr>
<tr>
<td>Age (years)</td>
<td>30–68</td>
<td>23–88</td>
<td></td>
<td>23–88</td>
</tr>
<tr>
<td>Median</td>
<td>55</td>
<td>58</td>
<td>NS</td>
<td>57</td>
</tr>
<tr>
<td>Single/Multiple EM</td>
<td>7/0</td>
<td>78/3</td>
<td>NS</td>
<td>85/3</td>
</tr>
<tr>
<td>Tick bite reported Y/N</td>
<td>3/4</td>
<td>55/25</td>
<td>NS</td>
<td>58/29</td>
</tr>
<tr>
<td>EM size (cm)</td>
<td>5–30</td>
<td>3–37</td>
<td></td>
<td>3–37</td>
</tr>
<tr>
<td>Median</td>
<td>15</td>
<td>10</td>
<td>NS</td>
<td>10</td>
</tr>
<tr>
<td>Borrelia antibody pos/n analysed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6 ab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mos</td>
<td>4/7</td>
<td>39/81</td>
<td>NS</td>
<td>43/88</td>
</tr>
<tr>
<td>6 mos</td>
<td>4/7</td>
<td>36/78</td>
<td>NS</td>
<td>40/85</td>
</tr>
<tr>
<td>Borrelia IgM ab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mos</td>
<td>7/7</td>
<td>48/81</td>
<td>NS</td>
<td>55/88</td>
</tr>
<tr>
<td>6 mos</td>
<td>6/7</td>
<td>43/78</td>
<td>NS</td>
<td>49/85</td>
</tr>
<tr>
<td>Borrelia IgG ab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mos</td>
<td>5/7</td>
<td>51/81</td>
<td>NS</td>
<td>56/88</td>
</tr>
<tr>
<td>6 mos</td>
<td>7/7</td>
<td>46/78</td>
<td>NS</td>
<td>53/85</td>
</tr>
<tr>
<td>Allergy Y/N</td>
<td>1/6</td>
<td>25/56</td>
<td>NS</td>
<td>26/62</td>
</tr>
<tr>
<td>EM duration prior to treatment (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>2–215</td>
<td>0–63</td>
<td>0–215</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>4</td>
<td>6</td>
<td>NS</td>
<td>6</td>
</tr>
</tbody>
</table>

f, female; m, male; EM, erythema migrans; Y, yes; N, no; pos, positive; n, number; mos, months; NS, non-significant; Ig, immunoglobulin; C6, a synthetic peptide; ab, antibodies.

Six of the symptomatic patients were treated with amoxicillin and one with doxycycline. None of them reported diseases or medication, which could interfere with the immune system. When comparing, by logistic regression, all clinical variables listed in Table 10 between patients with and without symptoms at the six-month follow-up, no significant differences were found. Nor were there any significant differences between the two groups, by comparison with Fisher’s exact test (sex, allergic trait, B. burgdorferi serology at three and six months post-treatment and reported tick bites) or Mann-Whitney U-test (age, EM-size, -duration and -number). Nor did the received antibiotic treatment significantly differ between the symptomatic and asymptomatic patients.
RESULTS AND DISCUSSION

Figure 18. The monthly distribution of erythema migrans (EM) among the study participants (n=88), from the Åland Islands, Finland, from May 2002 to December 2004.

Asymptomatic patients included in the immunohistochemical analysis
Eighteen patients, who were asymptomatic at the six-month follow-up, were included in the IHC analysis of cytokine expression in skin biopsies. Ten of these were originally chosen to match the symptomatic patients (n=10, before exclusion of three of them) for age and gender, and eight were unmatched. The asymptomatic subjects (n=18) were considered to be representative of the larger asymptomatic group (n=81) in terms of reported symptoms at baseline and at the 3-month follow-up (data not shown). No statistically significant differences in clinical characteristics were found between the asymptomatic subjects (n=18) and the symptomatic patients (n=7) (Table 11).
RESULTS AND DISCUSSION

Table 11. Characteristics of the symptomatic and asymptomatic patients included in the immunohistochemical analysis of cytokine expression in erythema migrans skin biopsies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with symptoms at 6 mos Included in IHC (A)</th>
<th>Patients with no symptoms at 6 mos Included in IHC (C)</th>
<th>Statistical comparison (A vs C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Sex f/m</td>
<td>5/2</td>
<td>11/7</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years) Range</td>
<td>30–68</td>
<td>23–73</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>55</td>
<td>49</td>
<td>NS</td>
</tr>
<tr>
<td>Single/Multiple EM</td>
<td>7/0</td>
<td>16/2</td>
<td>NS</td>
</tr>
<tr>
<td>Tick bite reported Y/N</td>
<td>3/4</td>
<td>14/4</td>
<td>NS</td>
</tr>
<tr>
<td>EM size (cm) Range</td>
<td>5–30</td>
<td>5–16</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>15</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>Borrelia antibody pos/n analysed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6 ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mos</td>
<td>4/7</td>
<td>9/18</td>
<td>NS</td>
</tr>
<tr>
<td>6 mos</td>
<td>4/7</td>
<td>7/18</td>
<td>NS</td>
</tr>
<tr>
<td>Borrelia IgM ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3mos</td>
<td>7/7</td>
<td>11/18</td>
<td>NS</td>
</tr>
<tr>
<td>6mos</td>
<td>6/7</td>
<td>10/18</td>
<td>NS</td>
</tr>
<tr>
<td>Borrelia IgG ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3mos</td>
<td>5/7</td>
<td>14/18</td>
<td>NS</td>
</tr>
<tr>
<td>6mos</td>
<td>7/7</td>
<td>12/18</td>
<td>NS</td>
</tr>
<tr>
<td>Allergy Y/N</td>
<td>1/6</td>
<td>7/11</td>
<td>NS</td>
</tr>
<tr>
<td>EM duration prior to treatment (days) Range</td>
<td>2–215</td>
<td>0–45</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>4</td>
<td>7</td>
<td>NS</td>
</tr>
</tbody>
</table>

f, female; m, male; Y, yes; N, no; pos, positive; n, number; mos, months; Ig, immunoglobulin; NS, non-significant; IHC, immunohistochemical; EM, erythema migrans; ab, antibodies.

B. burgdorferi subspecies in skin biopsies (Paper I)
Seventy-five (81.5%) of the 92 initially screened EM biopsies were B. burgdorferi DNA positive (Table 12). However, in only 48 of these could the B. burgdorferi subtype be sequenced, resulting in 36 (48%) B. afzelii, 11
RESULTS AND DISCUSSION

(15%) B. garinii and one (1%) B. burgdorferi s.s, the latter detected in an asymptomatic patient. Seventeen (18.5%) of the EM biopsies were B. burgdorferi DNA negative. In the initial screening of 87 control biopsies from unaffected skin, 20 (23%) were B. burgdorferi DNA positive (data not shown). However, further sequencing of the amplicons turned out to be negative. The persistent symptoms post-treatment cannot be explained by the infecting B. burgdorferi subtype, since no significant differences in the occurrence of the different subtypes were found between the symptomatic and asymptomatic patients (comparison with asymptomatic subjects as a whole and with those included in IHC) (Table 12). The results are in line with previous European, Swedish and Finnish (from the Åland Islands) studies, in which B. afzelii was the most commonly found subspecies in EM biopsies, although the subspecies were detected at much higher frequencies compared to the findings in this study. A contributing factor to the lack of sequence results in many of the EM biopsies and the false positive results in the control biopsies in this study may be that the DNA samples were not of sufficiently good quality to allow a thorough analysis to be performed. However, the samples were analysed throughout blindly without knowledge of the clinical outcome. Consequently, evaluation of true and false positive samples has not been carried out.

Table 12. B. burgdorferi subtypes in erythema migrans skin biopsies from patients with and without symptoms six months post-treatment.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with symptoms at 6 months (A) included in IHC</th>
<th>Patients without symptoms at 6 months (B)</th>
<th>Statistical comparison (A vs B)</th>
<th>Patients without symptoms at 6 months (C) included in IHC</th>
<th>Statistical comparison (A vs C)</th>
<th>All included (A + B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (patients)</td>
<td>7</td>
<td>81</td>
<td></td>
<td>18</td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>n (EM biopsies)</td>
<td>7</td>
<td>85</td>
<td></td>
<td>20</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>PCR positive</td>
<td>6</td>
<td>42</td>
<td>NS</td>
<td>11</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Subtypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. afzelii / garinii / sensu stricto</td>
<td>5/1/0</td>
<td>31/10/1</td>
<td>NS</td>
<td>6/5/0</td>
<td>NS</td>
<td>36/11/1</td>
</tr>
<tr>
<td>Undetectable*</td>
<td>1</td>
<td>43</td>
<td></td>
<td>9</td>
<td></td>
<td>44</td>
</tr>
</tbody>
</table>

* Includes both PCR positive biopsies, in which the B. burgdorferi subtype was undetectable (n=27) and PCR negative (n=17) biopsies.

B., Borrelia; EM, erythema migrans; IHC, immunohistochemistry; n, number; PCR, polymerase chain reaction; NS, non-significant.

Interestingly, in a study comparing the occurrence of post-treatment symptoms in EM patients with positive and negative B. burgdorferi skin cultures, patients in the culture-positive group were shown to have a significantly worse clinical outcome and more severe symptoms at the follow-up time points compared to the culture-negative group. Cultures were not per-
formed in this study, but the culture results may well be compared with the PCR-findings, showing that six (86%) of the symptomatic and 42 (49%) of the asymptomatic patients were PCR-positive, without any significant difference between the groups.

**Cytokine expression in erythema migrans skin biopsies in relation to clinical outcome (Paper I)**

The expression of IFN-γ was lower in EM biopsies from symptomatic patients compared to patients without symptoms six months post-treatment (p=0.003, Figure 19 B). In addition, the symptomatic patients had decreased expression of IL-12p70 in the EM lesions compared to the asymptomatic patients (p=0.013, Figure 19 A), but the difference did not reach statistical significance after manual Dunn-Bonferroni correction for multiple variables. The expression of IFN-γ was, in addition, correlated with IL-12p70 in both EM and control biopsies (p≤0.01 for both, rho=0.69 and rho=0.77, respectively), which supports the results, since IL-12p70 induces and maintains IFN-γ secretion.265
RESULTS AND DISCUSSION

Figure 19 A–D. Immunohistochemical analysis of the number of cytokine expressing cells/μm² in erythema migrans skin biopsies from patients with (n=7) and without (n=18) persistent symptoms six months post-treatment. The diagrams illustrate the expression of interleukin (IL)-12p70 (A), interferon (IFN)-γ (B), IL-4 (C), and IL-10 (D) in erythema migrans (erythema) biopsies. The Mann-Whitney U-test and a manual Bonferroni correction were used for statistical analysis. P<0.0125 was considered significant. Horizontal lines denote the median.

The two patient groups did not differ significantly regarding the expression of IL-4 and IL-10 in either EM (Figure 19 C, D) or in control biopsies (Figure 19 G, H), and the number of IL-4- and IL-10-expressing cells was low in general. Neither did the expression of IL-12p70 or IFN-γ differ in unaffected (control biopsies) skin between the two groups (Figure 19 E, F; respectively). Overall, the expression of IFN-γ was slightly higher in EM compared to control biopsies, though no statistically significant differences in expression of any of the four cytokines were found between erythematous and unaffected skin, which is surprising. No correlations between cytokine expression in the skin and cytokine levels in serum and plasma, respectively, were found.
Figure 19 E–H. Immunohistochemical analysis of the number of cytokine expressing cells/μm² in control (unaffected) skin biopsies from patients with (n=7) and without (n=18) persistent symptoms six months post-treatment. The diagrams illustrate the expression of interleukin (IL)-12p70 (E), interferon (IFN)-γ (F), IL-4 (G), and IL-10 (H) in unaffected skin. The Mann-Whitney U-test and a manual Bonferroni correction were used for statistical analysis. P<0.0125 was considered significant. Horizontal lines denote the median.

The finding of decreased Th1-type inflammatory cytokine expression in EM biopsies from symptomatic compared to asymptomatic patients support the hypothesis that a strong Th1-type response is necessary early in the course of infection with B. burgdorferi in order to successfully eradicate the spirochetes, to be able to downregulate the inflammatory response later on and consequently, to ensure a favourable outcome of LB. Other studies have also demonstrated a predominant Th1-type inflammatory response in EM lesions, but follow-up studies correlating early cytokine expression in EM to the development of symptoms post-treatment have not previously been conducted. The symptomatic patients had overall low cytokine expression in both the EM and control biopsies, which is somewhat surprising. However, they did not differ significantly from the asymptomatic patients in
RESULTS AND DISCUSSION

terms of clinical characteristics in general or allergic traits or prior immuno-suppressive treatment in particular. Nor did they differ with regard to technical or analysis procedures and they did not have diseases that could interfere with the immune system.

Another surprising finding, which is difficult to explain, is the lack of differences in cytokine expression between erythematous and unaffected skin. The skin is, besides a physical barrier, in fact an important innate immune organ possessing various defence mechanisms. Thus, speculatively, the equal cytokine expressions in erythematous and unaffected skin could be explained by a temporary increase in the general, steady state cytokine expression in whole skin elicited by the *B. burgdorferi* infection. However, these speculations are contradicted by studies showing that healthy skin in general is devoid of cytokine expression.\textsuperscript{341-343} Another, though less probable explanation could be that the equal cytokine expressions are due to some methodological factor (bias). Although it is reasonable to assume that the results then would have been quite the opposite, *i.e.* significantly lower cytokine expression in unaffected skin. Furthermore, the risk of bias was minimized by using anonymous samples and blinded analyses throughout the study.

The decreased early inflammatory response in the EM lesions in the symptomatic patients was not systemically reflected, since any differences in cytokine levels in serum or plasma were found between the two groups. Whether the difference in local cytokine expression between the symptomatic and asymptomatic patients could be due to other factors, such as polymorphisms of TLRs or genetic diversity in immune genes, is not known, but is conceivable on the basis of previous studies in, for example, leprosy,\textsuperscript{344} cutaneous leishmaniasis\textsuperscript{345} and sepsis.\textsuperscript{346} Furthermore, mutations in the TLR2 gene have been shown *in vitro* to impair *B. burgdorferi* lipoprotein recognition by the receptor with subsequent reduction in NF-κB activation.\textsuperscript{347} In patients with LB, the ability to elicit an optimal immune response to *B. burgdorferi* may well partly depend on genetic factors. However, such factors are not likely to have a major impact on spirochete killing *per se*, since all patients were treated with antibiotics and none of them showed signs of treatment failure. Instead, an insufficient pro-inflammatory response, not generating proper downregulatory feed-back mechanisms at the site of inflammation, could possibly generate a low-grade inflammatory response that continues despite eradication of the spirochetes, causing subtle tissue damage and persistent symptoms. An additional skin biopsy post-treatment from the site of the EM lesions could in part have been able to answer this hypothesis. Further studies are warranted to shed light on any as-
RESULTS AND DISCUSSION

...sociation between regulation of cytokine responses and the development of persistent symptoms after antibiotic treatment of LB.

Innate immune responses to B. garinii in relation to clinical outcome (Paper II)

In Paper II, innate cytokine secretion by both B. garinii-stimulated, autologous DCs and whole blood cells was analysed and related, retrospectively, to the clinical outcome of LB.

Cytokine secretion in B. garinii-infected dendritic cells

All cytokines (i.e. TNF, IL-4, IL-10, IL-12p70 and IFN-γ) were detectable with the ELISPOT assay (Figure 20). The number of TNF-secreting DCs was overall high in all patient groups, whereas the number of DCs secreting IL-4, IL-10, IL-12p70 and IFN-γ was low in all groups. The asymptomatic B. burgdorferi-seropositive individuals had significantly more B. garinii-induced, TNF-secreting DCs in comparison with the subacute NB patients and the seronegative controls (p=0.048 and 0.018, respectively) (Figure 20 A). A slight tendency for subgrouping of TNF-secreting DCs was observed in the chronic patient group; one with high (>200 DCs) and one with low (<100 DCs) numbers of TNF-secreting DCs (Figure 20 A), though the patients were few in total. The B. garinii-induced secretion of IL-4, IL-10, IFN-γ and IL-12p70 by DCs did not differ significantly between the groups (Figure 20 B–E, respectively). The mean age was lower among the B. burgdorferi-seronegative controls compared to the other groups, but no significant correlation between age and number of TNF-secreting DCs was found. Three outliers, one each in the subacute, asymptomatic and seronegative group, had high numbers of cytokine-secreting DCs throughout all measurements (Figure 20 A–E). However, these individuals did not differ from the other subjects in terms of clinical characteristics. It should also be noted that non-parametric tests were used consistently for statistical analyses, and hence outliers should not have influenced the results.
RESULTS AND DISCUSSION

Figure 20. The median (of triplicates) number of *B. garinii*-specific, tumour necrosis factor (TNF)- (A), interleukin (IL)-4 (B), IL-10- (C), interferon (IFN)-γ (D)- and IL-12p70 (E)- secreting cells/20 000 dendritic cells (DC) from patients with different clinical outcomes of Lyme borreliosis and from *B. burgdorferi*-seronegative controls, analysed with the ELISPOT assay. The *B. garinii*-specific cytokine secretion was obtained by subtracting the number of spots in unstimulated wells from the number of *B. garinii*-stimulated wells. Horizontal lines denote the median.
RESULTS AND DISCUSSION

*B. garinii*-induced secretion of innate cytokines in whole blood cells

All the measured innate cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF) in *B. garinii*-stimulated whole blood cell supernatants were detectable with the CBA. Significantly higher levels of IL-12p70 were found in whole blood supernatants from asymptomatic *B. burgdorferi*-seropositive individuals compared to *B. burgdorferi*-seronegative controls (p=0.031) (Figure 21). No significant differences in the levels of IL-1β, IL-6, IL-8, IL-10 or TNF were found between the four groups. The levels of IL-12p70 did not correlate with age.

![Figure 21. IL-12p70 levels (median values in pg/ml) in *B. garinii*-stimulated whole blood cell supernatants from patients with different clinical outcomes of Lyme borreliosis and from *B. burgdorferi*-seronegative controls. Asympt, asymptomatic. Horizontal lines denote the median.](image)

*B. garinii*-induced secretion of chemokines in whole blood cells

No significant differences in chemokine levels (MIP-1α, MIP-1β, MCP-1 and eotaxin) in *B. garinii*-stimulated whole blood cell supernatants were found between the four groups. Detailed information on the cytokine and chemokine levels in the whole blood cell supernatants is provided in Paper II.

The finding that asymptomatic subjects were shown to respond with pro-inflammatory cytokine secretion (i.e. TNF and IL-12p70) to stimulation with live *B. garinii* sprochetes are in agreement with previous cytokine data on asymptomatic individuals, using *B. afzelii* lipopeptides (OF) for stimulation of autologous T cells, and support the view that pro-inflammatory and
RESULTS AND DISCUSSION

Th1-inducing responses early during infection with \textit{B. burgdorferi} results in optimal resolution of LB. Both IL-12p70 and TNF are important innate cytokines with pro-inflammatory and antibacterial effects.\textsuperscript{267,348} IL-12p70 also induces the production of IFN-\(\gamma\), favours the differentiation of Th1 cells, and forms a link between innate resistance to pathogens and adaptive immunity.\textsuperscript{267} Interestingly, high TNF levels in the CSF have been shown to be associated with a successful clinical outcome of NB in humans.\textsuperscript{299} TNF also seems to be important, beyond concomitant treatment with antibiotics, in spirochete elimination in mice infected with \textit{B. burgdorferi}.\textsuperscript{349}

Several cell types, such as DCs, macrophages and monocytes have been shown to secrete IL-12p70 and/or TNF in response to stimulation with live \textit{B. burgdorferi} spirochetes.\textsuperscript{272,278,350} In this study, live spirochetes, as compared to ultra-sonicated ones and OF were shown to elicit the most potent cytokine responses in DCs. The findings are consistent with results from other studies.\textsuperscript{281,328} and are assumed to best mimic the \textit{in vivo} immune response to \textit{B. burgdorferi}.

The asymptomatic individuals are an interesting patient group from a clinical and an immunological point of view. They have both antibodies- and T-cell responses to \textit{B. burgdorferi},\textsuperscript{154} which indicates previous exposure to the pathogen, but they have no history of clinical signs or symptoms of LB. The fact that they also respond with increased numbers of TNF-secreting DCs and increased levels of IL-12p70 elicited by whole blood cells in response to live spirochetes, suggests that they have an optimal immune response to \textit{B. burgdorferi}. In light of this assumption, it is surprising that the number of TNF-secreting DCs or the levels of IL-12p70 in whole blood cells did not differ between the asymptomatic subjects and the patients in the so called chronic group, which unfortunately was heterogeneous in its composition, including both patients with post-NB symptoms and post-treatment ACA. However, the \textit{B. garinii}-elicited TNF response in DCs differed between the asymptomatic subjects and the subacute patients, although the significance was weak. Interestingly, these two groups differ clinically with respect to symptom development; the subacute patients develop clinical signs of LB, whereas the asymptomatic subjects do not. Nevertheless, the mechanisms leading to subclinical, asymptomatic \textit{B. burgdorferi} infection are poorly studied and most probably depend on several factors, of which the early immune response to \textit{B. burgdorferi} is likely to be one important part.

The ELISPOT assay used in this study was developed for analysis of innate cytokine responses generated exclusively by \textit{B. garinii}-stimulated DCs. All the cytokines measured in the ELISPOT assay, \textit{i.e.} IL-4, IL-10, IL-12, IFN-\(\gamma\)
RESULTS AND DISCUSSION

and TNF, are known to be produced by DCs in various amounts in response to different stimuli.\textsuperscript{351,352} The lack of effector cells (\textit{i.e.} T cells) in the assay may, however, be reflected in the low number of IL-4, IL-10, IL-12p70 and IFN-\(\gamma\) secreting-DCs, since DCs and T cells are dependent on cross-talk with each other to maintain their cytokine secretion.\textsuperscript{23,353,354} The secretion of TNF by DCs, on the other hand, is independent of such cross-talk.\textsuperscript{354} Although IL-12p70 secretion, which was low in all four groups measured with the ELISPOT assay, is enhanced by T cell-derived IFN-\(\gamma\), Filgueira \textit{et al.} was able to demonstrate T-cell independent IL-12p70 production by \textit{B. burgdorferi}-infected DCs.\textsuperscript{272} The discrepancy between the results is, however, unknown. Although the whole blood samples contained T cells, a comparison between the actual IL-12p70 responses in the ELISPOT (\textit{i.e.} number of cytokine-secreting DCs) and whole blood assay (levels of cytokines) is not feasible.

On the basis of the results from Papers I and II, it may be concluded that the early immune response to \textit{B. burgdorferi} seems to have an impact on and to be associated with the clinical outcome of LB. More specifically, decreased early inflammatory cytokine secretion in EM skin lesions is associated with persistent symptoms post-treatment, whereas pro-inflammatory cytokine responses to live spirochetes are associated with a subclinical, asymptomatic course of \textit{B. burgdorferi} infection.

Clinical, immunological and diagnostic aspects of persistent symptoms post-neuroborreliosis (Paper III–IV)

\textbf{Effects of doxycycline on persistent symptoms and quality of life post-neuroborreliosis (Paper III)}

In this unique, randomized, placebo-controlled double-blind treatment trial, no significant impact of doxycycline on objective neurological signs, severity of persistent symptoms, or on quality of life (QOL) post-treatment was found. The outcome of the neurological examinations, the SSS and assessment of QOL are summarized in Table 13.

\textbf{Outcome of neurological examinations}

In cases of a deviant neurological examination at baseline or at the follow-ups, the facial nerve, balance, sensibility or tendon reflexes were affected. None of the patients developed new, objective neurological deficits or experienced worsening of current neurological signs during the study period (Table 13). Four patients improved in regard to Romberg’s test, whereof one

95
RESULTS AND DISCUSSION

improved during doxycycline-treatment and three during placebo-treatment. Most of the patients had unchanged outcome on neurological examination throughout the study (Table 13). No significant changes in neurological examinations were found either within or between the doxycycline and placebo group.

**Assessment of symptom severity**

With doxycycline treatment, four (no. 2, 5, 9, 10) patients improved with respect to the symptom with the highest severity score (spt1), whereas one (no. 8) patient improved with respect to spt1 with placebo-treatment, without any significant differences between the groups (Table 13). Five (no. 2, 6, 7, 8, 10) patients improved with doxycycline treatment with respect to the other one of the two symptoms (spt2) with the highest severity scores, whereas four (no. 5, 8, 10, 12) improved with placebo-treatment, without any significant differences between the groups. In all, two patients got worse regarding one of the symptoms with doxycycline treatment, and four with placebo treatment (Table 13). However, no significant differences were found when comparing changes in severity of the two symptoms within or between the doxycycline and placebo group.

**Assessment of quality of life (SF-36)**

The PCS improved in seven (no. 1, 4, 5, 8, 9, 10, 15) patients with doxycycline treatment and in four (no. 2, 12, 13, 15) with placebo-treatment (Table 13). The MCS improved in seven (no. 1, 3, 9, 10, 11, 13, 14) with doxycycline treatment and in eight (no. 2, 5, 7, 8, 9, 10, 11, 15) with placebo-treatment. Six patients (no. 3, 6, 11, 12, 13, 14) got worse in PCS with doxycycline treatment, and six (no. 1, 7, 8, 9, 10, 11) with placebo. The MCS got worse in six patients (no. 4, 5, 6, 8, 12, 15) with doxycycline treatment, and in three (no. 1, 12, 13) with placebo (Table 13). No significant differences were found in change of PCS or MCS either within or between the two treatment groups. Notably, only one patient had unchanged QOL, during placebo treatment. However, in comparison with the general Swedish population (matched for mean age)\(^{355}\), the patients had significantly decreased PCS mean values at time of inclusion (\(p=0.002\), data not shown), a finding that is consistent with previous\(^{213}\) and recent data\(^{218,356}\).
RESULTS AND DISCUSSION

Table 13. Outcome of the neurological examinations, symptom severity scores and assessments of quality of life with doxycycline and placebo treatment.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>DOX 3 weeks</th>
<th>PBO 3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NL exam</td>
<td>SSS</td>
</tr>
<tr>
<td></td>
<td>spt1/spt2</td>
<td>PCS/MCS</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+/+</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>+/-</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0/+</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0/+</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0/+</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>+/NA</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>+/-</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0/NA</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

DOX, doxycycline; PBO, placebo; NL exam, neurological examination; SSS, symptom severity score; spt, symptom; SF-36, Short Form-36; PCS, physical component summary; MCS, mental component summary; NA, not analysed; + indicates improvement; 0 indicates unchanged; – indicates impairment. The outcome was assessed by calculating the difference between the values at the end of and prior to treatment with DOX and PBO.

Effects of doxycycline on systemic cytokine responses (Paper III)

No doxycycline-mediated effects on cytokine responses in either PBMC or in serum were found.

Antigen-stimulated cytokine responses in mononuclear cells

In line with previous studies, most post-NB patients had an OF-specific IFN-γ response (Figure 22 A) in peripheral blood, whereas an OF-specific IL-4 and TGF-β response was observed in only a few patients (Figure 22 B, C), analysed with the ELISPOT assay. The number of OF-specific IL-12p70-secreting cells corresponded to those found in a study by Jarefors et al. (Figure 22 D). No significant differences were found in either unstimulated or OF-stimulated IFN-γ, IL-4, IL-12p70 or TGF-β responses in PBMC within or between the doxycycline and placebo treatment groups.
Figure 22. The number of outer-surface fraction (OF)-specific IFN-γ (A), IL-4 (B), TGF-β (C) and IL-12p70 (D) secreting peripheral blood mononuclear cells from patients with persistent symptoms post-NB (n=9), detected by ELISPOT, before and after treatment with doxycycline (DOX) and placebo (PBO). The OF-specific cytokine secretion was obtained by subtracting the number of spots in unstimulated (spontaneous) wells from the number of spots in OF-stimulated wells. Values, which represent medians of triplicates, are given as number of cytokine-secreting cells/100 000 lymphocytes. The dotted lines in graph A (=15 cells/100 000) and B (=10 cells/100 000) indicate the cut-off for OF-specific cytokine secretion, as previously described. No significant differences in change of the number of cytokine secreting cells were found within or between doxycycline and placebo treatment. Horizontal lines denote the median.
RESULTS AND DISCUSSION

IL-1β, IL-6, IL-8, TNF and IL-10 were detectable in most of the OF- and LPS-stimulated PBMC-supernatants and, except for IL-8, undetectable in unstimulated (spontaneous) samples (Table 14). No significant differences in change of antigen-stimulated or unstimulated cytokine levels in PBMC-supernatants were found either within or between the doxycycline and placebo treatment groups.

**Spontaneous cytokine levels in serum**

The median levels of circulating IL-1β, IL-6, IL-8, GM-CSF and IL-10 in serum are presented in Table 14. The levels of IL-2, IL-4, IL-5, IFN-γ and TNF were very low or undetectable (data not shown). IL-8 was detectable in all serum samples. No significant differences in change of circulating, spontaneous cytokine levels in serum were found either within or between the two treatment groups.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Antigen</th>
<th>Doxycycline median pg/ml (IC range)</th>
<th>Placebo median pg/ml (IC range)</th>
<th>Detectable (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>OF</td>
<td>0.43 (0.13-1.50)</td>
<td>0.60 (0.13-1.10)</td>
<td>0.52 (0.13-0.70)</td>
</tr>
<tr>
<td>IL-6</td>
<td>OF</td>
<td>0.19 (0.19-1.60)</td>
<td>0.19 (0.19-2.70)</td>
<td>0.19 (0.19-2.70)</td>
</tr>
<tr>
<td>IL-8</td>
<td>OF</td>
<td>0.15 (6.12-12.9)</td>
<td>9.76 (7.70-14.3)</td>
<td>11.6 (7.40-14.9)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>OF</td>
<td>0.75 (0.75-9.85)</td>
<td>0.75 (0.75-9.10)</td>
<td>0.75 (0.75-16.2)</td>
</tr>
<tr>
<td>IL-10</td>
<td>OF</td>
<td>0.15 (0.15-1.80)</td>
<td>0.15 (0.15-1.72)</td>
<td>0.15 (0.15-1.30)</td>
</tr>
</tbody>
</table>

The hypothesis in Paper III was based on many years of clinical observations indicating subjective improvement of persistent symptoms with repeated courses of doxycycline treatment in post-NB patients. However, the symptoms often reappeared after completion of treatment. This raised the question whether the improvement of symptoms could be due to a true doxycycline-mediated effect or merely a placebo-effect. In this pilot study, neither anti-inflammatory nor symptom-modifying effects of doxycycline could be
RESULTS AND DISCUSSION

demonstrated. Neither was a significant placebo-effect observed. Besides the double-blind randomization, an additionally important difference between the clinical observations and this study is the fact that the patients might have been influenced by different information prior to treatment, which may have resulted in different expectations at the start of treatment. Thus, a placebo-effect may not be excluded as an explanation for improvement in symptoms in individual cases in the previous clinical observations. Several studies have highlighted the occurrence of subjective complaints after treatment of different clinical features of LB, but hitherto studies addressing clinical and immunological effects of antibiotic treatment on well-characterized post-NB symptoms have been missing.

Most of the patients had unchanged neurological status throughout the study, with no significant differences between the two treatment sets. However, the neurological signs were only roughly evaluated and no assessment of nerve function could hence be done. In accordance with other follow-up studies, the remaining neurological findings after treatment of NB consisted mainly of ataxia, balance and sensory disturbances as well as discomfort from persistent facial nerve palsy, which is also a known sequelae after treatment of NB in children. Fatigue, memory disturbances and concentration difficulties were reported by many patients; symptoms that were shown by Eikeland et al. to differ significantly between post-NB patients and healthy, matched controls. Headache, which is also a common complaint after enteroviral and other forms of acute aseptic meningitis, was present in most of the patients at inclusion.

At inclusion, both the PCS and MCS scores were decreased and the PCS score was significantly decreased in the post-NB patients compared to the general Swedish population, indicating an impaired QOL, caused by deterioration of physical health, in patients with post-NB symptoms. Similar results were obtained by Eikeland et al. and Klemper et al. However, no significant impact of doxycycline on QOL was found. Depression, diagnosed prior to inclusion, was present in three of the patients. Psychiatric comorbidity has been suggested as one of many possible causes of PLDS. However, in the Norwegian follow-up study, no difference in psychiatric comorbidity was found either between post-NB patients as a whole and matched controls, or between post-NB patients with incomplete and complete recovery.

Aside from IL-8, the circulating cytokine levels in serum were low. A treatment duration of three weeks was expected to be sufficient to influence systemic cytokine responses, since reduction in systemic cytokine responses and anti-inflammatory effects in humans have already been observed within
RESULTS AND DISCUSSION

a few hours after doxycycline administration. In addition, reduced levels of IL-6, IL-8, IL-13 and GM-CSF have been found after two weeks of doxycycline (50-300 mg/day) treatment. In this study, IL-1β, IL-6, IL-8, IL-10 and TNF were all detectable in antigen-stimulated PBMC-supernatants, but no significant doxycycline-mediated changes in cytokine levels could be observed. The cause of this remains to be elucidated and further studies on post-NB patients are needed to confirm these results. The lack of improvement of the persistent symptoms with additional antibiotic treatment is consistent with other trials addressing this issue, studying patients with various case definitions. Patients with prolonged convalescence after treatment of NB tend to be middle-aged or older, as in this study, in which the mean age among the study participants was 59 years. In fact, the risk of developing long-lasting symptoms after treatment of NB has been shown to increase with age. It is possible that some of the remaining symptoms are due to subtle damage on nervous tissue caused by the inflammatory response to B. burgdorferi, and that such damage has poorer regenerative capacity with increasing age. This reasoning is supported by the fact that persistent symptoms after treatment of NB are quite uncommon in children. Although the findings of this pilot study can contribute additional knowledge of PLDS and are important information to caregivers dealing with LB, the complete mechanisms of the phenomenon need to be further investigated.

MRI-findings in patients with persistent symptoms after treatment of neuroborreliosis (Paper IV)

WML and lesions in the basal ganglia were seen in both patients and matched controls, without any significant differences between the groups (Table 15). There was also no difference between patients and controls regarding the three subtypes of WML (periventricular, central and subcortical). Subependymal lesions were more frequently found and were more advanced in their extent (Figure 23) among the patients (median degree=1, as compared to 0.5 in controls), without being statistically significant (Table 15). WML were detected in controls ≥43 years of age and subependymal lesions were found in controls ≥56 years. In contrast, in patients the corresponding lesions were found down to the ages of 42 and 25 (Figure 24), respectively. A significant correlation was found between age and the total number of lesions, in both patients (rho=0.83, p<0.01) and controls (rho=0.61, p<0.05) (data not shown). In addition, the degree of subependymal lesions was also significantly correlated to age in both patients (rho=0.66) and controls (rho=0.73) (for both p<0.01). Neither the number of WML nor the degree of subependymal lesions was correlated to the duration of symptoms post-treatment (rho=0.35 and 0.44, respectively). No focal gray matter or cranial nerve lesions were found.
### Table 15. Magnetic resonance imaging (MRI) findings in patients with persistent symptoms after treatment of neuroborreliosis.

<table>
<thead>
<tr>
<th>Pair number</th>
<th>Number of WML</th>
<th>Periventricular WML</th>
<th>Central WML</th>
<th>Subcortical WML</th>
<th>Degree of subependymal lesion</th>
<th>Lesions in basal ganglia</th>
<th>Lesions in gray matter</th>
<th>Cortical atrophy</th>
</tr>
</thead>
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<tr>
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<td>n</td>
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</tr>
</tbody>
</table>

Cases with positive MRI findings: 12/10 median 1 median 0.5 2 1 0 0 2 1

Mc Nemar test: $p=0.50$ $p=1$ $p=1$ $p=0.29$ $p=0.12$* $p=1$ 11 11

* Wilcoxon signed-rank test.

**WML, white matter lesions; P, patient; C, control; y/n, yes/no. Uncountable defined as >100 WML.***

### Results and Discussion

- **Degree of subependymal lesion:**
  - 0 = none
  - 1 = slight
  - 2 = moderate
  - 3 = severe

- **Lesions in basal ganglia:**
  - Present = y
  - Absent = n

- **Lesions in gray matter:**
  - Present = y
  - Absent = n

- **Cortical atrophy:**
  - Present = y
  - Absent = n
Figure 23. An 83-year old female with persistent symptoms >12 years after treatment of neuroborreliosis (patient no. 4, Table 8). The axial fluid-attenuated inversion recovery (FLAIR) image demonstrates severe (grade 3) subependymal capping along with lesions in the white matter in the periventricular, central and subcortical regions. Cortical atrophy is also present.

Figure 24. A 25-year old man, with symptom duration >3 years post-treatment of neuroborreliosis (patient no. 5, Table 8). The axial fluid-attenuated inversion recovery (FLAIR) image demonstrates slight subependymal capping (grade 1).
RESULTS AND DISCUSSION

Taken together, no specific MRI-findings were seen in patients with long-term, persisting symptoms following treatment of NB. Comparative MRI studies of post-treatment NB patients are lacking. Morgen et al.\(^{247}\) characterized MRI-findings in a heterogeneous group of patients with PLDS and they found nonspecific (compared with healthy controls), subcortical white matter hyperintensities in the patients, and the findings correlated with symptom duration, but not with age. Another study by Fallon et al.,\(^{358}\) demonstrated objective abnormalities in functional brain activity (cerebral blood flow and metabolism) in patients with memory impairment after treatment of LB (Lyme encephalopathy), compared to matched controls. In this study, five patients (33%) reported memory deficits and three (20%) reported concentration difficulties, but the complaints had not been objectively assessed with memory tests prior to inclusion. No correlation between these complaints and MRI-findings was found either. Previous studies investigating MRI-lesions particularly in NB have mainly focused on the acute or untreated, late stage of the infection,\(^{126,359-362}\) with highly variable diagnostic criteria applied for the included patients. In addition, the MRI-findings have been reported differently and controls have not consistently been included. Thus, we aimed to systematically include all kinds of previously described MRI-findings in NB in the study protocol. We included several sequences, including IV contrast as well as diffusion-weighted imaging and the fluid-attenuated inversion recovery (FLAIR), which is considered more sensitive in detecting WML.\(^{363,364}\) The previously reported findings of high-signal WML\(^{126,247,365}\) were also found to some extent in this study, and are also in agreement with previous age-related MRI-findings.\(^{334}\) Surprisingly though, subependymal lesions were found in a patients as young as 25 years of age. The cause and importance of these findings, and whether they may represent low-grade inflammation or tissue damage post-treatment, as has been proposed,\(^{254,297,366,367}\) is not known. However, the lesions did not correspond to findings in other diseases (e.g. MS), nor did they correlate with the symptom duration. The non-significant findings may be related to the small study population and the widespread age range (23-83 years, Table 8). However, the study is unique in its kind and would reasonably have revealed any evident differences between the patients and controls.
CONCLUDING REMARKS

EM is an early manifestation of LB with a good prognosis following antibiotic therapy. Nonspecific symptoms, mainly in the form of musculoskeletal pain and fatigue were shown to persist in some EM patients for more than six months post-treatment, but tended to disappear with time. We found an association between the post-treatment symptoms and a decreased early expression of inflammatory, Th1-type cytokines in EM skin biopsies from the symptomatic patients. No correlation between clinical characteristics, allergic predisposition, B. burgdorferi genotype or serology and the development of symptoms post-treatment was found.

Asymptomatic B. burgdorferi-seropositive individuals are interesting from both clinical and immunological perspectives, since they have apparently encountered B. burgdorferi without developing symptoms of LB. These individuals were found to display a significantly increased innate inflammatory immune response to live B. garinii spirochetes, induced by DCs and whole blood cells, in comparison with patients with a history of subacute NB, and healthy controls. Whether this is the optimal immune response to B. burgdorferi remains to be determined.

A randomized, placebo-controlled trial showed that three weeks of doxycycline therapy did not improve objective neurological signs, subjective symptoms or quality of life in NB patients with persistent symptoms post-treatment. Nor could any doxycycline-mediated effects on systemic cytokine responses be demonstrated. Consequently, the use of doxycycline for treatment of persistent symptoms post-NB is not recommended.

Brain MRI-findings in NB patients with persistent symptoms post-treatment were shown to be nonspecific and to correlate with age, but not with the duration of symptoms. Thus, brain MRI does not facilitate diagnosis of, or provide an explanation for the persistent symptoms.

Finally, I conclude this thesis by stating that the clinical outcome of LB is most probably dependent on a complex interplay between B. burgdorferi, the host's immune response and vulnerability, and diverse effects of antibiotic therapy. Future studies may shed light on further details of this interaction.
CONCLUDING REMARKS

FUTURE STUDIES

In view of the results of this thesis, it would be interesting to develop and further pursue some of the matters arising. In fact, several important studies addressing other clinical, immunological and diagnostic aspects of LB are already underway in our research group. For instance, comparison of the occurrence of single-nucleotide polymorphisms in TLR between healthy controls and patients with persistent symptoms after treatment of NB has been carried out in a small patient population and will be re-evaluated in a larger population, with additional comparison with asymptomatic B. burgdorferi-seropositive subjects.

A prospective MRI-study of patients with early NB, with follow-up MRI on two occasions after antibiotic treatment, is running and will be completed in a few years. The aim and expectation of this study is to be able to follow the development of any structural brain lesions in NB and to relate them to clinical, immunological and microbiological characteristics, as well as to the outcome of the infection.

A personal goal ahead is also to develop a comprehensive database of all patients with NB in the county of Östergötland, Sweden, and to create a biobank with blood and CSF samples, which can be used for future research. In addition, it would be interesting to conduct a follow-up study of patients with NB and enterovirus meningitis, in order to compare the clinical outcome of these two infections and to relate any persistent symptoms to a matched control population.

LB diagnostics are subject to continuous development, why it is important to review, in an interdisciplinary and evidence-based manner, the local laboratory routines at our hospital in the near future. A well-functioning cooperation in the region has already been established.

At last, the national “STING study” is underway. It is unique in that it includes tick-bitten patients and the corresponding tick, subjecting both to extensive analysis and follow-up. The study will be able to resolve epidemiological, microbiological, clinical, diagnostic, immunological and prognostic issues of tick ecology and tick-borne infections in Scandinavia.
GENERAL ADVICE ON LYME BORRELIOSIS TO HEALTHCARE PROVIDERS

LB is a tick-borne infection with, in most cases, a characteristic clinical picture and a good prognosis after antibiotic treatment. Long-term, nonspecific complaints, without objective manifestations, are seldom caused by *B. burgdorferi*. To facilitate and optimize the diagnostics, treatment and follow-up of patients with suspected LB, it is desirable that the following advice is taken into account in meeting with the patient:

The local diagnostic guidelines for LB should always be followed. The Swedish guidelines for serologic testing are presented in Table 3.

Diagnosis of NB should *never* be made without doing lumbar puncture and analysis of cells, *B. burgdorferi* antibodies and other inflammatory markers in the CSF. An uncertain diagnosis complicates the future course of the patient – especially if symptoms persist after antibiotic treatment.

It is important to adhere to evidence-based treatment recommendations for LB, in order to avoid complications of the infection and the treatment, as well as to minimize ecological impacts. The Swedish treatment recommendations for LB are presented in Table 4.

A patient with LB, who remains unwell after treatment, should be discussed with a specialist in the field or be referred to a clinic for infectious diseases for further evaluation. The importance of giving correct information on LB to the patient, as well as carrying out evidence-based investigation and treatment cannot be stressed enough.

Last, but not least, patients who suffer from persistent symptoms after adequate diagnosis and treatment of LB should be further investigated to exclude treatment failure and other causes of the symptoms, as well as be offered symptom-relieving treatment and support. Repeated antibiotic treatment of nonspecific, persistent symptoms post-treatment is not recommended.
ACKNOWLEDGEMENTS

I would like to bring this thesis to an end by expressing my sincere gratitude to all the people, who in one way or another, have contributed to its completion. In particular, I would like to thank:

**Pia Forsberg**, my dear supervisor, mentor and good friend. Your charisma, positive attitude and clinical experience have been very valuable to me during my time as a PhD-student. You introduced me to the fascinating world of tick-borne infections and made me realize the benefits of combining clinical and pre-clinical research. Your attitude, that "everything is possible", has strengthened and encouraged me both as a physician and researcher. It has been a pleasure to work by your side! You are a true role model for me!

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****

113
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