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Neuroborreliosis in Childhood

Clinical, Immunological and Diagnostic Aspects

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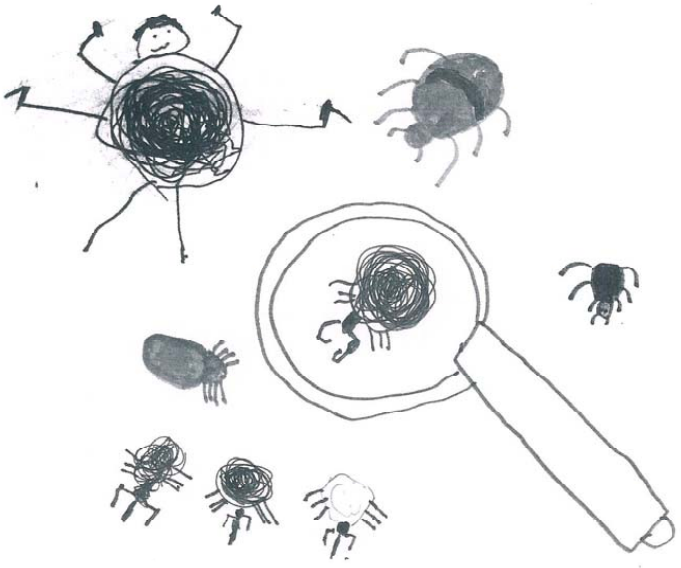
To Stefan



”Ticks and other weird bugs…”, by Otto, Bobo and Balder

ABSTRACT

Lyme Borreliosisis is a multi-organ infectious disease caused by the spirochete *Borrelia burgdorferi*. The spirochete is transmitted to humans by tick bites. Neuroborreliosis (NB) is a disseminated form of the disease, in which the spirochetes invade the nervous system. In children, subacute meningitis and facial nerve palsy are typical clinical manifestations of NB.

The aim of this thesis was to study clinical, immunological and laboratory characteristics in children being evaluated for NB in a Lyme endemic area of Sweden, in order to identify factors of importance for prognosis and clinical recovery. A total of 250 patients and 220 controls were included during 1998-2005, with a prospective and a retrospective part.

Less than half (41%) of children with signs and symptoms indicative of NB got the diagnosis confirmed by detection of *Borrelia* specific flagella antibodies in CSF (clinical routine method). Surprisingly few patients were diagnosed as having other infectious or neurologic diseases and consequently, many patients ended up with an uncertain diagnosis. However, four new *Borrelia* antigens (DbpA, BBK32, OspC, IR6) were evaluated and performed well in laboratory diagnostics. If they were combined in a panel, together with the flagella antigen, the sensitivity was 82% and the specificity 100%, leading to improved diagnostic accuracy in children with NB, as compared to using the routine flagella antibody test alone.

Clinical recovery at the 6-month follow-up (n=177) was generally good and nonspecific symptoms, such as headache and fatigue, were not more frequently reported in patients than in controls. No patient was found to have recurrent or progressive neurologic symptoms.
However, permanent facial nerve palsy was found in 22% of patients at the 2-year follow-up, with consequences such as eye-closing problems, excessive tear secretion, pronunciation difficulties and cosmetic complaints.

When cellular immune responses were investigated, the number of *Borrelia*-specific IL-4 and IFN-γ secreting cells in CSF was found to be more prominent in children with NB than in controls. Furthermore, a much stronger IL-4 response in CSF was seen in children as compared to adults with NB. This cytokine profile of children with NB is believed to represent an effective and balanced type1/type2 response in a relevant compartment, and could contribute to the less severe course of the disease seen in children as compared to adults with NB.

No prognostic factors were found to influence the outcome in patients with “Confirmed NB” or facial nerve palsy. Nor was any specific cytokine profile, or antibody response to new *Borrelia* antigens in CSF, correlated to a less favorable clinical outcome.

An NB prediction score test, based on clinical features on admission, is suggested to help physicians to determine whether to start early antibiotic treatment, before results from *Borrelia* antibody tests are available.

Results in this thesis support the notion that mononuclear pleocytosis in CSF, in patients being evaluated for NB, indicates that they are true NB cases despite the fact that an antibody response cannot yet be visualized with the routine flagella test. Consequently, early antibiotic treatment in NB seems to be the correct cours of action and over-treatment is not a substantial problem.

SAMMANFATTNING PÅ SVENSKA

Borrelia-infektion hos barn och vuxna är den vanligaste fästingburna infektionen i Sverige och orsakas av en bakterie som heter *Borrelia burgdorferi.* Den sprids till människa via fästingbett och kan orsaka besvär från hud, leder, hjärtmuskel och nervsystem. När nervsystemet är infekterat kallas det Neuroborrelios.

Denna avhandling handlar om Neuroborrelios hos barn i syd-östra Sverige, ett område med hög Borrelia-förekomst. Jag har studerat symtom, laborativa provsvar och tillfrisknande hos 250 barn med misstänkt Neuroborrelios under åren 1998-2005 och jämfört med friska barn. Dessutom har jag tittat närmare på vissa signalsubstanser inom immunförsvaret i blod och ryggvätska och vilken roll signalsubstanserna spelar för förlopp och utläkning av infektionen. Avhandlingen innehåller också en utvärdering av fyra nya diagnostiska test vid misstänkt Neuroborrelios hos barn.

Det visar sig att mindre än hälften (41%) av barnen med misstänkt Neuroborrelios får diagnosen säkerställd med det befintliga Borrelia-testet (baserat på ett protein som kallas flagellin) som används rutinmässigt. Dock förblir diagnosen oklar för många barn (59%).
De fyra nya Borrelia-testen (baserade på protein som kallas DbpA, BBK32, OspC och IR6) visar sig fungera bra och om man kombinerar dem med befintligt Borrelia-test, kan man säkerställa Neuroborrelios hos 82% av barnen med misstänkt infektion. Jag hoppas att dessa nya Borrelia-test i framtiden kan leda till förbättrad diagnostik hos barn som utreds för misstänkt Neuroborrelios.

Immunförsvarets signalsubstanser, som analyserades i ryggvätska och blod, visade sig ha en viss profil hos barn med Neuroborrelios jämfört med barn utan Borrelia-infektion, men även jämfört med vuxna med Neuroborrelios. De immunologiska T cellerna producerade två olika sorters signalsubstanser, som kallas ”Interferon-γ” och ”Interleukin-4”. Denna immunologiska profil verkar fördelaktig och kan möjligen bidra till den i allmänhet goda utläkning av Neuroborrelios som man ser hos barn jämfört med vuxna.

De vanligaste symtomen vid en Borrelia-infektion i nervsystemet är huvudvärk, trötthet, dålig aptit, feber och ont i nacken. Ansiktsförlamning är det vanligaste specifika neurologiska symtomet. Antibiotikabehandling ges till 69% av barnen och vid en 6 månaders uppföljning rapporterar patienterna god utläkning av de olika symtomen. Inget barn hade återkommande eller allvarliga neurologiska symtom vid uppföljningen. Däremot, barn med ansiktsförlamning visade sig få kvarstående besvär i viss utsträckning. När de undersöktes 2 år efter sin ansiktsförlamning förekom mild till måttlig kvarstående förlamning i 22% av fallen. Patienterna uppgav besvär av ökat tårflöde, sluddrigt tal, svårigheter med att stänga ögat och dessutom rapporterade många patienter att snedheten i ansiktet var kosmetiskt störande.

Inga specifika symtom, laborativa prov, immunologiska signalsubstanser eller diagnostiska test visade sig vara kopplade till ökad risk för kvarstående besvär efter Neuroborrelios i allmänhet och inte eller hos patienter med ansiktsförlamning.

En checklista har utarbetats med olika symtom som är typiska för barn med Neuroborrelios. Den föreslås kunna användas som beslutsunderlag för start av tidig antibiotikabehandling, redan innan svar på Borrelia-testen finns tillgängliga.

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ABBREVIATIONS

ab = antibodies
ACA= Acrodermatitis chronicum atrophicans
B. b = Borrelia burgdorferi
BBK32 = protein named after it's gen
BCG = calmette tuberculosis vaccination
BSK = Barbour-Stroenner-Kelly
CBMC = cord blood mononuclear cell
CDC = Centers for Disease Control and prevention
CMV = cytomegalo virus
CNS = central nervous system
CSF = cerebrospinal fluid
CSF-L = cerebrospinal fluid lymphocyte
CSF-MNC = cerebrospinal fluid mononuclear cell
CT = computer tomography
d = days
DbpA = decorin binding protein A
DNA = deoxyribonucleic acid
EBV = Epstein Barr virus
ELISA = enzyme-linked immunosorbant assays
ELISPOT = enzyme-linked immunospot assay
EM = erythema migrans
ENT specialist = Ear-Nose- and Throat specialist
f = female
Fp = facial nerve palsy
Healthy = adults with no proven infection
HGA = human granulocytic anaplasmosis
HGE = human granulocytic ehrlichiosis
HLA = human leukocyte antigen
HSV = Herpes Simplex virus
i.v. = intravenous
IFN-γ = interferon gamma
LB = Lyme borreliosis
LFA = leukocyte function-associated antigen
Ig = immunoglobulin
IL = interleukin
IR6 = invariable region 6 peptide
m = male
m = month
N.d = not determined
n.s. = non significant
NB = neuroborreliosis
OD = optical density
OF = outer surface protein-enriched fraction
OND = other neurologic diseases
Osp = outer surface protein
OspC = outer surface protein C
p.o. = peroral
PBL = peripheral blood lymfocyte
PBMC = peripheral blood mononuclear cell
PCR = polymerase chain reaction
PcV= phenoxylmethyl penicillin
PHA = phytohaemagglutinin
Poss. NB = possible Neuroborreliosis
r = recombinant
rho = correlation coefficient
RNA = ribonucleic acid
PRR = pattern recognition receptor
RT = room temperature
SPSS = statistical products and service solution
Susp. NB = suspected NB
TBE = Tick-borne encephalitis
TCM = tissue culture medium
TGF = transforming growth factor
Th1 = T helper lymphocyte type 1
Th2 = T helper lymphocyte type 2
TLR = Toll-like receptor
TNF = tumor necrosis factor
VlsE = variable protein-like sequence expr. site
VP-shunt = ventriculo-peritoneal shunt
VZV = Varicella Zoster virus
WB = western blotting
w = week

ORIGINAL PAPERS

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

I. **B H Skogman**, S Croner, L Ödkvist: Acute facial palsy in children – a 2-year follow-up study with focus on Lyme Neuroborreliosis, Int J of Ped Oto-Rhino-Laryngology (2003) 67; 597-602

II. M Widhe, **B H Skogman,** S Jarefors, M Eknefelt, G Eneström, M Nordwall, Christina Ekerfelt, S Croner, S Bergström, P Forsberg, J Ernerudh: Up-regulation of *Borrelia* specific IL-4 and IFN-γ secreting cells in cerebrospinal fluid from children with Lyme Neuroborreliosis, Int Immunology (2005) 10; 1283-1291.

III. **B H Skogman**, S Croner, P Forsberg, J Ernerudh, P Lahdenne, H Sillianpää, I Seppälä: Improved Laboratory Diagnostics of Lyme Neuroborreliosis in Children by Detection of antibodies to New Antigens in Cerebrospinal Fluid. Ped Inf Dis J, accepted

IV. **B H Skogman**, S Croner, M Eknefelt, M Norwall, J Ernerudh, P Forsberg: Lyme Neuroborreliosis in Children – a Prospective Study of Clinical Outcome and Prediction of Diagnosis. Ped Inf Dis J, submitted

INTRODUCTION

Lyme Borreliosis

Lyme Borreliosisis (LB), also named Lyme disease (LD), is a multi-organ infectious disease caused by the spirochete *Borrelia burgdorferi* (Steere 1989). The spirochete is transmitted to humans by hard ticks primarily in the temperate zones of the northern hemisphere. In Sweden, LB is the most important vector-borne infection (Berglund et al. 1995; Gustafson et al. 1990). The skin, joints, heart or nervous system can be involved and symptoms can be localized, early disseminated or late persistent (Evans 2000). Neuroborreliosis (NB) is one of the disseminated forms of the disease in which the spirochetes invade the nervous system (Bingham et al. 1995; Garcia-Monco et al. 1995; Oschmann et al. 1998).

Historical notes

The first clinical manifestations of LB were described in 1883 (Buchwald 1883). The German physician Buchwald presented a case of diffuse idiopathic skin atrophy, as a suggested cutaneous manifestation of a tick born infection. It was later described as Acrodermatitis chronica atrophicans (ACA), a typical skin lesion in LB (Herxheimer et al. 1902). In 1910, a Swedish dermatologist described a tick bite associated, annular, red, skin lesion as; an Erythema chronicum migrans Afzelius (ECMA) (Afzelius 1910). In the following year (1911) the third cutaneous manifestation of LB, the lymphocytoma, was described, but not characterized in detail until in 1943 by a Swedish dermatologist (Bäfverstedt 1943). Neurologic symptoms connected to tick bites, were first suggested by the French neurologists Garin and Bujadoux (Garin et al. 1922). Later, the triad of meningitis, cranial nerve palsy and radicular pain, the "Bannwarth's syndrome" was described (Bannwarth 1941). In the 1950s, penicillin was presented as a curative treatment for EM and meningitis (Hellerström 1951; Hollström 1951). Not until 30 years later, on the opposite side of the Atlantic, was the causative agent identified. In 1972 in a small town called Lyme in south Connecticut USA, a cluster of children and young adults presented with arthritis of unknown etiology. Alan Steere later described Lyme arthritis (LA) (Steere et al. 1977). The causative agent, the spirochete *Borrelia burgdorferi*, was identified in 1982 by William Burgdorfer (Burgdorfer et al. 1982) and from this point, the term Lyme Borreliosis (LB) was commonly used.

Borrelia burgdorferi - the spirochete

The *B. burgdorferi* is a large gram-negative, helical shaped, highly motile but slowly reproducing spirochete, 5-20 μm long and 0.2- 0.3 μm wide. It has a protoplasmic cylinder with a linear chromosome and several linear and circular plasmids in the cytoplasm (Casjens 2000), a periplasmic space with 7-11 attached flagella and a trilaminar outer surface membrane (Figure 1). The main structural component of the flagella is flagellin, a 41 kDa protein (Shapiro et al. 2000) which have often used as the major immunological antigen in diagnostic tests (van Dam 2001).

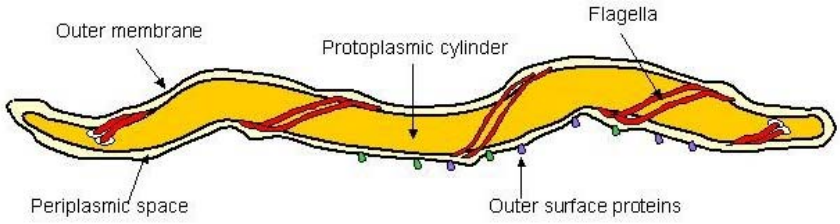


Figure 1 The *Borrelia* spirochete

On the outer surface, the spirochete has mainly lipoproteins (OspA, B, C, E, F), which are strongly immunogenic (Ma et al. 1993) but also genetically highly variable (Embers et al. 2004; Ma & Weis 1993; Zhang et al. 1997). This is advantageous for the spirochete when it comes to evading the immune response in the host (de Silva et al. 1998) and probably also the main reason for difficulty in finding sensitive diagnostic tests.

Mainly three subspecies are pathogenic to humans and associated with clinical presentations in the family of *B. burgdorferi* sensu lato (Wang et al. 1999). *B. burgdorferi* sensu stricto is connected mainly to arthritis, *B. afzelii* to cutaneous manifestations and *B. garinii* to neurologic disease (van Dam et al. 1993). However, all subspecies may cause EM or the different clinical manifestations and all three subspecies have been identified in CSF in paitents with NB (Ornstein et al. 2002).

Ticks - the vectors

Different species of ticks act as vectors for *Borrelia* spirochete around the world; *Ixodes scapularis* or *Ixodes Pacificus* in Northern America, *Ixodes persulcatus* in Asia and *Ixodes ricinus* in Europe (Gern et al. 2000; Miyamoto et al. 1991; Piesman 2006). It has been suggested that *Ixodes uriae* transmits the *Borrelia* spirochete around the world by residing on seabirds (Gylfe et al. 2001; Olsen et al. 1993). The life cycle of the *Ixodes ricinus* is a complex process involving four stages: egg, larva, nymph and adult (Figure 2-4) (Parola et al. 2001).

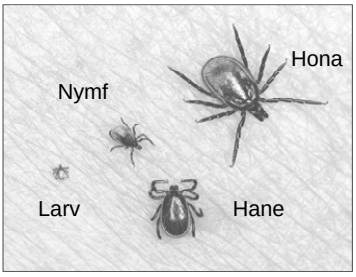


Figure 2. Four stages of ticks

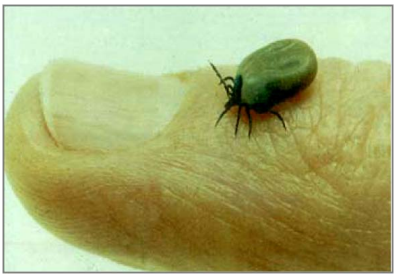


Figure 3. Female tick after a blood meal

A blood meal is required to develop from one stage into the next stage in the life cycle. Female adults need to feed on extra large blood meals to lay eggs. During winter, ticks are resting in diapause but when the air temperature exceeds 4-6° C they become active (Duffy et al. 1994). In southern Sweden, a tick normally completes its life cycle in 3-4 years.
They prefer areas with high humidity and are often found on grass, 10-50 cm above ground, waiting for a host to pass by. The ticks are eyeless but have special sensory organs that are believed to detect heat radiation, movements, butyric acid and carbon dioxide (Parola & Raoult 2001). Ticks seek hosts, such as different mammals, birds and reptiles (Anderson 1989) whereas humans are incidental hosts for the ticks. Preferentially, larvae feed on small rodents, nymphs on birds and medium-size mammals and adult ticks on large animal hosts, such as deer (Parola & Raoult 2001) .
The tick's mouthparts are specially adapted for firm attachment to the host's skin and for blood sucking. The tick is able to maintain the *Borrelia* spirochetes, or other potential pathogens ingested with a blood meal, and can transmit the pathogen to a new host during a subsequent blood meal. The transmission of the spirochete from ticks to humans is estimated

to take 24-48 hours since the spirochetes reside in the mid-gut of the tick and need to migrate to its salivary glands (Piesman et al. 1987a). The tick's saliva contains anticoagulants, local anesthetics, antihistamines and different enzymes which facilitate the migration of the *Borrelia* spirochete in the tissue and hide (Figure 7) (Piesman et al. 1987b).

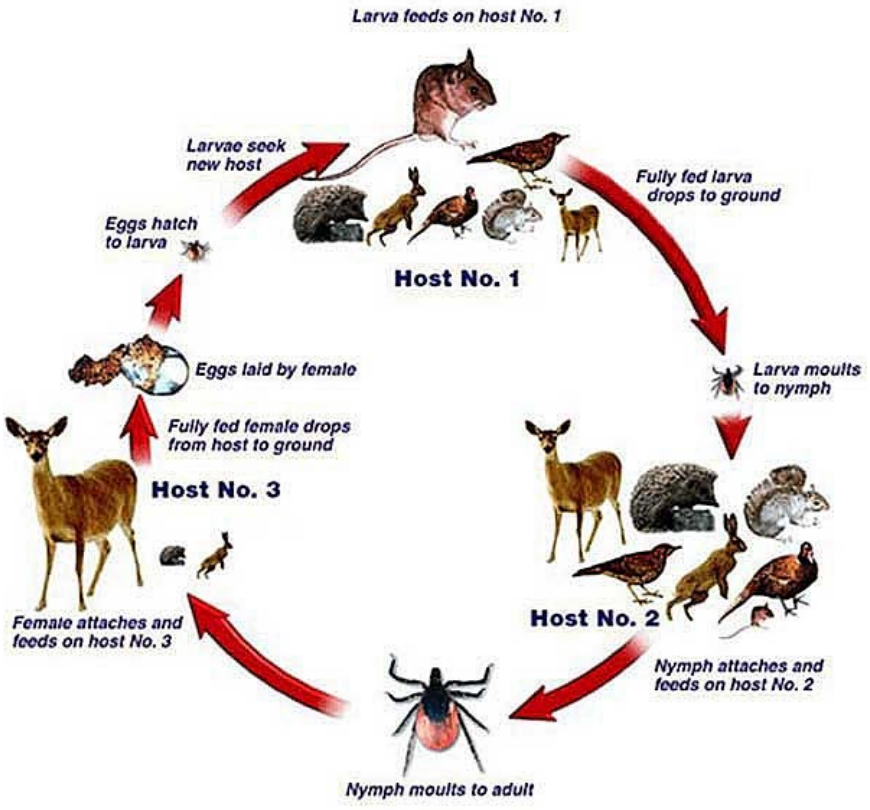


Figure 4. The life cycle of the tick

Epidemiology, risk and prevention of Lyme Borreliosis

LB is the most common tick-borne infection in Europe and the USA (Stanek et al. 2003; Steere 2006; Steere 1989). Epidemological studies in Scandinavia have shown that, in high endemic areas along the coast or on islands in the archipelago, 19 - 26 % of inhabitants were positive to IgG *Borrelia* antibodies in serum (Carlsson et al. 1998; Gustafson et al. 1990). In older age groups and with a female dominance, an increase in seroprevalence has been reported (Carlsson et al. 1998). Seroprevalence in children was 2.6 % in a Lyme endemic area in Germany, (Christen et al. 1993) and 15 % in Slovenia (Cizman et al. 2000).

In a 1-year prospective population based study in southern Sweden, the overall incidence of LB was 69 cases per 100.000 inhabitants per year, with considerable variation between different counties in the study area (Berglund et al. 1995). A peak at 5 - 9 and 60 - 74 years of age was reported. A slight male predominance in younger ages, with a clear female predominance in older ages was seen (Berglund et al. 1995). Interestingly, exactly the same pattern was found in a Lyme endemic area in Germany in 1996 – 97, but with a generally higher incidence rate of 110 per 100,000 inhabitants (Huppertz et al. 1999).
The yearly incidence of NB in childhood was 5.8 per 100,000 children (0 to 13 years of age) in Lower Saxony in Germany (Christen et al. 1993).

Risk factors for human exposure to ticks and tick-borne diseases depend upon tick abundance and geographic distribution of ticks in the area (Randolph 2001; Robertson et al. 2000).
It is suggested that climate factors are of great importance in the short and long perspective variations (Bennet et al. 2006b; Lindgren et al. 2000; Randolph et al. 1999). Furthermore, behavior in the countryside, light colored clothing, daily tick body checks and early removal of ticks from the skin can reduce the risk of aquiring Lyme Borreliosis (Stjernberg et al. 2005a; Stjernberg et al. 2001; Stjernberg et al. 2002).
A vaccine based on recombinant OspA for immunization against *B. burgdorferi s.s.*, was developed in 1998 and introduced in the USA. It was proven to be effective and safe in adults (Sigal et al. 1998) and in children (Sikand et al. 2001). However, four years later, the vaccine was withdrawn from the market due to limited acceptance. The reasons were probably the relatively high cost and need of frequent booster doses, in combination with the concern that, in rare cases, the vaccine might trigger an autoimmune arthritis (which was seen in mice but never proven in humans) (Hanson et al. 2003; Steere 2006).

Other tick-borne infections

Tick-borne encephalitis (TBE)

TBE is caused by a flavivirus and is the second most important tick-borne infection in Europe (Charrel et al. 2004). It is a spring-summer disease with a typical biphasic course in the majortity of patients. Initially, there is an influenza-like phase lasting about one week, which is followed by one relatively asymtomatic week. In the second phase the patient experiences symptoms of meningitis with high fever, headache, neck stiffness and nausea, which in severe cases, is acompanied by convulsions, encephalitis and neurological deficits. The majority of patients recover completely, but in adults both neurologic and neuropsyciatric sequelae have been reported in up to 30% of cases (Mickiene et al. 2002). Children seem to have a less severe course of the disease (Cizman et al. 2000; Cizman et al. 1999; Logar et al. 2000). Total recovery was reported in 371 children with TBE in Slovenia (Lesnicar et al. 2003) but in rare cases, sequelae may occur (Cizman et al. 1999).
There is a safe and effective vaccine (Wittermann et al. 2008) and, for children living in TBE endemic areas, immunization is recommended from 7 years of age (Skogman et al. 2004).

Human granulocytic anaplasmosis (HGA)

As a veterinary disease, tick-borne fever (ehlichiosis) has been known since the 1930s. In humans, the disease, human granulocytic ehrlichiosis (HGE) was not recognized until 1994 in the USA (Bakken et al. 1994; Chen et al. 1994) and a few years later in Eurpoe (Petrovec et al. 1997). The gram-negative obligate intracellular bacteria invades granulocytes and resides in membrane bound vacuoles, referred to as morulae (Carlyon et al. 2003). These morulae can be seen in blood-smears under a microscope, which could be used as a visual diagnostic test (Bakken et al. 2000). However, the sensitivity is too low, and therefore PCR or serological testing has been preferred (Bjoersdorff et al. 1999; Brouqui et al. 2001). In 2001 the bacteria was renamed *Anaplasma phagocytophilium* (Bakken et al. 2001b; Dumler et al. 2001) and consequently the infectious disease is now called human granulocytic anaplasmosis (HGA) (Brouqui et al. 2004). Typical clinical manifestations are fever, malaise, chills, myalgias and arthralgia. Laboratory findings show leucopenia, thrombocytopenia, elevated liver enzymes and occasionally, low red blood cell count (Bakken et al. 2001a). In most cases the disease is mild, but can be fatal. Doxycycline is the drug of choice for treatment and the efficacy of alternative antibiotics is uncertain (Lantos et al. 2002). Since doxycycline may cause staining

of the tooth enamel (Grossman et al. 1971), it should be avoided for young children, but rifamin or flouroquinolones could possibly be used instead (Dumler et al. 2007).

Rickettsioses

Rickettsioses is one of the oldest known vector-borne diseases (Parola & Raoult 2001). It is caused by an obligate intracellular bacteria, belonging to the genus *Rickettsia*, and in 1989 the Rocky Mountain spotted fever (*R. rickettsii*) was the first rickettsiosis described. Seven to ten days after a tick bite, symptoms such as fever, headache, rash, lymphadenopathy and a typical inoculation eschare ("tache noire") occurs along with thrombocytopenia, leukocytopenia and elevated liver enzymes (Brouqui et al. 2004; Parola & Raoult 2001). First line antibiotic treatment is doxycycline, but several other antibiotics are effective (Parola & Raoult 2001). Different rickettsioses have been described in children (Bitsori et al. 2002), including patients with meningitis or facial nerve palsy (Vander et al. 2003).

Rickettsia helvetica is so far the only rickettsial species isolated from ticks in Sweden (Nilsson et al. 1999) and it has been diagnosed by serologic testing in patients with previous tick bites, as well as in blood donors (Elfving et al. 2008; Nilsson et al. 2005b).

Pediatric patients have not been studied in Sweden.

Clinical characteristics of Lyme Borreliosis

Clinical signs and symptoms form the basis for the recognition of the disease. LB can be divided into three stages: early localized, early disseminated and late persistent (Eppes 2003; Evans 2000). Clinical features are different depending on the subtype of *Borrelia* causing the disease (van Dam et al. 1993). *B. burgdorferi* s.s. is the only subspecies present in the USA and consequently, LB in Europe and the USA differ in clinical picture (Steere 2006). EM has a faster expansion in the skin and is associated with more frequent systemic symptoms when caused by *B. burgdorferi s.s* (USA). Furthermore, the chronic skin lesion Acrodermatitis chronicum atrophicans, ACA, is mainly caused by *B. afzelii* and consequently rarely reported in the USA. Finally, carditis occurs more often in the USA due to *B. burgdorferi s.s*. In addition, clinical manifestations of LB differ between children and adults (Berglund et al. 1995; Huppertz et al. 1999), as shown in Table 1.

Table 1. Proportions of clinical manifestations of LB in children and adults in Europe and USA

	Sweden Berglund 1995	**Germany** Christen 1995	**Germany** Huppertz 1999	**USA** Asch 1994	**USA** Gerber 1996
Children	**n=232**	**n=208**	**n=62**	**n=51**	**n=201**
EM	65 %	13 %	77 %	47 %	66 %
Multiple EM	-	3 %	-	-	23 %
Lymphocytoma	7 %	2 %	5 %	-	-
NB	28 %	78 %	7 %	29 %	4 %
Arthritis	-	4 %	11 %	41 %	7 %
Carditis	-	-	-	-	<1 %
Adults	**n=1239**		**n=251**	**n= 169**	
EM	74 %		92 %	77 %	
Lymphocytoma	2 %		1 %	-	
NB	14 %		2 %	29 %	
Arthritis	7 %		3 %	41 %	
ACA	3 %		2 %	-	
Carditis	<1 %		<1 %	6 %	

Erythema migrans (EM)

The skin is the most frequently affected organ in early localized LB and EM is the most typical skin lesion. It occurres in all ages, in both sexes and is considered pathognomonic for LB (Stanek & Strle 2003). Days to weeks after a tick bite, a small red macula appears on the skin. As the red skin leasion slowly enlarges, central clearing usually begins, resulting in a ring-shaped patch with marked linings. Interestingly, it has recently been shown that the Borrelia subspecies in combination with the sex of the patient influence the appearance of the EM (Bennet et al. 2006a).Women infected with *B. afzelii* more often presented with a non-annular EM. In children, the EM is more often located in the head and neck area whereas in adults, the lower parts of the body (mainly legs) often are involved (Berglund et al. 1995; Christen et al. 1993). There might be difficulties in correctly diagnosing EM in the axilla, the face or above hairline (Figure 5). Differential diagnoses are other insect bites, eczema and bacterial or fungal skin infection.

Lymphocytoma

The *Borrelia* lymphocytoma is a solitary redish-blue indured skin leasion (Figure 6), most frequently located on the earlobe in children, but on rare occasions it can also occur in the areola mammae in adults (both sexes) (Stanek & Strle 2003). Breast lymphocytomas can cause differential diagnostic problems, such as suspected malignancy with a risk of being operated on false grounds, instead of being treated with antibiotics and cured from a benign lumphocytoma (Strle et al. 1992).

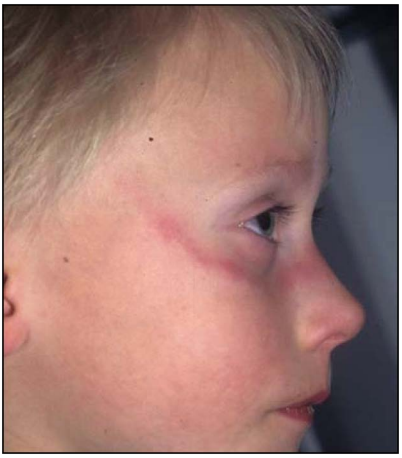


Figure 5. Erythema migrans

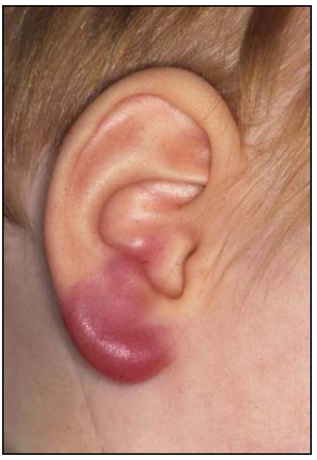


Figure 6. Lymphocytoma

Multiple EM

Bloodstream dissemination (spirochetemia) can result in multiple EM often accompanied by systemic symptoms such as fever, arthralgia, myalgias, headache or fatigue (Gerber et al. 1996). Children with multiple EM are younger and have a longer incubation period (22 vs. 13 days) as compared to children with single EM (Arnez et al. 2003b). Pleocytosis in CSF was found in 25.7 % of children with multiple EM, providing evidence of dissemination and inolvment of the nervous systeme without meningeal symptoms (Arnez et al. 2002). Furthermore, ”flu-like” nonspecific symtoms such as fever, fatigue, headache and neck pain can be seen, predominantly in the USA, in children without EM or neurological symptoms (Feder et al. 1993). The absence of respiratory or gastrointestinal symptoms can help in differential diagnostics between early LB and viral-type illness (Feder et al. 1993).

Neuroborreliosis

Typical clinical features of early disseminated NB are subacute meningitis and involvement of cranial or peripheral nerves (Kristoferitsch 1993; Oschmann et al. 1998). Facial nerve palsy is the most common peripheral nerve involvment in NB and is seen more often in children than in adults (Cook et al. 1997; Ljostad et al. 2005; Peltomaa et al. 1998; Shapiro et al. 1997).

Carditis

If the heart muscle is involved, conduction adnormalities are seen in terms of atrioventricular conduction blocks or bundle branch blocks, but myocarditis or congestive heart failure are rare (Klein et al. 1991; Woolf et al. 1991). In the USA, electrocardiographic (ECG) abnormalities are seen in 29 % of children with LB (Woolf et al. 1991). In Eurpope, the condition is rare and ECGs are not routinely performed in children being evaluated for LB.

Other manifestations

Rare case reports of keratitis, iridocyclitis, myositis, osteomyelitis and fasciitis are published and interpreted as associated with LB (Mikkila et al. 2000; Stanek & Strle 2003). Congenital LB infection, with transmission through the placenta from a *Borrelia* infected mother to the faetus, has been suggested, but no evidence has been found (Sood 2006; Strobino et al. 1993).

Acrodermatitis chronicum atrophicans, ACA

The chronic skin leasion ACA**,** as a manifestation of late persistent LB, is mainly caused by *B. afzelii* occurs only in older patients (median of 65 years of age). It is often accompanied by muscle weekness, radiacular pain or dysestesias but symptoms resolve with antibiotic treatment. (Asbrink 1985; Asbrink et al. 1986).

Arthritis

Myalgias, migratory and recurrent arthralgias and periarticular pain can develop weeks after the tick bite and belongs to early dissiminated LB but the more typical Lyme arthritis with one or several swollen and painful joints, occuring months to years after a tick bite, should be classified as late persistent LB. Differential diagnoses are different forms of rheumatic or reactive arthritis, rather than septic arthritis (Gerber et al. 1998). Recurrent or chronic arthritis, despite antibiotic treatment, is seen in one-third of patients with Lyme arthritis in Germany

(Huppertz et al. 1995). Furthermore, untreated Lyme arthritis may, in rare cases, develop into late persistent NB (Szer et al. 1991). The diagnosis LB arthritis should always be confirmed by elevated titres of *Borrelia* specific IgG antibodies in serum (Huppertz et al. 1995; Sood 2006).

Late persistent NB

Prolonged neurologic manifestations of LB (such as headache, memory difficulties and other cognitive impairement) have been reported in adults but rarely in children (Bingham et al. 1995; Garcia-Monco & Benach 1995; Kristoferitsch 1993; Wang et al. 1998).
In addition, persistent symptoms after NB, despite antibiotic treatment, has been reported from more than half of adult patients (Vrethem et al. 2002) which were significantly more frequent than in a control group. Furthermore, in a 5-year follow-up, both children and adults with NB were included (Berglund et al. 2002). Children reported sequelae in 15 % of cases with facial nerve plasy, ataxia, parestesia and concentration disorders. Adults reported facial nerve plasy, neuropathy, dementia, ataxia, paresthesia or concentration disorders in 30 % of cases. Authors conclude that persistent symptoms after NB exist and are more frequent in adults than in children, but that pathologic mechanisms are not fully understood. Suggested mechanisms are summerized under “Severity of disease” in this thesis.
In larger studies, long-term neuropsychological disorders attributable to Lyme disease are not seen in children (Adams et al. 1999; Wang et al. 1998; Vazquez et al. 2003) , in contrast to adults, where cognitive disorders and persistent or recurrent neurological symptoms are found (Cairns et al. 2005; Halperin 2007; Picha et al. 2006; Shadick et al. 1994; Weber 2001). However, prolonged antibiotic regiment has not been proven to reduce symptoms in long-term follow-up studies (Cairns & Godwin 2005; Halperin 2007; Halperin et al. 2007).
The term “Post-Lyme syndrome” are now used to describe longstanding persistent symptoms after antibiotic treatment of NB (Cairns & Godwin 2005).

Treatment of Lyme Borreliosis

Treatment guidelines for children with LB differ between and within countries since few studies are conducted. Traditions also vary from country to country (Halperin et al. 2007; Sood 1999; Stanek & Strle 2003; Wormser et al. 2006). Randomized prospective studies demonstrate that peroral **doxycyline**, **penicillin**, **amoxicillin** and **cefuroxime axetil** are effective in a 10-14 day treatment of EM (Arnez 2007; Luft et al. 1996; Nadelman et al.

1992). In the USA and Europe, arthritis and lymfocytoma are treated with oral regimen for a 14-28 day period (Wormser et al. 2006). Swedish recommendations are shown in Table 2 (MPA 1998). However, there is a tradition of treating NB with parenteral antibiotics, in the USA. Parenteral **penicillin** and **ceftriaxone** are shown to be equivalent in treatment of NB in children (Mullegger et al. 1991) but ceftriaxone has the advantage of a once daily dosage. In Europe, **oral doxycycline** is the drug of choice for adults, adolescents and children > 8 years of age since it has been proven to be effective, safe and easily administered (Borg et al. 2005; Dotevall et al. 1999a; Kohlhepp et al. 1989). 10 day doxycyline regimens have been shown to be as effective as 14 days (Dotevall & Hagberg 1999a; Thorstrand et al. 2002). However, tooth enamel can be stained in younger patients, and therefore doxycycline should be avoided (Grossman et al. 1971). These children receive parenteral ceftriaxone or penicillin instead (Table 2) (MPA 1998). Finally, doxycycline is the drug of choice if an co-infection with HGA is suspected, which has been highlighted lately (Wormser 2006; Wormser et al. 2006).

Table 2. Antibiotic treatment in Lyme Borreliosis, as recommended in Sweden

	Children 0-8 years	**Children 8-12 years**	**Adults**
EM	PcV 12,5 mg/kg x 2-3 10 days, p.o.	PcV 12,5 mg/kg x 2-3 10 days, p.o.	PcV 1g x 2-3 10 days, p.o.
Lymphocytoma	PcV 25 mg/kg x 3 14 days	Doxycycline 4 mg/kg x 1 14 days	Doxycycline 200 mg x 1 14 days
Arthritis/ACA	Amoxicillin 20 mg/kg x 2 20 days	Doxycycline 4 mg/kg x 1 20 days	Doxycyclin 200 mg x 1 20 days
NB	Ceftriaxone 100 mg/kg x 1 14 days, i.v. or Bensyl pc 50 mg/kg x 3-4 14 days, i.v.	Doxycycline 8 mg/kg x 1 (first 2 days), p.o. Doxycycline 4 mg/kg x 1 12 days, p.o. or Ceftriaxone 100 mg/kg x 1 14 days, i.v. or Bensyl pc 50 mg/kg x 3-4 14 days, i.v.	Doxycycline 400 mg x 1 (first 2 days), p.o. Doxycycline 200 mg x 1 12 days, p.o. or Ceftriaxone 2 g x 1 14 days, i.v. or Bensyl pc 3 g x 3-4 14 days, i.v.

Lyme Neuroborreliosis in children

Case definition

According to Europe case definitions (Stanek et al. 1996), neurological symptoms shall be combined with pleocytosis in CSF and detection of *Borrelia* specific antibodies in CSF.
A serum/CSF index is used in the serologic ELISA methods in order to ensure intrathecal synthesis of *Borrelia* antibodies (Hansen et al. 1991). A flagella antigen is routinely used in Swedish laboratories in comfirming the NB diagnosis (Ekerfelt et al. 2004).

Clinical features

Among children with NB, the vast majority of patients present with facial nerve palsy or subacute meningitis (Belman et al. 1993; Christen et al. 1993). However, in smaller children, the nonspecific symptoms of fatigue, loss of apetite or changes in mood may dominate the clinical picture and the NB diagnosis can easily be missed (Christen 1996).

In facial nerve palsy due to NB, pleocytosis in CSF is present in most cases (Belman et al. 1997; Sandstedt et al. 1985). However, cranial and peripheral nervous system affection, without inflammation in CSF, may occur (Garcia-Monco & Benach 1995; Kruger et al. 1991) Studies on histopathologic mechanisms have not been conclusive and diagnosis may remain uncertain in such cases (Meurers et al. 1990; Roberts et al. 1998). Among all children with facial nerve palsy, the causative agent is *Borrelia* in 33 - 65 % of cases (Albisetti et al. 1997; Christen et al. 1993; Cook et al. 1997; Peltomaa et al. 1998; Tveitnes et al. 2007) whereas in adults the proportion of LB is 10 - 20% (Ljostad et al. 2005; Olsson et al. 1988; Roberg et al. 1991). Other etiologic agents for facial nerve palsy are Herpes Simplex virus (HSV), Varicella Zoster virus (VZV), Epstein Barr virus (EBV), or enterovirus (Christen et al. 1993). Tick borne encephalitis (TBE) virus has not been found to cause isolated facial nerve palsy (Lesnicar et al. 2003; Mickiene et al. 2002)

Several authors have studied Lyme meningitis as compared to viral meningitis and found that low grade fever, longer duration of symptoms, facial nerve palsy, papilledema and mononuclear dominance in pleocytosis in CSF are factors significantly more often associated with Lyme meningitis in children (Eppes et al. 1999; Shah et al. 2005; Tuerlinckx et al. 2003).

Other manifestations of NB are recurrent headache, meningoencephalitis, abducens or oculomotorial nerve palsy. Peripheral nerves are rarely involved but both sensorial and motorial impairments have been described. Occasionally, peripheral radiculoneuritis is combined with facial nerve palsy and meningitis (Christen et al. 1993), earlier described as "Bannwarth's syndrome" in adult patients {Oschmann, 1998 #198; Bannwarth, 1941 #616}. Case reports are published of rare conditions associated with NB, such as myoclonus (Vukelic et al. 2000), ataxia (Ylitalo et al. 1994), trochlear nerve palsy (Muller et al. 1998), cranial polyneuritis (Huisman et al. 1999), acute transverse myelitis (Huisman et al. 1999), Guillain-Barré syndrome (Shapiro 1998), hemiparesis (Klingebiel et al. 2002; Wilke et al. 2000) and sensorineural hearing loss ("sudden deafness") (Peltomaa et al. 2000).

Furthermore, "pseudotumor cerebri", has been identified as a rare manifestation of late LB in children. It is characterized by increased intracranial pressure in the absence of any intracranial space-occupying lesion, as the name indicates (Kan et al. 1998). Clinical symptoms are headache, papilledema and diplopia due to sixth cranial nerve palsy, sometimes accompanied by EM, myalgia or arthralgia. When a lumbar puncture is performed, increased intracranial pressure is found and pleocytosis and/or increased CSF protein may occur (Belman et al. 1993). It is suggested that the pathofysiologic mechanisms of "pseudotumor cerebri" are caused by a direct infectious or inflammatory infiltration (ie immunecomplexes) at the site of the arachnoid villi and consequently disturbed cerebrospinal fluid outflow, or possibly by a secondary autoimmune mechanism (Garcia-Monco et al. 1988; Kan et al. 1998). Treatment with antibiotics, acetazolamide (a carbonic anhydrase inhibitor) and in rare cases ventricular drainage, provides an excellent outcome (Kan et al. 1998).

Clinical outcome

Clinical outcome after antibiotic treatment of NB has been reported as excellent in children (Hansen et al. 1992; Mullegger et al. 1991; Thorstrand et al. 2002; Vazquez et al. 2003).
A long term retrospective study suggests that even if left untreated, long-term clinical recovery is favorable (Niemann et al. 1997). However, since rare cases of late NB with persistent clinical symptoms are reported (Bloom et al. 1998; Kan et al. 1998; Wilke et al. 2000), antibiotic treatment is recommended in NB.
With focus on clinical outcome after facial nerve palsy, 75 - 82 % recovery rate has been reported in children (Niemann et al. 1997; Peltomaa et al. 1998) whereas a less favourable outcome (54 - 77%) has been seen in adults (Bagger-Sjoback et al. 2005; Bjerkhoel et al.

1989; Ljostad et al. 2005). Furthermore, older age has been associated with less favorable outcome (Danielidis et al. 1999) and idiopathic facial nerve palsy of unknown origin, Bell's palsy, has been reported to have a poorer prognosis than Lyme associated facial nerve palsy (Peltomaa et al. 1998). Finally, steroid treatment of Bell's palsy has been shown to improve clinical recovery in adults (Adour et al. 1996; Holland et al. 2004; Sullivan et al. 2007; Taverner et al. 1971) but not in children (Ashtekar et al. 2005; Salman et al. 2001; Unuvar et al. 1999).

Four cases of childhood Neuroborreliosis

Case 1.

A **10-year old boy** with right-sided facial nerve palsy and pain behind right ear since 2 days. Previous tick bite 3 years ago. Normal lumbar puncture, no *Borrelia* antibodies in CSF or serum. No treatment.

One week later, no improvement of the facial nerve palsy. Headache. Starts steroid treatment at the ENT clinic for "Bell's palsy". A few days later, pain around elbow, diagnosis "epicondylitis". One week later, acute left-sided facial nerve palsy.

Lumbar puncture with pleocytosis with 273 x 10^6/L mononuclear cells in CSF. Starts peroral antibiotic treatment with doxycycline. Elevated IgM *Borrelia* antibodies in CSF.

Diagnosis: Neuroborreliosis (with bilateral facial nerve palsy and radiculitis).

Outcome: Total recovery of left-sided facial nerve palsy but persistent right-sided facial nerve dysfunction.

Case 2.

A **2-year old girl** with EM on the left cheek. No previous tick bite. Starts peroral penicillin treatment. 6 days later acute left-sided facial nerve palsy. EM in regress. Normal lumbar puncture, no *Borrelia* antibodies in CSF. Slightly elevated IgM *Borrelia* antibodies in serum. No treatment. Persistent facial nerve palsy. 10 days later repeated lumbar puncture with normal CSF. No *Borrelia* antibodies in CSF or serum. No treatment.

Diagnosis: Peripheral Neuroborreliosis (with normal CSF x 2)?

Outcome: Total recovery of the facial nerve palsy.

Case 3.

A **7-year old girl** with left-sided acute facial nerve palsy. No previous tick bite. Lumbar puncture with 600 x 10^6/L mononuclear cells in CSF. Starts intravenous antibiotic treatment with ceftriaxone. One week later abdominal pain, physical examination reveals slightly enlarged liver and spleen and some small lymph nodes.

Diagnosis: Acute lymphatic leukemia (with facial nerve palsy due to CNS involvement).

Outcome: Survival.

Case 4.

A **16-year old girl** presented with soar throat and fever, CRP 29 and Strep A neg. Diagnosed as "viral infection". 5 days later headache, nausea, vertigo, vision disturbancies and right-sided acute facial nerve palsy with hyperestesia. Physical examination shows bilateral papilledema. Normal computer tomography (CT). No lumbar puncture was performed.
No improvement during 10 days, repeated normal CT. Lumbar puncture with normal cell count, but high intracranial pressure. Receives a ventricular drainage and starts treatment with steroids and acetazolamide (a carbonic anhydrase inhibitor). Negative serology for CMV and EBV but positive for IgG *Borrelia* antibodies in CSF. Receives intravenous ceftriaxone, gradual clinical improvement.

Diagnosis: Neuroborreliosis ("pseudotumor cerebri").

Outcome: Total recovery.

Immunity to infection

Fighting the enemy

The immune system is like an army, designed to fight invading pathogens. The primary objective is destruction. During the battle, tissue damage in the host is inevitable, either due to substances from the invading microbe, lysis of infected cells or by release of substances from the host's immune system. This destructive potential must be kept under control and therefore a rapid, effective and limited immune response to eliminate the intruder is preferable to minimize host damage (Janeway 2005).

Innate recognition of pathogens

Cells in the innate immune system (e.g. dendritic cells, monocytes/macrophages and granulocytes) are able to recognize invading pathogens by pattern recognition receptors (PRRs), for example various Toll-like receptors (TLRs) (Medzhitov et al. 2000). They bind, alone or in combination, to invariant, conserved molecular patterns on the microbal surface (e.g. lipoprotein or lipopolysaccarides shared by large groups of microbial pathogens).
By this mechanism, many different microbes can rapidly be identified by the immune cells and an early innate immune response can be initiated (Janeway 2005).
By this innate recognition combined with signals from surrounding cells in the tissue, the dendritic cells can perform phagocytosis, digest the pathogen, and on HLA molecules, express antigen fragments on their cell surface. Together with co-stimulatory molecules, this activates T cells. As the dendritic cells become antigen-presenting cells (APCs), they mature and migrate to the lymph nodes. T lymphocytes interact with the APCs, are stimulated by different cytokines, and thus polarise them into different subsets of activated T cells (Figure 4). In addition, complement is important in early inflammation, as part of the innate immunity, but it also interacts with the adaptive immune responses (Longhi et al. 2006; Song et al. 2000). Furthermore, it seems to play a role in the protection against the *Borrelia* spirochete and has been found to be activated in the central nervous system compartment in patients with NB (Henningsson et al. 2007).

Cytokines

Cytokines are small proteins that are secreted from different immune cells, as well as from other cells. They act as signal molecules, and are most important in the interaction between immune cells and crucial for the activation and regulation of different immune responses

(Borish et al. 2003). Cytokines are divided into different groups according to their main effector functions. Thus, there are pro-inflammatory and anti-inflammatory cytokines but they can also be categorized into type 1 and type 2 cytokines (Mosmann et al. 1996). Furthermore, certain cytokines are known to have opposite effects in different situations (Cavaillon 2001) and there are also different effector functions in humans as compared to mice (Glickstein et al. 2001; Shtrichman et al. 2001). Important pro-inflammatory cytokines in humans include interleukin 1 (IL-1), tumor necrosis factor alfa (TNF-α), interferon gamma (IFN-γ) and IL-12, whereas anti-inflammatory cytokines include tumor growth factor beta (TGF-β) and IL-10 (Borish & Steinke 2003). The anti-inflammatory cytokines act with the aim of regulating or inhibiting the pro-inflammatory responses (McGuirk et al. 2002).
Furthermore cytokines, whose main task is to act as a chemotactic agent at the inflammation site, are called chemokines. They recruit neurophils as well as mononuclear cells and may play an important roll in CSF in patients with NB or in dermatological *Borrelia* manifestations (Mullegger et al. 2007; Rupprecht et al. 2005a).

Type1/type2 immune responses

The T helper (Th) lymphocytes are subdivided into different subsets depending on the cytokines they secrete. Th1 lymphocytes secrete mainly IFN-γ and Th2 lymphocytes mainly IL-4 (McKenzie 2000; Spellberg et al. 2001). Not only T cells, but also cytotoxic cells, NK and other cells, can be categorized according to the Th1/Th2 dichotomy. Therfeore, the term type 1/type 2, or Th 1/Th 2-like, immune response is sometimes used.
In addition to Th 1/Th 2, there are also various types of T regulatory lymphocytes (T regs); natural thymus derived T regs as well as T regs induced in the periphery (Rouse 2007).
T regs act both by cell-cell interaction and cytokine secretion, e.g. IL-10 and TGF-beta (McGuirk & Mills 2002). The Th 1/Th 2 subsets are to a great extent mutually antagonistic and a balance between the two subsets is believed to be of importance in the immune regulation during for example a *Borrelia* infection. The T regs inhibit both type 1 and type 2 responses and it is therefore an important anti-inflammatory component (Belkaid 2007; Cavani et al. 2000; Cottrez et al. 2000; Demengeot et al. 2006; Simpson 2008). Furthermore, a new group of T cells that produce a pro-inflammatory cytokine IL-17 is found and hypothesized to be of importance in prolonged inflammatory immune responses and tissue damage (Schmidt-Weber et al. 2007; Steinman 2007).

The adaptive immune systeme

The humoral immune response, leading to the production of specific antibodies, is aimed at eliminating the pathogen (Janeway 2005). In Borreliosis it follows the general pattern of IgM preceding IgG, but in rare patients, a detectable antibody response may be delayed or even absent (Steere 1989). IFN-γ stimulates B-lymphocytes to produce opsonizing antibodies and activate complement which leads to phagocytosis and the formation of membrane attack complexes, whereas IL-4 stimulates mast-cells and basophiles (Romagnani 1996; Snapper et al. 1987). Different IgG-subclasses are also believed to be of importance for protection against infection (Widhe et al. 1998) and IFN-γ apears to be associated with IgG1 and IgG3 antibody production (Janeway 2005) although the mechanisms behind the isotype switches are not fully understood (Manis et al. 2002).

Immune responses in early life

During pregnancy the mother's immune system is believed to be type 2-deviated in order to prevent rejection of the fetus (Wegmann et al. 1993) but this is to some extent inconclusive (Chaouat et al. 2004; Jonsson et al. 2006; Matthiesen et al. 1998; Wegmann et al. 1993).
At birth and during the first years of life, the child's immune system is prone to respond with a type 2-like response and consequently, the production of IFN-γ in newborns is suppressed (Bottcher et al. 2002; Langrish et al. 2002; Marodi 2002; Shu et al. 1994). Newborns with atopic heredity show even higher IL-4 production from cord blood mononuclear cells (CBMCs) than newborns without heredity (Gabrielsson et al. 2001). Gradually, during childhood, the type 2 polarization changes to type 1 with an improved capacity to produce IFN-γ (Bottcher et al. 2002; Hoffmann et al. 2005; Holt 1995; Prescott et al. 1999).

In epidemiological studies, various markers for increased infectious burden during early life have been associated with decreased prevalence of atopic disease during childhood (Matricardi et al. 1997; Matricardi et al. 2002) . It is possible that early exposure to infectious agents causes a change in the type 1/type 2 balance, which could be beneficial for the child with atopic heredity (Strachan 1989; von Mutius 2007). It has been suggested that viral infections decrease atopic manifestations (Matricardi et al. 2002) but results are controversial (Benn et al. 2004). It has also been suggested that atopic disposition is associated with more severe symptoms in respiratory syncytial viral infection (Stensballe et al. 2006). Furthermore, cytomegalovirus (CMV) and Ebstein-Barr virus (EBV) might influence the immune responses

during early life and be associated with a reduced risk of IgE-sensitization (Nilsson et al. 2005a; Sidorchuk et al. 2004).
Futhermore, the question of whether vaccination against different infectious agents in childhood is beneficial or not for the individual child or in a larger society prospective, is controversial and has been discussed for decades (Enriquez et al. 2007; Garpenholt et al. 1998; Gruber et al. 2001; Nilsson et al. 1998; Trollfors 1994; Trollfors 2007). Recently, a meta-analysis failed to find an association between BCG- or perussis-whole-cell-vaccination, and asthma (Balicer et al. 2007). Thus, the discussion on childhood vaccinations and the influences on allergic and/or immunologic mechanisms will probably continue.

Immune responses in Lyme Borreliosis

Type 1/type 2 immune responses in Lyme Borreliosis

Membrane lipoproteins on the *Borrelia* spirochetes cell surface exhibit stimulatory properties via the TLR 2 on macrophages, in order to raise strong inflammatory response in the host (Hirschfeld et al. 1999) . The type 1 response is characteristic for patients with LB (Grusell et al. 2002; Pohl-Koppe et al. 1998). High numbers of *Borrelia*-specific IFN-γ secreting cells have been found in CSF and blood in adult patients with NB (Ekerfelt et al. 1997a), but no IL-4. In addition, in mixed-age studies of LB, an explicit type 1 response in blood has been demonstrated (Bauer et al. 2001; Oksi et al. 1996). The type 1/type 2 cytokine secretion in CSF in children with NB has not earlier been studied.

It is a matter for discussion whether this type of immune response is pathogenic or protective. Several studies of experimental LB have been performed on animal models with contradictory results (Kang et al. 1997; Keane-Myers et al. 1995), although an initial strong type 1 response, followed by type 2, seems plausible for eradication of spirochetes.
Where NB is concerned, mice models have not been available because the *Borrelia* infection does not involve the nervous system in rodents. In studies on non-human primates though, strong inflammatory responses to the *Borrelia* spirochete were demonstrated in CNS (Pachner et al. 1998). In patients with NB, it has been suggested that an initial strong type 1 response with IFN-γ followed by a later switch to a type 2 response is associated with a better prognosis (Figure 7) (Widhe et al. 2004).

Concluding remarks on immunity to Lyme Borreliosis

In conclusion, the innate response is aimed at recognizing lipoproteins on the cell surface (Osps) of the *Borrelia* spirochete and thereby rapidly limiting replication and spread of the spirochete in the tissue. The adaptive response, with somewhat slower but more specific properties, is aimed at final destruction and elimination of the spirochete in order to prohibit a chronic infection. Importantly, the innate and adaptive components in the immune system are closely integrated and need to synergize in the clearance of the spirochete.
A strong type 1 response followed by a switch to type 2, is suggested as beneficial in LB but immune responses in children with NB have not been studied previously.

Defense mechanisms of the *Borrelia* spirochete

The *Borrelia* spirochete is highly invasive and can migrate through the extra cellular matrix by destroying tissue and binding to various components such as proteoglycans (Isaacs 1994). It can enter endothelial cells (Comstock et al. 1989) and bind to platelets and red blood cells (Coburn et al. 1994), which might be important for spirochetemia and further passage through the blood brain-barrier in NB (Garcia-Monco et al. 1990). The *B. burgdorferi* can evade the host's immune defenses due to an incredible potential for antigenic variation (de Silva et al. 1998; Zhang et al. 1997) and the spirochetes preferably localize in selected immunologically privileged sites (Barthold et al. 1991; Embers et al. 2004) . The outer surface proteins (Osps) are environmentally regulated and limited surface exposure of certain immunogenic antigens helps the *Borrelia* spirochete to remain unidentified by the host (Figure 7) (Cox et al. 1996). Furthermore, it has been proposed that, spirochetes can hide intracellularly in host cells (Girschick et al. 1996; Hu et al. 1997; Pachner et al. 1995a) or transform into cystic inactive forms (Brorson et al. 2001; Kersten et al. 1995; Murgia et al. 2002). Whether this is a mechanism for persistence of disease or not, is still controversial (Seiler et al. 1996).
In addition, the *Borrelia* spirochete can bind factor H to outer surface protein E (OspE) and thereby protect itself from host complement binding, membrane attack complexes and phagocytosis (Alitalo et al. 2001; Hellwage et al. 2001; Kraiczy et al. 2001a; Kraiczy et al. 2001b; Stevenson et al. 2002).
In tick saliva, a substance has been found that can bind to OspC and thereby protect the spirochete from antibody-mediated killing (Ramamoorthi et al. 2005).
Finally, the *Borrelia* spirochete lacks physiological iron and by this mechanism could possibly be resistant to parts of the innate and adaptive immune responses (Posey et al. 2000).

Interaction between the tick, the *Borrelia* spirochete and the human host

The tick:

Mouthparts are specially designed for firm attachment to host and for blood sucking.

The saliva of the tick contains components that prevent blood clotting and histamine secretion in the host. It helps the tick to remain unremoved by the human host and it facilitates for the *Borrelia* to migrate through tissue and into small vessels.

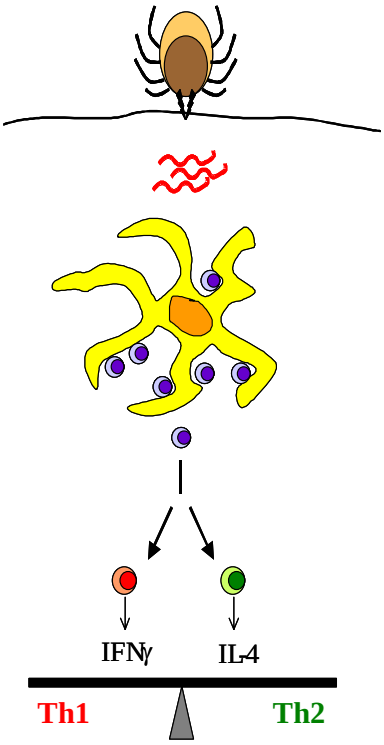
The hosts immune system:

The immature dendritic cell samples environment. Through innate recognition receptors and stimulation by cytokine signals, it can perform phagocytosis and express foreign antigens on cell surface. This activation will trigger maturation and migration to lymph nodes.

In the lymph node, the dendritic cell presents antigens to naïve T-cells. Secreted cytokines act as signals, telling T-cells to polarize towards type 1 or type 2 response. B-cells start producing specific antibodies.

The innate and adapted immune responses are closely working together and the primary task is to eliminate the pathogen.

A strong inflammatory type 1 response with IFN-gamma is believed to be beneficial for eradication of the *Borrelia* spirochete but can also cause extensive tissue damage. A counterbalancing type 2 response with IL-4 is probably needed to turn-off the inflammatory response.



The *Borrelia* spirochete:

The spirochete gets activated in the mid gut of the infected tick during the blood meal.

It migrates to the salivary glands and is transmitted to the host during the meal. This process takes 24-48 hours.

The *Borrelia* spirochete binds to extra cellular matrix and migrates through the tissue. It binds to platelets or red blood cells and can spread via the blood stream to different organs, including the CNS. The spirochete can probably also locally invade CNS by cranial nerves.

Antigenic variation of outer surface proteins (Osps) and limited surface exposure of certain immunogenic antigens helps the *Borrelia* spirochete to remain unidentified in the host.

Furthermore, it has been proposed that it can hide intracellularly in host cells or transform into cystic inactive forms.

The *Borrelia* spirochete can bind factor H to outer surface E (OspE) and thereby protect it self from host complement binding, membrane attack complexes and phagocytosis.

In addition, the *Borrelia* spirochete lack physiological iron and can possibly be resistant to parts of the innate and adaptive immune responses by this mechanism.

Figure 7. Interaction between the tick, the *Borrelia* spirhochete and the human host

Severity of disease

Many similarities between *Borrelia burgdorferi* and *Treponema pallidum* (Syphilis) have been described and *Borrelia* infection in CSN has been called the “Great Imitator” (Pachner 1988). What actually causes prolonged symptoms in LB is still uncertain (Pachner 1988; Pachner et al. 1995b; Picha et al. 2006; Steere 2006). However, several possible pathogenic mechanisms have been suggested to explain persistent symptoms in LB.

Persistent or reactivated spirochetal infection, despite antibiotic treatment, might possibly cause residual symptoms (Weber 2001). This hypothesis is supported by isolation of *Borrelia* spirochetes in CSF in patients without clinical symptoms (Pfister et al. 1989) and in skinbiopsies from previously healed EM (Kuiper et al. 1994). Furthermore, spirochetal DNA was found with PCR technique, years after treatment, but whether or not this finding indicates viable spirochetes in the site, is unclear (Pachner et al. 1995b; Strle et al. 1995). It has been proposed that the spirochetes, by transforming into inactive cystic forms, can avoid antibiotic effects and thereby cause prolonged symptoms ((Brorson & Brorson 2001; Kersten et al. 1995; Murgia et al. 2002).

Another hypothesis is that persistent symptoms are caused by an autoimmune mechanism (Sigal 1997). This is supported by sequence homology between an epitop of the *Borrelia* protein OspA and human leukocyte function-associated antigen (LFA)-1 in LB arthritis (Gross et al. 1998) and similarities beween *Borrelia* flagellin and myelin basic protein in NB (Weigelt et al. 1992). Markers for nervous tissue damage have been reported in late NB (Dotevall et al. 1999b; Garcia Monco et al. 1993) and *Borrelia* specific T cell clones in CSF from patients with NB, were found to be reactive to human proteins (Hemmer et al. 1999).

It has also been suggested that the genetic properties of the host influence clinical outcome, and some associations with HLA-DR alleles have been found in persistent arthritis (Steere et al. 1990; Steere et al. 2006). In NB, such associations have been discussed (Halperin et al. 1991; Hendrickx et al. 2006; Kruger et al. 1991; Wokke et al. 1988) and suggested as factors for suceptiability but they have not proven to influence outcome (Garcia-Monco & Benach 1995; Steere 2006). Different *B. burgdorferi* strains have been connected to different clinical pictures and severity in LB (Baranton et al. 2001; Isogai et al. 1996; Strle et al. 1999; Wang et

al. 2002), probably by different invasioness and antigenic variation in different strains but exact mechanism are still not known (Embers et al. 2004).

Furthermore, pathogenic properties of the spirochete, in interaction with the elicited immune responses in the host, are believed to be of importance for the course of the disease and the outcome (Seiler & Weis 1996; Wooten et al. 2001). A balanced type 1/type 2 immune response is suggested as beneficial for the resolution of symtoms in Lyme Borreliosis (Widhe et al. 2004; Wooten & Weis 2001). It is proposed that T cells with cytolytic properties are involved in tissue damage in persistent NB (Ekerfelt et al. 2003), and both T regulatory cells and T cells producing IL-17 are probably part of the pathogenic mechanism, but this needs further study (McGuirk & Mills 2002; Steinman 2007). If no inflammation is observed, persistent signs and symptoms after NB may simply be the result of an incompletely healed lesion, a sequele (Garcia-Monco & Benach 1995; Oschmann et al. 1998).

Finally, if a tick-borne co-infection occurs simultaineously with the *B. burgdorferi*, for example an HGA infection with *Anaplasma phagocytophilium*, changes in the hosts immune response are reported, which might contribute to a less favorable clinical outcome (Evans 2000; Hilton et al. 1999; Holden et al. 2005; Thomas et al. 2001; Zeidner et al. 2000). Why children seem to have a more benign course of the disease, and a reduced risk of persistent symptoms after LB, has not, to my knowledge, been studied previously.

Laboratory diagnostics in Lyme Borreliosis

The diagnosis of LB should be based on typical clinical features in combination with laboratory testing to support the diagnosis. However, skin manifestations such as EM are considered pathognomonic for LB and laboratory testing is not necessary, in particular since most cases lack antibodies at an early stage. In contrast, arthritis and neurological manifestations are often nonspecific and confirmation with laboratory analyses are mandatory for verification of diagnosis (Brouqui et al. 2004).

Culture

The golden standard for diagnosis of infectious disease is isolation of the infectious agent by culture. However, culturing *B.burgdorferi* is difficult, probably due to the scarcity of bacteria in clinical samples, slow reproduction and suboptimal media conditions (Brouqui et al. 2004;

Wilske 2003; Wormser et al. 1998). A specific culturing medium, Barbour-Stoenner-Kelly (BSK) is used for incubation of the spirochete. It takes 2-3 weeks to obtain a positive result but the success rate is generally low. Spirochetes are found in skin biopsies from EM (Strle et al. 1996), in blood samples (Arnez et al. 2003b; Wormser et al. 1998), in synovial fluid (Eiffert et al. 1998), in myocardium (Stanek et al. 1990) and CSF (Karlsson et al. 1990). Thus, culturing is a specific but time-consuming method where negative result does not exclude LB and therefore not used for clinical routine.

Polymerase chain reaction (PCR)

PCR-based techniques have been used to identify small numbers of *B. burgdorferi* in various tissues (Dumler 2001; Keller et al. 1992; Lebech et al. 2000; Nocton et al. 1994; Priem et al. 1997). The different PCR methods have varied considerably in analytic sensitivity and are not yet standardized in the laboratory diagnostics of LB. The most frequently used targets for amplification are the plasmid-encoded OspA and the chromosomal 16S rRNA. Many other gene targets have been tested in different patient samples in Europe or in the USA, but none found superior. It is concluded in a meta-analysis, that sensitivities are rather high in skin biopsies (68%) and synovial fluid (73%) whereas in blood and CSF, sensitivities are low (29% and 18% respectively) (Dumler 2001). Persistent B. burgdorferi DNA is found in samples years after antibiotic treatment, and consequently, a positive PCR result is not always indicative of active infection with viable spirochetes (Strle et al. 1995). Furthermore, a negative PCR result does not exclude LB (Dumler 2001).
In summary, PCR methods perform with high specificity but they are not yet sensitive enough. Furthermore, they are not appropriate as a single confirmatory test in LB and therefore not suitable for routine clinical practice (Avery et al. 2005). However, a PCR analysis could be useful in rare cases of longstanding symptoms with negative serology (Avery et al. 2005; Holl-Wieden et al. 2007; Picha et al. 2005).

Serologic assays

Serologic assays measuring antibodies against *B. burgdorferi* have been the backbone of laboratory diagnostics of LB. Mainly assays with flagella antigen or whole cell lysate, have been used as routine methods (Hansen et al. 1989). The two most frequently used methods are enzyme-linked immunosorbant assay (ELISA) and Western Blotting (WB) (Wilske 2003). Serology testing is not standardized in Europe, thus different laboratories and different commercial kits show highly varied performances (Ekerfelt et al. 2004; Goossens et al. 1999).

IgM assays are generally more sensitive but may be cross-reactive with other spirochetal infections, EBV, CMV, rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) and therefore cause false positive results (Brown et al. 1999; Bunikis et al. 2002; Magnarelli 1995; Tugwell et al. 1997). Persistent high IgG antibody titres may reflect earlier *Borrelia* infections (Ekerfelt et al. 1999) and prolonged antibody responses are seen years after antibiotic treatment (Hammers-Berggren et al. 1994).
Two-tier testing protocol has been recommended from the Centers for Disease Control and prevention (CDC) in the United States and in Europe (Ledue et al. 1996; Wilske 2003). When a positive screening result in serum or CSF is found, an immunoblot or other second step-test could be performed to confirm the initial result (Brouqui et al. 2004; Ledue et al. 1996; van Dam 2001; Wilske 2003; Wilske 2005). The procedure improves specificity but is dependent on technically advanced laboratories, is costly, may be subjective in interpretation of results and might not influence decision-making (Bacon et al. 2003; Blaauw et al. 1999).
Many of the diagnostically relevant outer surface proteins of *B. burgdorferi* have a considerable interspecies variability, which leads to unpredictable reactivity in serology testing. It is also important to take into account the greater antigenic diversity of the different strains in Europe, as compared to the Northern America, when discussing diagnostic laboratory procedures (Steere 2006; Wilske 2003).

In order to improve diagnostic performance in serologic testing, several new recombinant protein and peptide antigen candidate have been successfully used in immunoblots and ELISAs. Examples are decorin binding protein A (DbpA) and DbpB, OspC, BBK32 (Goettner et al. 2005; Heikkila et al. 2005; Heikkila et al. 2002c; Panelius 2002; Panelius et al. 2007; Schulte-Spechtel et al. 2004), BBA50, BBA25 (Fikrig et al. 2004; Rupprecht et al. 2005b), p100, p58, p41i or BmpA (Goettner et al. 2005; Schulte-Spechtel et al. 2004; Schulte-Spechtel et al. 2003). Furthermore, an immunodominant peptide from *Borrelia* protein VlsE, called invariable region 6 (IR6), or commercially C6, has been in focus as a promising antigen for improved serology and also as an indicator of therapy outcome (Bacon et al. 2003; Liang et al. 1999; Peltomaa et al. 2004; Philipp et al. 2003; Schulte-Spechtel et al. 2004; Schulte-Spechtel et al. 2003; Skarpaas et al. 2007; Tjernberg et al. 2007; Wilske 2003).
Since the clinical picture as well as the immune responses in LB differ between children and adults (Holt 1995; Huppertz 2001; Pohl-Koppe et al. 2001; Widhe et al. 2005), it is also important to focus on pediatric patients when evaluating new serologic tests for LB (Heikkila et al. 2005).

Additional diagnostic methods

B. burgdorferi usually induces a cellmediated immune response which can be analyzed by the lymphocytic proliferation test (Dattwyler et al. 1986). This test proved useful in seronegative patients (Huppertz et al. 1996; Tugwell et al. 1997) but the method has not gained acceptance because its low sensitivity and large numbers of false positive reactions due to cross reactivity against other microbes (Zoschke 1992).

New assays for detection of antigen-antibody immune complexes (Brunner et al. 2001; Schutzer et al. 1999) or cytokine/chemokine secretion (Rupprecht et al. 2005b) have been developed but need to be further analyzed in different clinical settings (Segal et al. 2005).

The enzyme-linked immunospot assay (ELISPOT) is used to detect cytokine secretion to an antigen in T cell immune responses on a single cell level (Forsberg et al. 1995a). The method has been used to study adult patients with NB (Ekerfelt et al. 1998; Forsberg et al. 1995a) and is described in detail under “Methods” in this thesis.
Finally, the usefulness of different laboratory methods is summarized in Table 3.

Table 3. Usefulness of different laboratory methods in Lyme Borreliosis

Method	Advantages	Disadvantages
Culture	Golden standard for diagnosis Research High specificity	Time-consuming Fastidious organism Low sensitivity
PCR	Confirmatory test Research Quick	High sensitivity only in skin biopsies May reflect unviable organisms High variablility between PCR methods
ELISA	Routine analysis for serum and CSF Quick Inexpensive	Low sensitivity in early LB Cross-reactivity with other infections High interlaboratory variability
WB	Confirmatory test High specificity Research	Time-consuming Subjective interpretation Cross-reactivity with other disease
ELISPOT	Confirmatory test Research High sensitivity	Time-consuming Advanced laboratory handling No standard protocol exists

INITIATION OF THE STUDY

In 1997-98, as a young assistant physician at the Pediatric clinic in Linköping, a Lyme endemic area of Sweden, I met several children with clinical features indicative of NB.
We always performed a lumbar puncture on admission, in most cases non-traumatic, with the help of midazolame and excellent nurses, and eagerly awaited results from the cell count in CSF. If there was an elevated mononuclear cell count in CSF, we started antibiotic treatment according to national guidelines, which implied intravenous antibiotics if the child was younger than 8 years of age. Parents were often worried and asked many questions.
The doctor's answer was in most cases: let's wait for the *Borrelia* antibody test results.
One week later, when test results were available, quite a few patients proved **not to** have elevated *Borrelia* antibody titres in CSF. Further questions were posed by patients and parents, which were even more difficult to answer. Was it a *Borrelia* infection? Was it not? If not, what was it? Should the child continue the course of antibiotic treatment?
Questions arose in the mind of the doctor, such as: do we treat young children unnecessarily with parenteral antibiotics? Is a 14 days regimen necessary? Are we missing viral infections? Are there prognostic factors of importance for clinical recovery?
The impression was that most children responded promptly to treatment and that recovery seemed acceptable. However, children with facial nerve palsy appeared, in some cases, to be troubled by persistent facial nerve dysfunction. Literature available at that time, gave me a few answers but not sufficient.
My supervisor at the Pediatric clinic, Stefan Croner, encouraged me to look closer into medical files of children who had been evaluated for Neuroborreliosis during the previous years. We could always learn something. Furthermore, we started planning a follow-up study of children with facial nerve palsy in co-operation with the specialist Lars Ödkvist at the Ear-Nose-and Throat clinic. We could always learn something more!
Last but not least, we set up a meeting with a very enthusiastic person at the clinic of Infectious diseases, professor Pia Forsberg…

AIMS OF THE STUDY

The aim of this thesis was to study clinical, laboratory and immunologic characteristics and clinical outcome in children being evaluated for Neuroborreliosis, in a Lyme endemic area in Sweden.

Objectives:

- To study the long term clinical outcome in children with facial nerve palsy (Paper I)

- To study immune responses, with focus on IFN-γ and IL-4, in CSF and blood in children with NB, in order to understand their impact on clinical features and recovery (Paper II)

- To study reactivity four new *Borrelia* antigens (DbpA, BBK32, OspC, IR6) in CSF and serum in children with NB, and to evaluate their usefulness as diagnostic tests (Paper III)

- To study clinical and laboratory characteristics in children being evaluated for NB, in order to identify factors of importance for prediction of diagnosis and recovery (Paper IV)

MATERIALS AND METHODS

Subjects (Paper I-IV)

Patients (Paper I-IV)

All and all, 250 patients with signs and symptoms indicative of NB were included (Figure 8). Primarily, twenty-seven patients with a history of facial nerve palsy (n=27) were included in a clinical 2-year follow-up study in 1998. They were re-examined by a pediatrician (BHS) and an ENT-specialist at the University Hospital in Linköping, in order to evaluate the recovery after acute facial nerve palsy and the severity and nature of sequelae (Paper I).

The majority of patients (n=177) were included in a prospective clinical study during the period 2000 to 2005 (Paper IV). Five pediatric clinics in the counties of Östergötland, Jönköping and Kalmar in southeast Sweden took part in the study. Patients with signs and symptoms indicative of NB were included from Norrköping (n=45), Linköping (n=95), Motala (n=6), Jönköping (n=27) and Västervik (n=4). All patients were examined by a pediatrician, underwent a lumbar puncture and gave a blood sample. Patients with facial nerve palsy were referred to an ENT-specialist for oto-neurologic examination including the House-Brackmann grading system for facial nerve palsy (House et al. 1985). All blood and CSF samples were drawn before the start of antibiotic treatment.

Table 4. Subjects included in the thesis

Diagnosis	**Paper I** Facial palsy follow-up (n=27)	**Paper II** Immune responses (n=64)	**Paper III** New Borrelia antibody tests (n=57)	**Paper IV** Clinical prospective (n=177)
Confirmed NB	11	34	24	72
Possible NB	10	30	16	46
Not determined	6	-	17	59
Controls	-	10	36	174
Total	27	74	93	351

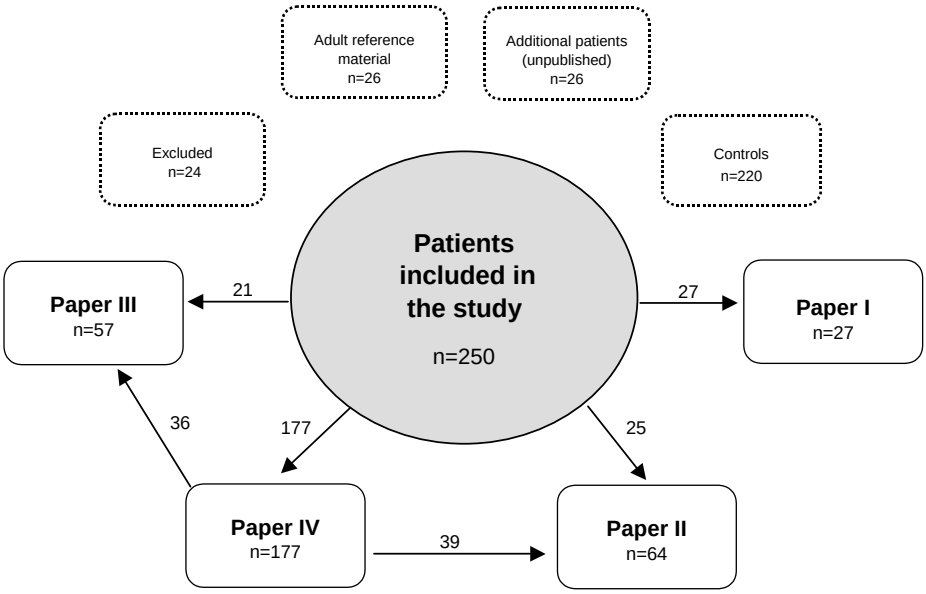


Figure 8. Subjects in the thesis

Some of the 177 patients in Paper IV also contributed with CSF and serum samples for studies on immune responses (n=39) (Paper II) and new *Borrelia* antibody tests (n=36) (Paper III) (Figure 5). Additionally, 25 patients were included in the study of immune responses (Paper II) and 21 patients in the study of *Borrelia* antibody tests (Paper III). These 46 children were mainly recruited from pediatric clinics in Norrköping and Linköping and were being evaluated for NB between 1998- 2002.

Controls (Paper II, III, IV)

Altogether, 220 controls were included (Table 4, Figure 8).

In Paper I, the recovery rate of patients with facial nerve palsy was evaluated in a 2-year follow-up, and consequently no controls were needed.

In the study of immune responses (Paper II), 10 children with other neurologic diseases (OND) (n=4) or ventriculoperitoneal (VP)-shunt (n=6) were included as controls (Non-NB). The OND-patients were diagnosed as Bell's facial palsy (n=2), multipel sclerosis (n=1) and tension-type headache (n=1), all of them without clinical or laboratory signs of *Borrelia* infection.

In Paper III, the study of new *Borrelia* antibody tests, thirty-six controls were included (n=36). Those were children with OND (n=18) or VP-shunt (n=2) or adults with no proven *Borrelia* infection (n=16). The children with OND were diagnosed as having epilepsy (n=8), viral meningitis (n=10) or hydrocephalus (n=2), all of them without clinical or laboratory signs of *Borrelia* infection.

Controls in the clinical prospective study (Paper IV) were children from a random sample of Swedish population obtained from the Swedish National Register of Statistics (n=177). These controls were matched for sex, age and geographic location. However, controls who reported a former *Borrelia* infection, were excluded (n=3).

Excluded subjects (Paper I, II, IV)

In all, 24 subjects were excluded from the study (Figure 8).

Ten patients in the facial nerve palsy follow-up study (Paper I) did not want to participate because “the child had recovered” (n=5), two families had moved (n=2) and three did not respond to invitation (n=3).

In the immune response study (Paper II), five children from the control group were excluded because they had clinical features putative of Lyme Borreliosis such as suspected EM (n=4) or pleocytosis in CSF (n=1).

No patient was excluded from the study of new *Borrelia* antibody tests (Paper III) whereas in the clinical prospective study (Paper IV), nine patients were excluded due to verified viral meningitis (n=3), rheumatoid arthritis (n=1), sarcoidosis (n=1), missing data at follow-up (n=3) or personal reasons (n=1).

Classification of patients (Paper I-IV)

Classification of patients with signs and symptoms indicative of NB was based on laboratory findings as shown in Table 5. The strict definition of “Confirmed NB” with pleocytosis and *Borrelia* specific antibodies in CSF follows European guidelines (Stanek et al. 1996). Patients who did not meet the criteria for “Confirmed NB” were divided into two groups, “Possible NB” and “Not determined” (Table 4 and 5).

Table 5. Classification of patients being evaluated for NB (Paper I-IV)

Diagnosis	Laboratory findings
Confirmed NB	- Pleocytosis in CSF [*] - IgG and/or IgM *Borrelia* antibodies in CSF [§]
Possible NB	- Pleocytosis in CSF [*] - No anti-flagella antibodies in CSF - May have IgG and/or IgM *Borrelia* antibodies in serum
Not determined	- No pleocytosis in CSF - No anti-flagella antibodies in CSF - May have IgG and/or IgM *Borrelia* antibodies in serum

[*] Pleocytosis: ≥ 5 x 10^6/L cells in CSF (> 90% mononuclear cells)
[§] Intratechal *Borrelia* specific anti-flagella antibody synthesis in CSF

In all papers, classification of patients is the same, but there are some differences in terminology, which need to be clarified. **Confirmed NB** is referred to as **Verified NB** in Paper I and **Definite NB** in Paper III**. Possible NB** is called **Suspected NB** in Paper I.

Representativity of patient sample (Paper IV)

Patients in the clinical prospective study (n=177) are believed to be representative of all children being evaluated for NB in the area during the period 1998-2005, by comparison to 82 patients not included in the study, as described in Paper IV. No differences in sex, age and classification of diagnosis were found. Furthermore, a comparison was made between patients in the prospective study (n=177) and all other patients in the thesis (Figure 8), and no differences in age, sex or diagnostic group were identified.

The selection bias should be low, because patients were always referred to pediatric clinics for investigations involving a lumbar puncture.

Adult reference material (Paper II)

In order to understand the importance of different types of immune responses in NB, a clinical reference material of adult NB patients (n= 26) was used in Paper II for comparison with pediatric NB patients (Figure 8).

Methods (Paper I-IV)

Laboratory investigations in CSF (Paper I-IV)

Pleocytosis was defined as total white cell count in CSF $\geq 5 \times 10^6$/L, with mononuclear cells > 90% of the total count (Shah et al. 2005). Data on pleocytosis in CSF was available for all patients included in the study. Protein levels (g/L) in CSF were not analysed routinely and therefore not included in presentations on laboratory findings in CSF. In most cases sample volumes of CSF from patients were unfortunately too low to give priority to supplementary analyses. Further more, lactate in CSF was not routinely analysed in our patients. In an earlier investigation of CSF in children being evaluated for NB, we did not find differences in levels of lactate in CSF between patients with "Confirmed NB" as compared to other patients (unpublished data). With this knowledge, and the fact that lactate is seldom referred to in literature on diagnosis of NB, we did not include complementary analyses on lactate in CSF in present studies. Nor did we prior to analyse oligoclonal bands in CSF and serum.

Borrelia serology (Paper I-IV)

Laboratory diagnostics for detection of *Borrelia* infection was based on the routine anti-flagella ELISA by DAKO (kit K6028 and K0416, Glostrup, Denmark) (Hansen 1994).
An index > 0.3 was used to ensure intrathecal synthesis of Borreia specific anti-flagella antibodies in NB (Hansen & Lebech 1991). With a positive *Borrelia* specific antibody index in CSF, the NB diagnosis was confirmed, but additional information on antibody titers in serum alone was not always available. Therefore, data on serum antibodies in the "Confirmed NB" group is not shown in the tables.
When the *Borrelia* specific antibody index in CSF was negative, results of *Borrelia* serum titers were always available and consequently presented in the tables. Data on seroconversion over time was not available since patients did not routinely give blood samples at the follow-up. *Borrelia* antibodies in serum are difficult in diagnostics because IgM is a non-specific test with a high degree of false positive results (Brown et al. 1999; Bunikis & Barbour 2002; Hansen 1994; Tugwell et al. 1997) and IgG in serum may reflect an earlier *Borrelia* infection (Ekerfelt et al. 1999).Thus subgrouping of patients after IgM and IgG in serum is precarious and therefore not done in this study.
For controls in Paper III, the anti-flagella ELISA method was modified by our co-authors in Finland by a titer end point determination (Seppala et al. 1994) .

Preparation of cells in blood and CSF (Paper II)

Blood and CSF samples from patients and controls were collected. Peripheral blood mononuclear cells (PBMC) were separated by gradient centrifugation, washed and resuspended in cell culture medium. Cells were counted under a phase-contrast microscopy using a Bürker chamber, and the lymphocyte concentration was adjusted to 1 x 10^6/mL. CSF-mononuclear cells (CSF-MNC) were counted under a phase-contrast microscopy, using a Jessen chamber, before centrifugation, followed by resuspension in cell medium culture. Details in cell preparation are further described in Paper II.

Preparation of Borrelia outer surface protein antigen (Paper II)

An outer surface protein (Osp) enriched fraction (OF), mainly containing OspA and OspB, was prepared from of *Borrelia garinii* strain Ip90, as previously described (Bergstrom et al. 1991; Magnarelli et al. 1989). *Borrelia* OF antigen was used in a optimized concentration of 10 µg/mL. Stimulation of cells (PBMC and CSF-MNC) with *Borrelia* OF antigen has previously been shown to discriminate between adult patients with NB and patients with other neurologic disease (OND) as well as healthy controls (Ekerfelt et al. 1997b; Forsberg et al. 1995b). The ELISPOT (enzyme-linked immunospot) assay was used for detection of secretion of the cytokines IFN-γ and IL-4, which is further described below.

The ELISPOT method for detection of cytokine secretion (Paper II)

The ELISPOT assay, originally described by Czerkinsky et al (Czerkinsky et al. 1988), was used for analysis of *Borrelia* OF antigen stimulated and unstimulated cytokine secreting cells. ELISPOT is a sensitive method to visualize cytokine secretion at a single cell level. In our study, the method was slightly modified and optimized for IL-4 and IFN-γ spots as described previously (Ekerfelt et al. 1997b; Forsberg et al. 1995b).

In short, nitro-cellulose-bottomed micro titre cell culture plates were coated with mouse-anti-human IL-4 or mouse-anti-human IFN-γ (Mabtech, Stockholm, Sweden). Cell suspensions with PBMCs and CSF-MNCs were added to wells in triplicates (or duplicates or single wells depending on cell supply). *Borrelia* OF antigen (as described above) was added for stimulation of cells to secrete cytokines. Wells without *Borrelia* OF antigen were also prepared for detection of unstimulated, spontaneous cytokine secretion. Phytohaemagglutinin (PHA) was used as a positive control and tissue culture medium (TCM) as a negative control. Plates with cells were cultured, washed and then biotinylated detector mouse-anti-human IL-4 or IFN-γ (Mabtech AB, Sweden) was added. Plates were further incubated, washed and

prepared with alkaline phosphatase-conjugated streptavidin. Plates were again washed and "spots" were developed for 15 minutes by adding BCIP –NBT in development-buffer (AP conjugate substrate kit, BioRad, Hercules, CA). The colour development was stopped, by rinsing with deionized water. One spot is like the "footprint" of one cytokine-secreting cell. Finally, the spots were counted manually under a dissection microscope or automatically with computer software. Two people, blind to the patient diagnosis, counted the spots. Data from our laboratory showed that there was a strong count correlation between them (rho=0.95: $p<0.01$ for IFN-γ and rho=0.88: $p<0.01$ for IL-4, calculated with Spearman's rank correlation test). Intra-assay variation for IFN-γ was 7% to 23% and for IL-4 25%. Inter-assay variation was 31% for IFN-γ and 38% for IL-4. The method is shown in Figure 9 and more details are given in Paper II (Ekerfelt et al. 1997a).

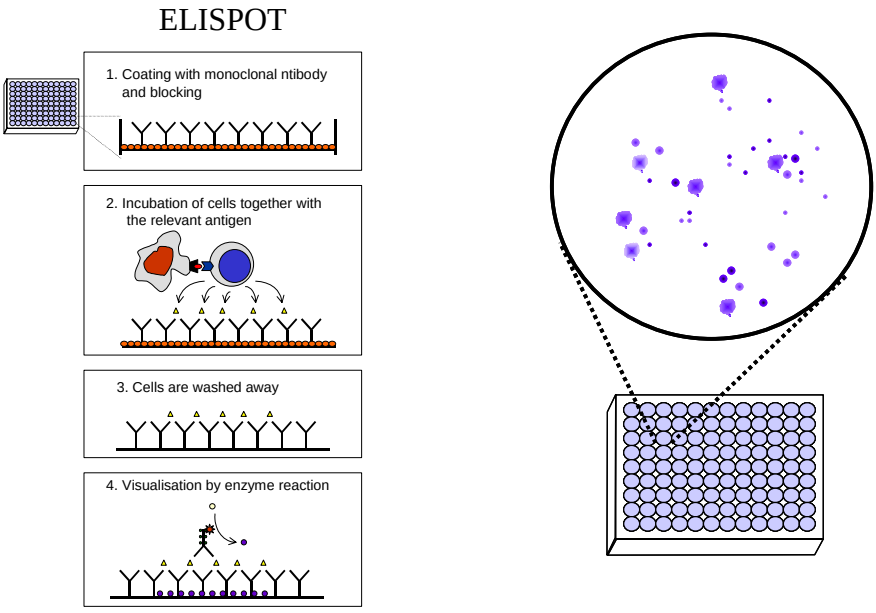


Figure 9. The ELISPOT method

Clarifications of calculations in the ELISPOT method (Paper II)

The number of spots in CSF was re-calculated as the number of spots/100 000 CSF-lymphocytes (CSF-L), in order to enable comparisons to be made between different patient samples, and is referred to as cytokine secretion in CSF. The number of spots in PBMC wells is designated cytokine secretion in blood. The mean of triplicates or duplicates was used when analyzing the cytokine results. *Borrelia*-stimulated secretion refers to the number of spots in *Borrelia* OF antigen-stimulated wells. The number of spots in wells with no *Borrelia* OF antigen added is referred to as unstimulated spontaneous cytokine secretion or background cytokine secretion. To achieve *Borrelia*-specific secretion, the unstimulated cytokine secretion was subtracted from the *Borrelia*-stimulated secretion. As described in Paper II, we found it justifiable to do this calculation in order to achieve the true number of *Borrelia* specific spots.

In this thesis, we also wanted to evaluate the ELISPOT method as a diagnostic test for NB in children (not included in Paper II). We have calculated test accuracy on data from Paper II (64 patients and 10 controls). Cut-off was set at 0, since all positive values imply *Borrelia* specific cytokine secretion and negative values mean that unspecific cytokine secretion was higher than the *Borrelia*-specific (in fact negative values indicate that the *Borrelia* antigen induced inhibition of response). Results are shown in Table 16.

Borrelia strains and new antigens (Paper III)

Three *Borrelia* strains (*B. afzelii* A91 and *B. garinii* 40 and *B. burgdorferi* s.s. IA) were used as described earlier (Junttila et al. 1999) for cloning genes, and the production and purification of the recombinant proteins DbpA, BBK32 and OspC (Heikkila et al. 2002a; Heikkila et al. 2002b; Heikkila et al. 2002c; Panelius 2002; Panelius et al. 2002). The OspC proteins can be assigned to groups B4 (IA), A1 (A91), and G2 (40) according to the classification of Lagal et al (Lagal et al. 2003). The two antigens DbpA and OspC were each used in three variants (*B. afzelii, B. garinii, and B. burgdorferi* s.s.) due to heterogeneity between subspecies. The antigen BBK32 was used only in one variant (*B. afzelii)* due to high homogeneity (Panelius 2002). The VlsE peptide (IR6), deduced from the *B. garinii* IP90 sequence, was produced by our co-authors at the Core Facility of the Haartman Institute, University of Helsinki, Finland (Liang et al. 1999).

New Borrelia antibody tests (Paper III)

An ELISA-method was used for detection of IgG antibodies to the new recombinant antigens DbpA, BBK32 and OspC and the peptide antigen IR6 in CSF and serum. In short, the optical density (OD) values were obtained at 405 nm Multiscan photometer (Thermo Labsystems, Helsinki, Finland). The highest OD value of each of the three subspecies (*B. afzelii, B. garinii,* or *B. burgdorferi* s.s.) for the antigens DbpA and OspC, was used in calculations as well as the OD values from BBK32 (*B. afzelii)* and IR6 (*B. garinii*). The cut-off was set as mean + 3 SD in CSF, and as mean + 2 SD in serum, both from controls. Results were expressed as ratios of the OD values divided by the calculated cut-off, in order to compare the different ELISAs. OD/cut-off >1 was considered a positive test.

Furthermore, when antibody results were combined in a panel, reactivity to ≥ 2 antigens were considered as postivite test, as described in paper III.

The method is more closely described by Panelius et al (Panelius 2002).

The House-Brackmann scale (Paper I)

In the oto-neurological examination of patients in Papers I and IV, an objective evaluation of grade and nature of patients with acute facial nerve palsy was made using a validated grading system called House-Brackmann scale (House & Brackmann 1985). Each of the three branches of the facial nerve is evaluated separately and 1 is normal function whereas 6 is total loss of function.

The questionnaire (Paper IV)

On admission patients (n=177) in our clinical prospective study were asked to answer a standardized questionnaire with questions on duration and character of different specific symptoms, known previous tick bites, EM, lymphocytoma and the basic health of the child. The pediatrician registered data from the clinical examination, and laboratory findings in CSF and serum. At the 2 and 6-month follow-up, a pediatric nurse telephone interviewed patients, or their parents, with questions from a structured questionnaire focusing on previously reported symptoms, recovery time, persistent symptoms and whether or not daily activities were affected. Some cases (n=28) were missed at the 6-month follow-up but with the patient personal identification numbers, complementary information concerning recovery was retrieved from medical files. Controls (n=174) were sent a slightly modified questionnaire, still including the same questions as the NB patients, about occurrence and

character of different specific symptoms, affected daily activities, known previous tick bites, EM, lymphocytoma and the basic health of the child.

The NB prediction score test (Paper IV)

In one previous study, authors have introduced a prediction test, based on clinical data on admission, analyzed in a logistic regression with a soft-wear model available for use (Avery et al. 2006). Unfortunately, this model was not suitable for our setting because it was aimed to distinguish between Lyme meningitis and viral meningitis, which was not our purpose primarily and this will be further discussed later in the thesis.

Thus, the NB prediction score test was constructed with the aim of helping the pediatrician to determine whether to start early treatment, before results from *Borrelia* antibody tests were available. We focused on signs, symptoms and laboratory findings present on admission and compared the frequency in patients being classified as "Confirmed NB" with patients in the "Not determined" group. A logistic regression analysis was done to find the relevant variables for prediction of diagnosis. Subsequently, these variables were included in a score table as will be described more closely in "Results and discussion". Controls were not used in this context because they were not evaluated with laboratory data.

Additional patients and methods (unpublished)

Additional patients

Since it is known that viruses in the Herpes virus family can stay latent in the nervous system and become re-activated, for different reasons, causing facial nerve palsy (Billue 1997; Christen et al. 1993; Davies et al. 2005; Furuta et al. 2005; Lee et al. 2006), we wanted to investigate if there could be unidentified viral infections among children being evaluated for NB. In addition, we wanted to check for enteroviral meningitis (Thoren et al. 1994). Material on children being evaluated for NB (n=99), but not meeting the criteria for "Confirmed NB", was used. Some unpublished results from this study will be discussed in this thesis as they could be of interest in understanding etiology and the nature of patients not meeting the criteria for "Confirmed NB". Clinical features were very similar to patients in Paper I-IV and will therefore not be described separately. Of these 99 patients, 73 were already included in Paper I-IV. Consequently as shown in Figure 8, 26 additional patients will be part of the discussion in the thesis without being included in Paper I-IV.

Viral diagnostics

Polymerase-chain-reaction (PCR) methods were used for the detection of viral antigens in CSF. A commercial total nucleic acid kit (Roche MagnaPure Compact instrument) was used for the extraction of virus RNA or DNA in all CSF samples. HSV 1 and 2 and VZV DNA were analyzed in a real-time PCR (Light Cycler instrument) (Legoff et al. 2006; Mengelle et al. 2004) where as enteroviral RNA was analysed in routine semi-nested PCR (Glimaker et al. 1993; Thoren & Widell 1994). For diagnostics of TBE, serum samples from patients were analyzed with a commercial routine ELISA kit, Immunozym FSME IgG/IgM (Progen Biotechnik GMBH, Heidelberg) (Treib et al. 1998). Cut-off levels were set as recommended by the manufacturer.

Statistics

Statview software (Paper I) and SPSS software, versions 10 and 15.0 for Windows (Paper II, III and IV) were used for statistical calculations. The non-parametric Kruskal Wallis and Mann Whitney U tests were used when comparing continuous data between diagnostic groups (Paper II, IV). Chi^2 and Fishers exact test were used for non-continuous data (Paper I-IV). Wilcoxon signed ranks test was used for paired samples when comparing data within groups and for correlation analyses, Pearson's correlation test was used (Paper II). Finally, a logistic regression was used in paper IV to find relevant variables for the NB prediction score test. Graph-Pad Prism (Graph Pad Software Inc.) was used for figures in Paper III.
Levels of significance were determined as $p<0.05$ (*), $p<0.01$(**) or $p<0.001$(***).
Accuracy of diagnostic and predictive tests was presented as sensitivity and specificity (Paper II, III, IV) but also as positive and negative predictive values (Paper IV).
Sensitivities were calculated on "Confirmed NB" and "Possible NB" patients together whereas specificities were calculated on controls (Paper II, III).

Ethics

Approvals were obtained from the Regional Ethics Committees of the Faculty of Health Sciences at Linköping University, Sweden, and at Helsinki University Central Hospital, Helsinki, Finland. Informed written consent was received from patients (or parents) and controls.

RESULTS AND DISCUSSION

Clinical features (Paper IV)

Patients being evaluated for NB

Characteristics of patients with signs and symptoms indicative of NB (n=177) were studied in the clinical prospective study and results are described below (Table 6-9). It is a large study and patients are believed to be representative of all children being evaluated for NB in the area between 1998-2005, as described in "Materials and methods" and in Paper IV.

Diagnostic groups

Seventy-two out of 177 patients (41%) were found to meet the definition of "Confirmed NB" (Table 5 and 6). A large group of patients (n=105, 59%) did not meet these criteria and were subsequently divided into two groups: "Possible NB" (n=46) and "Not determined"(n=59). Both these groups contained patients with *Borrelia* antibodies in serum, previous tick bites and EM/lymphocytomas (Table 6 and 7). No patient was re-evaluated into another diagnostic group during follow-up.

Table 6. Laboratory findings on admission (n=177)

Laboratory findings	**Confirmed NB** (n=72)	**Possible NB** (n=46)	**Not determined** (n=59)
Pleocytosis[&] in CSF, median (range)	220 (7-1280)	68 (5-575) ***	1 (0-4) ***
Borrelia antibodies in CSF[§]			
- IgG, n (%)	22 (30)	0 (0) ***	0 (0) ***
- IgM, n (%)	20 (28)	0 (0) ***	0 (0) ***
- IgG + IgM, n (%)	30 (42)	0 (0) ***	0 (0) ***
Borrelia antibodies in serum			
- IgG, n (%)	-	4 (9)	3 (5)
- IgM, n (%)	-	12 (26)	7 (12)
- IgG + IgM, n (%)	-	9 (20)	6 (10)

[&] Pleocytosis: ≥ 5 x 10^6/L cells in CSF (> 90% mononuclear cells)

[§] Intratechal *Borrelia* specific anti-flagella antibody synthesis in CSF

Differences, as compared to "Confirmed NB", are shown as *** (p<0.001)

Laboratory findings

Pleocytosis in CSF was most prominent in the "Confirmed NB" group and mononuclear cells dominated (>90% of total cell count) in all patients (Table 6). This mononuclear cell dominance has been seen in earlier studies and found to differentiate between children with Lyme meningitis and children with viral infection (Eppes et al. 1999; Huppertz 2001).
Borrelia specific antibodies in CSF were present in all 72 patients in the "Confirmed NB" group since they were classified according to guidelines (Stanek et al. 1996).
In our study, no child had *Borrelia* antibodies in CSF without also having pleocytosis, but such cases are described in literature (Bingham et al. 1995; Kan et al. 1998). In serum, *Borrelia* antibodies occurred in 25 of the children (55 %) in the "Possible NB" group and in 16 children (27 %) in the "Not determined" group, as shown in Table 6.

Age and sex

Age-distribution in different diagnostic groups is shown in Figure 11.
Median age on admission was 8 years but patients classified as "Not determined" were older (median 12)(Table 7). Boys and girls were equally represented among the 177 patients but when comparing the "Confirmed NB" group with "Possible NB" or "Not determined", males were in significant majority (62%) in "Confirmed NB". Median age and male predominance are in concordance with earlier studies on LB in children (Christen et al. 1993).

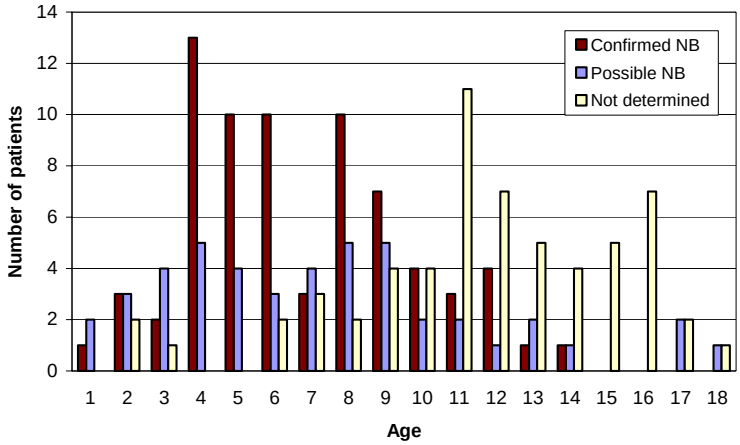


Figure 11. Age-distribution in different diagnostic groups (n=177)

Tick bites and season for illness

Occurrence of tick bites was reported in 56 % of the patients (n=177) with a median interval of 1-2 months between bite and onset of neurologic symptoms. No differences between diagnostic groups were found. Among controls (n=174), 52% reported tick bites "the last 5 summers". Consequently, information on previous tick bites cannot help us in the prediction of diagnosis. The majority of cases were admitted to hospital from May to November (Figure 9) which is in line with studies from Germany (Christen et al. 1993). In the "Not determined" group, a few cases occurred between December and April (Figure 12) but differences were not significant when comparing different diagnostic groups over the year.

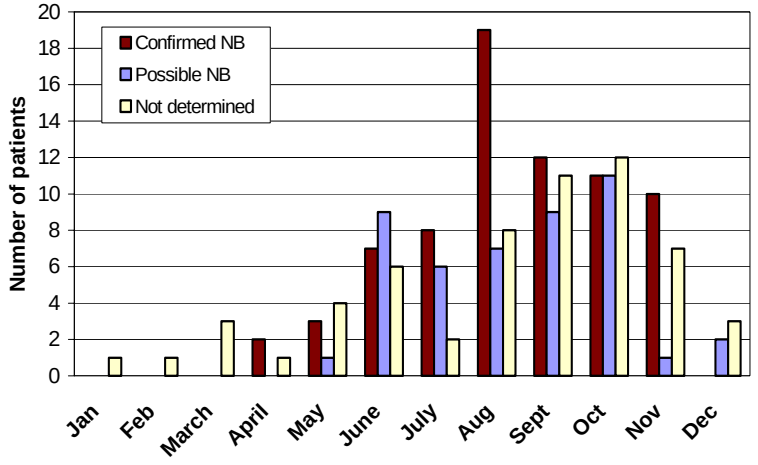


Figure 12. Seasonal distribution of illness in different diagnostic groups (n=177)

Duration of symptoms

Duration of neurological symptoms on admission varied from 1 day to more than 6 months. Patients in the "Possible NB" group had shorter duration of symptoms than "Confirmed NB" patients, which probably explains why patients in the "Possible NB" group did not yet show *Borrelia* antibody production in CNS. Children in the "Not determined" group reported significantly longer duration of symptoms on admission (Table 7).

Table 7. Characteristics of children being evaluated for NB (n=177)

On admission		Confirmed NB (n=72)	Possible NB (n=46)	Not determined (n=59)
Age	Median, n (range)	6 (1-14)	7 (1-18)	12 (2-18) ***
Sex	Male, n (%)	44 (61)	19 (41) *	25 (42) *
Previous tick bite	Yes, n (%)	40 (56)	26 (57)	32 (54)
Season for illness	January - March, n (%)	0 (0)	0 (0)	5 (8) *
	April - June, n (%)	12 (17)	10 (22)	11 (19)
	July - September, n (%)	39 (54)	22 (48)	21 (36)
	October - December, n (%)	21 (29)	14 (30)	22 (37)
Duration of symptoms	< 1 week, n (%)	19 (26)	26 (57) **	21 (36)
	1-4 weeks, n (%)	45 (62)	19 (41)	17 (29)
	1-2 months, n (%)	7 (10)	1 (2)	9 (15)
	> 2 months, n (%)	1 (1)	0 (0)	12 (20) **
Clinical features	Headache, n (%)	50 (69)	26 (57)	43 (73)
	Fatigue, n (%)	62 (86)	29 (63)	28 (47) ***
	Facial nerve palsy, n (%)	43 (60)	35 (76)	20 (34) **
	Loss of appetite, n (%)	43 (60)	26 (57)	13 (22) ***
	Neck pain, n (%)	33 (46)	18 (39)	21 (36)
	Fever, n (%)	33 (46)	19 (41)	9 (15) ***
	Nausea, n (%)	19 (26)	12 (26)	20 (34)
	Pain in extremity, n (%)	21 (29)	8 (17)	13 (22)
	Vertigo/nystagmus, n (%)	12 (17)	9 (19)	22 (37) **
	Neck stiffness, n (%)	22 (30)	13 (28)	8 (14)
	Parestesia, n (%)	3 (4)	0 (0)	2 (3)
	Irritable/aggressive	2 (3)	3 (7)	2 (3)
	Oculomotorial palsy, n (%)	1 (1)	0 (0)	1 (2)
	Peroneal palsy, n (%)	0 (0)	0 (0)	1 (2)
	Sudden deafness, n (%)	0 (0)	0 (0)	1 (2)
	EM, n (%)	11 (15)	17 (40) *	7 (12)
	Lymfocytoma, n (%)	2 (3)	7 (15) *	0 (0)
Symptoms per patient	Median, n (range)	5 (1-10)	4 (1-9)	3 (1-9) **

Differences, as compared to “Confirmed NB”, are shown as * (p<0.05), **(p<0.01) or *** (p<0.001)

Clinical symptoms and signs

Headache and fatigue were the two most common symptoms on admission whereas acute peripheral facial nerve palsy was the most frequent neurologic sign (Table 7).

Fatigue, facial nerve palsy, loss of appetite and fever occurred more often among children in the “Confirmed NB” group as compared to the “Not determined” group (Table 7).

Headache, neck pain, neck stiffness and nausea were common symptoms equally presented in different diagnostic groups. Myalgias were not easily distinguished from radiant pain or artralgias in our pediatric patients but as many as 42 patients (24%) reported pain in extremities. No differences between groups were found. Furthermore, no arthritis was

reported or found. Vertigo or nystagmus occurred more frequently among “Not determined” patients (Table 7). A few uncommon signs and symptoms such as parestesia (n=5), oculomotorial nerve palsy (n=2), peroneal nerve palsy (n=1), sudden deafness (n=1), and changes in personality or aggressiveness (n=7) occurred in different diagnostic groups, as shown in Table 7. Finally, patients reported several coincidental symptoms in both the “Definite NB” and the “Possible NB” group whereas patients in the “Not determined” group had significantly fewer complaints (Table 7).

EM/lymphocytoma

EM (n=35) or lymfocytomas (n=9) were reported from, or found in several patients in all three diagnostic groups but were significantly more common in the “Possible NB” group (Table 7). Males and females were equally represented. The majority of cases (n=41, 93%) had occurred within two months prior to the onset of neurologic symptoms, which is in concordance with earlier findings (Arnez et al. 2002; Arnez et al. 2003b; Stanek & Strle 2003). No child presented with multiple EM in contrast to studies from Slovenia where multiple EM are more frequent (Arnez et al. 2002).

Facial nerve palsy

More than half of the children being evaluated for NB (55%) presented with facial nerve palsy, which is similar to previous studies (Christen et al. 1993; Huppertz 2001; Thorstrand et al. 2002). Most of them were classified as “Definite NB” and “Possible NB” (Table 8).
The duration of facial nerve palsy on admission did not differ between groups and the majority of patients came within 2 days of onset. The facial nerve function was examined by an Ear-Nose-Throat specialist in 44 patients (45%) (Table 8). The House-Brackmann scale was used in order to evaluate the severity of the palsy (House & Brackmann 1985) and patients showed mild to total loss of function on admission. Severity of the facial nerve palsy did not differ between diagnostic groups. Pleocytosis, as a sign of ongoing inflammation in CNS, was found in 78 patients (80%) with facial nerve palsy, some without signs of meningitis. Similar findings have been seen earlier (Christen et al. 1990; Cook et al. 1997; Sandstedt et al. 1985). Headache or meningeal symptoms, were reported by 59 patients (60%) in all three diagnostic groups, but significantly more often in “Confirmed NB”, as compared with “Not determined” ($p<0.05$). Further related symptoms are shown in Table 8.

Facial nerve palsy in combination with EM or lymphocytoma occurred more often among patients in “Confirmed NB” and “Possible NB” than in the “Not determined” group ($p<0.05$). It has been reported that tick bites, EM and lymphocytomas were more frequently found in the head and neck region in children, than in adults, and correlated to neurological manifestations to a higher extent in children (Berglund et al. 1995; Christen 1996; Jorbeck et al. 1987; Olsson et al. 1988). Our data cinfirmes these findings and the majority of EM and lymphocytomas were found in the head and neck region on the ipsilateral side (Table 8). One might suspect that the spirochete invades CNS through the facial nerve but no histo-pathological evidence is found for the invasion of CNS through pathways other than hematogenic spread through the blood-brain barrier.(Garcia-Monco et al. 1990).

Table 8. Children with facial nerve palsy on admission (n=98)

On admission		**Total** n (%)
Duration of facial nerve palsy	1 – 2 days	54 (55)
	3 – 6 days	35 (38)
	1 – 2 weeks	7 (7)
	3 – 4 weeks	2 (2)
Facial nerve dysfunction *	Mild	5 (5)
	Moderate	19 (19)
	Severe	12 (12)
	Total loss of function	8 (8)
	Not evaluated	54 (55)
Related signs and symptoms	Headache or meningeal symtoms	59 (60)
	EM/lymphocytoma (ipsilateral,	16 (16)
	head/neck)	9 (9)
	EM (other parts of body)	2 (2)
	Mediaotitis (ipsilateral, ear) [&]	3 (3)
	Vesicles (ipsilateral, ear/mouth) [&]	8 (8)
	Pain (ipsilateral, ear/face) [&]	13 (13)
	Vertigo/nystagmus	1 (1)
	Taste disturbance	3 (3)
	Bilateral facial nerve palsy	
Diagnostic groups	Confirmed NB	43 (44)
	Possible NB	35 (36)
	Not determined	20 (20)

* Evaluated by the House-Brackmann facial nerve palsy grading system (Hansen & Lebech 1991)

[&] No patient presented with contralateral signs or symptoms

Treatment

Antibiotic treatment was prescribed for patients with signs and symptoms indicative of NB in combination with pleocytosis in CSF. Mainly ceftriaxone (n=62) and doxycycline (n=56) were used according to national guidelines with an age limit of 8 years for doxycycline (Table 9) (MPA 1998; Thorstrand et al. 2002). In addition, five patients in the "Not determined" group with clinical features indicative of NB received doxycycline without having pleocytosis, thus diverging from clinical routines. One of these children had a EM, two reported previous tick bites and two patients had experienced a former *Borrelia* infection and demanded antibiotic treatment. Children were treated with antibiotics for 10-14 days depending on different routines at different clinics.

In addition, one child in the "Not determined" group got steroid treatment for Bell's palsy, two got antibiotic treatment for media otitis and three got antiviral treatment due to vesicles near the ear or the mouth.

Table 9. Antibiotic treatment

Treatment	**Confirmed NB** (n=72)	**Possible NB** (n=46)	**Not determined** (n=59)
Ceftriaxone i.v, n (%)	42 (58)	20 (43)	0 (0) ***
Penicillin G i.v, n (%)	2 (3)	3 (7)	0 (0)
Doxycycline p.o, n (%)	28 (39)	23 (50)	5 (8) ***

Differences, as compared to "Confirmed NB", are shown as *** ($p<0.001$)

Further discussion on clinical features

The overall impression is that clinical features are very similar in "Confirmed NB" and "Possible NB" but differ from the "Not determined" group. Children in the "Not determined" group seem to be either patients with facial nerve palsy of unknown origin with short duration of symptoms (Bell's palsy) or patients being investigated for non-specific neurological symptoms (i.e. headache, fatigue) with long duration of symptoms. A few patients in "Not determined" presented with EM and admittedly, some of these patients could have been NB patients without inflammation in CSF. In cases of very short duration of symtoms at first lumbar puncture and prolonged symptoms, or in rare cases with severe neurologic symptoms, a repeated lumbar puncture could be considered. It is also reported from earlier studies that

most symptoms resolve spontaneously in NB without antibiotic treatment (Niemann et al. 1997) but there is a risk of progression to late NB (Kan et al. 1998).
Most EM or lymfocytomas were untreated in our study (87%) and discovered on admission or from the history. A few children (n=5, 13%) had received oral penicillin 1 week to 2 months earlier. Obviously, these five children developed neurological symptoms despite antibiotic treatment of EM according to Swedish guidelines (MPA 1998). Possibly, antibiotic treatment with penetration to CNS should have been benefitial. In a large study on EM in southern Sweden, no such cases were seen and it was concluded that phenoxymethyl penicillin p.o. was the drug of choice (Bennet et al. 2003). Other studies support amoxicillin, azitromycin, cefuroxim-axetil or doxycyline for oral treatment of EM, in order to acheive prophylactic treatment of disseminated LB by drug penetration to CNS (Arnez 2007; Eppes et al. 2002; Sood 1999).

Bilateral facial nerve palsy has been considered pathognomonic for Lyme NB in children (Christen et al. 1993). Interestingly, we found bilateral facial nerve palsy in four patients in our study, three being classified as “Confirmed NB” (Paper I, IV) and one as “Not determined” (Paper IV). The latter was an eleven-year-old boy with right-sided media otitis and vesicles on the lips. A lumbar puncture was normal, showing no signs of inflammation and no *Borrelia* antibodies or PCR findings of HSV 1 or 2 were found. The boy received amoxicillin for the media otitis and recovered completely from the bilateral facial nerve palsy. Consequently, we suggest that all children with facial nerve palsy should be referred to an ENT-specialist in order to find differential diagnoses but also for evaluation of recovery.

Prediction of diagnosis (Paper IV)

Design of the NB prediction score test

With our NB prediction score test, we wanted to construct a practical, easy-to-use variant of predictive test for use in Lyme endemic settings, with a clear cut-off between high and low probability of NB. Facial nerve palsy, fatigue and fever on admission were symptoms associated with a higher probability of "Confirmed NB" in our statistical calculations (Table 7), including a logistic regression, and are therefore included in the NB prediction score test. Detailed data on fever was not available in our material but, as it is well known that low grade fever is strongly connected to LB (Tuerlinckx et al. 2003) we have chosen to define fever as 38-39° C for the test. The occurrence of EM or lymphocytoma did not differ significantly between "Confirmed NB" and other children in our calculations, but since EM and lymphocytoma are regarded as pathognomonic for Lyme Borreliosis (Eppes 2003), it was considered motivated to include them in the score test. Pleocytosis in CSF was the most prominent variable for predicting "Confirmed NB" according to our calculations and was therefore given 2 points in the NB prediction score test. Thus, the score test includes:

Acute facial nerve palsy (1p), Fever (1p), Fatigue (1p), EM and/or Lymphocytoma (1p) and **Pleocytosis in CSF (2p)** as shown in Figure 13.

Age is a variable that differs between "Confirmed NB" and "Not determined" but is not included in the test. We have choosen to show results of the test, divided into different age-intervals, in order to report test performance in more detail in the different age groups in our material (Table 10).

The performance of the test at different score-levels is described more closely in Paper IV. A cut-off at score ≥ 3 for a positive test was chosen. Consequently, start of early antibiotic treatment was recommended at score ≥ 3 while at score ≤ 2, the physician should wait for *Borrelia* antibody test results and more carefully consider other differential diagnoses (Figure 13).

The Neuroborreliosis prediction score test

At admission:

- **Acute facial nerve palsy** □ **1 p.**
- **Fever** □ **1 p.**
- **Fatigue** □ **1 p.**
- **Erythema migrans and/or Lymphocytoma** □ **1 p.**
- **Pleocytosis in CSF** □ **2 p.**

Total score (points): **____ p.**

If score ≥ 3: **High probability of Neuroborreliosis.**
Start antibiotic treatment.

If score ≤ 2: **Low probability of Neuroborreliosis.**
Consider other diagnoses or wait for Borrelia antibody test results. If Erythema migrans or Lymphocytoma occur, start antibiotic treatment for cutaneous manifestation of Lyme Borreliosis.

Definitions:
Fever: 38 - 39˚ C
Pleocytosis: ≥ 5 x 10^6 cells/L (> 90% mononuclear cells)
Erythema migrans: Typical annular red skin lesion ≥ 5 cm in diameter.
Lymphocytoma: Typical blue-red indured skin lesion on earlobe.
Skin lesions can be reported by patient or verified by physician.
The NB prediction score test should be used in Lyme endemic areas only.

Figure 13. The Neuroborreliosis prediction score test

Results of the NB prediction score test (Paper IV)

Results indicate that all patients in the "Confirmed NB" group should be recommended for early antibiotic treatment in all age groups (Table 10). Additionally, all patients in the "Possible NB" group should also recieve antibiotic treatment according to the NB prediction score test. This seems accurate considering they all have pleocytosis with mononuclear dominance in CSF and many of them (76%) have clinical features strongly indicating early NB. A few patients (n=2) in the "Not determined" group (in age interval 12-18 years) would also, according to the test, be recommended for early antibiotic treatment, despite the lack of pleocytosis in CSF. These two children probably represent peripheral nervous system Borreliosis (facial nerve palsy, fatigue, EM) and early disseminated LB (headache, fever, fatigue, EM). Finally, 57 patients in the "Not determined" group would not, as indicated by the NB prediction score test, receive antibiotic treatment. Admittedly, some patients in the "Not determined" could be NB patients without inflammation in CSF. In cases with very short duration of symptoms at the first lumbar puncture or in rare cases with severe neurologic symptoms, a repeated lumbar puncture could be considered by the pedatrician. It is also noteworthy that EM and lymphocytomas, if diagnosed on admission, should always be treated with antibiotics as recommended in guidelines (MPA 1998) for cutaneous manifestations of Lyme Borreliosis (Figure 13, Table 2).

Table 10. Results of the NB prediction score test in different age groups

Age interval	**The NB prediction score test**	**Confirmed NB** (n=72)	**Possible NB** (n=46)	**Not determined** (n=59)
0-5 years	Positive test (score ≥ 3) Negative test (score ≤ 2)	29 0	18 0	0 3
6-11 years	Positive test (score ≥ 3) Negative test (score ≤ 2)	37 0	21 0	0 26
12-18 years	Positive test (score ≥ 3) Negative test (score ≤ 2)	6 0	7 0	2 28

Concluding remarks on prediction of diagnosis

In our material, the NB prediction score test predicts NB in 120 of 177 (68%) patients being evaluated for NB, and consequently according to the test, these patients would have been recommended for antibiotic treatment. The majority of patients were in the "Confirmed NB and "Possible NB" groups and this finding supports the clinical routine that children with NB symptoms and pleocytosis in CSF should receive early antibiotic treatment despite the lack of *Borrelia* antibodies in CSF. The NB predicition score test seems to perform well in all age groups in our material (Table 10) but needs to be further evaluated in different clinical settings.

Clinical outcome and prognosis (Paper I, IV)

Reported recovery at follow-up (Paper IV)

At the 2-month follow-up, 117 patients (67%) reported total recovery. Further recovery at the 6-month follow-up in different diagnostic groups is shown in Table 11. In total, 140 patients (79%) recovered at the 6-month follow-up and 37 patients (21%) reported persistent symptoms. The major complaints were headache, fatigue and facial nerve palsy (Table 11).

Table 11. Clinical recovery at the 6-month follow-up (n=177)

At follow-up		Confirmed NB n (%)	Possible NB n (%)	Not determined n (%)
Time to recovery	< 1 w	15 (21)	10 (22)	3 (5)
	1-4 w	39 (54)	16 (35)	15 (25)
	1-2 m	5 (7)	6 (13)	8 (14)
	3-6 m	6 (8)	8 (17)	9 (15)
	> 6 m	7 (10)	6 (13)	24 (41) ***
Reported recovery	Recovered (n=140)	65 (90)	40 (88)	35 (59) ***
	Not recovered (n=37)	7 (10)	6 (13)	24 (41) ***
Major persistent symptoms	Headache (n=22)	2 (3)	2 (4)	18 (31) ***
	Fatigue (n=10)	2 (3)	2 (4)	6 (10)
	Facial nerve palsy (n=11)	5 (7)	4 (9)	2 (3)

Differences, as compared to Confirmed NB, are shown as *** ($p<0.001$)

Several patients reported several symptoms

Patients with headaches in the “Not determined” group (n=18) were classified as migraine (n=4), as psychiatric problems (n=3) or as unspecific headache (n=11). Children with persistent symptoms after facial nerve palsy (n=11) reported as eye-closing impairment (n=1), affected pronunciation (n=2), extensive tear secretion (n=2), cosmetic complaints (n=5) and social consequences (n=1).

Patients as compared to controls (Paper IV)

When comparing reported persistent symptoms in patients at follow-up with the matched control group, persistent facial nerve palsy occurred, as expected, only among patients whereas headache and fatigue were reported with surprisingly less frequency among patients as compared to controls (Table 12). Less common symptoms such as loss of appetite, neck pain, nausea and vertigo did not differ between patients and controls. Affected daily activities were reported in 24% of patients and 38% of controls (n.s, data not shown).

Table 12. Frequency of reported symptoms in patients (n=177) and controls (n=174)

Major reported symptoms at 6-month follow-up	**Patients** n (%)	**Controls** n (%)
Headache	22 (12)	41 (24) **
Fatigue	10 (6)	34 (20) ***
Facial nerve palsy	11 (6)	0 (0) ***
Loss of appetite	3 (2)	5 (3)
Neck pain	3 (2)	3 (4)
Fever	0 (0)	0 (0)
Nausea	4 (2)	0 (0)
Myalgias	1 (1)	0 (0)
Vertigo	4 (2)	1 (1)
Neck stiffness	2 (1)	0 (0)

Differences between patients and controls, shown as ** ($p<0.01$) or *** ($p<0.001$)

Several patients and controls reported several symptoms

Clinical outcome in patients with facial nerve palsy (Paper I)

At re-examination 2 years after an acute episode of facial nerve palsy, the recovery rate was 78 %. Six out of 27 children (22 %) were found to have permanent facial nerve palsy. The facial nerve dysfunction was mild to moderate and none of the children had persistent severe or total loss of nerve function. One child had bilateral facial nerve palsy. Two children reported pronunciation difficulties and a further 2 children had eye-closing impairment with abundant tear secretion (Table 13).

Table 13. Oto-neurologic data of children (n=6) with sequelae of facial nerve palsy (2-year follow-up)

Number	Facial nerve *	Frenzel	Microscopy	Symptoms	Diagnosis
1	R 1 1 2	Normal	Normal	-	Confirmed NB
2	R 1 1 2 &	Normal	Normal	Eye closing impairment	Possible NB
3	R 1 2 3	Normal	Normal	-	Confirmed NB
4	L 2 3 3	Normal	Normal	Pronunciation difficulties	Suspected HSV #
5	L 2 1 3	Normal	Normal	Eye closing impairment	Not determined
6	R 3 3 2 L 2 3 3	Normal	Normal	Pronunciation difficulties	Confirmed NB

* House-Brackmann scale 1-6 on forehead, eye and mouth (1 is normal function and 6 is total loss of function), L= left, R= right

& Synkinesy between eye and mouth

\# Vesicles behind the ear, no confirmation of viral diagnosis was found

Examination for nystagmus in Frenzel glasses, including the headshake test, was normal in all children, which excluded permanent unilateral loss of vestibular nerve function. Oto-microscopy was normal and no hearing loss was reported or found in any child. No other symptoms or health problems accompanied the sequelae of the facial nerve palsy. No patient was found to have progressive neurologic symptoms. Characteristics of sequelae are shown in Tabel 13. Recovery after facial nerve palsy in different diagnostic groups in Paper I and Paper IV, is visualized in Table14. Furthermore, no significant differences were found when comparing recovery of facial nerve palsy between different diagnostic groups.

Table 14. Recovery of facial nerve palsy in different diagnostic groups (Paper I, IV)

	Paper I: **Re-examination at 2-year follow-up** (n=27)			**Paper IV:** **Reported symptoms at 6-month follow-up** (n=98)		
Diagnostic groups	**Confirmed NB** (n=11)	**Possible NB** (n=10)	**Not determined** (n=6)	**Confirmed NB** (n=43)	**Possible NB** (n=35)	**Not determined** (n=20)
Completely recovered	73 %	90 %	67 %	88 %	89 %	90 %
Persistent facial palsy	27 %	10 %	33 %	12 %	11 %	10 %

Prognostic factors for recovery at follow-up (Paper IV)

When comparing variables such as the season of the year, previous tick bites, EM, lymphocytoma, and most symptoms on admission among patients in the clinical prospective study (n=177), none of them influenced clinical recovery at the 6-month follow-up. However, headaches and long duration of symptoms (> 2 months) on admission were more often seen in patients who reported persistent symptoms at follow-up ($p<0.01$ and $p<0.001$ respectively). These patients were mainly classified as "Not determined" (Table 7) and were later diagnosed as having migraine, unspecific headache or headache connected to psychiatric problems.

Severity or duration of the facial nerve palsy on admission was not correlated to persistent symptoms from the facial nerve palsy at the 6-month follow-up. Furthermore, among children with facial nerve palsy, no differences in recovery were seen between diagnostic groups. Among all patients who received antibiotic treatment (n=123), recovery rate was not influenced by duration of symptoms on admission, choice of antibiotic drug or duration of treatment.

In a earlier retrospective study, it has been concluded that a shorter time from the onset of symptoms until the start of antibiotic treatment is profitable for clinical outcome (Berglund et al. 2002). Admittedly, in our prospective follow-up study, we could not find evidence that early antibiotic treatment would be advantageous for clinical outcome. Patients in "Confirmed NB" and "Possbile NB" groups reported early response to treatment in most cases (Table 11) and those reporting persistent symptoms had similar duration of symptoms before treatment. Finally, when analyzing patients solely in the "Confirmed NB" group, no specific variables correlated to a less favorable course of the disease. This is in line with an earlier study in Sweden by Thorstrand, where no prognostic factors could be identified (Thorstrand et al. 2002).

Prognostic factors in patients with facial nerve palsy (Paper I)

When comparing variables such as gender, age, laboratory findings, diagnosis, treatment or related symptoms (such as EM, headache, vertigo) or other health problems among patients with sequelae (n=6) and patients without sequelae (n=21), none of these influenced clinical recovery at the 2-year follow-up.

Further discussion on clinical outcome and prognosis

Persistent symptoms after NB in children, is considered more of a minor problem than in adults (Berglund et al. 2002; Vrethem et al. 2002). However, we found that patients reported persistent symptoms in 21 % of cases at the 6-month follow-up. Major complaints were, headache and fatigue which are common nonspecific symptoms in children and adolescents (Laurell et al. 2006).However interestingly, matched controls reported these unspecific symptoms even more frequently. Consequently, headache and fatigue are important symptoms on admission, but at follow-up they should not be considered attributable to NB. Why controls more frequently report headache and fatigue is unclear. It can possibly be explained by the fact that all controls answered the questionnaire by mail and patients by telephone interview (structured questionnaire) or by the fact that controls answered the questionnaire in the month of November when it is dark in Sweden (Palinkas et al. 2000; Rastad et al. 2006; Swedo et al. 1995).

No patient was found to have progressive or recurrent neurological symptoms at follow-up. A few reported problems in school, which possibly could represent cognitive impairement as in Post-Lyme symdrome. However, patients did not differ from controls but, admittedly, patients and controls were not evaluated with neuropshychiatric tests in our follow-up.
As for adults, results are contradictory in this field (Fallon et al. 2007; Klempner et al. 2001; Oksi et al. 2007; Picha et al. 2006; Shadick et al. 1994). One author found cognitive impairement in children post-Lyme, but not in controls (Tager et al. 2001) whereas others did not (Adams et al. 1999; Vazquez et al. 2003). One author treated five children with persistent symptoms after NB with perenteral antibiotics (ceftriaxone) and documented improvement. However, after the end of the treatment, some patients relapsed in symptoms.
No conclusions could be drawn from such studies with a small number of children, no controls and short follow-up time. Furthermore, it has been shown that antibiotic treatment per se can exert an anti-inflammatory effect which can possibly expain why some patients experience improvement of symptoms during and shortly after a course of antibiotic treatment (Brooks et al. 2001; Labro 2000). In larger studies on adults no beneficial long lasting cognitive improvements in post-Lyme patients can be achieved with prolonged antimicrobial therapy (Halperin 2007).

Mild to moderate facial nerve dysfunction at follow-up was reported in 11 % (Paper IV) and found as an objective finding in 22 % (Paper I) of patients with facial nerve palsy. Differences between results could possible be explained by the discrepancy between subjective symptoms and objective findings as described by Bagger-Sjöbäck et al (Bagger-Sjoback et al. 2005). However, the assumption that recovery is total in children with facial nerve palsy is wrong. This has also been highlightened by the author in a long-term follow-up with thorough neurophysiological examination of children with facial nerve palsy (Bagger-Sjoback et al. 2005). Sequelae after facial nerve palsy seems to appear less frequently in children than in adults (Adour et al. 1978; Hyden et al. 1982; Ljostad et al. 2005) but dysfunctional as well as cosmetic problems are reported in this thesis (Paper I and IV). Earlier studies has indicated a more favorable outcome in children with facial nerve palsy due to Lyme Borreliosis as compared to facial nerve palsy of unknown etiology (Christen et al. 1993; Peltomaa et al. 1998). In our study (Paper I and IV), no significant differences in recovery rate between patients with “Confirmed NB” and other patients could be seen (Table 14). Furthermore, no prognostic factors of importance for clinical recovery after facial nerve palsy were identified in our study (Paper I, IV). Thus, it is still unknown why some patients get a permanent facial nerve dysfunction.

Immune responses in children with Neuroborreliosis (Paper II)

Cytokine secretion

In our study, when comparing results from ELISPOT analyses, children with “Confirmed NB” and “Possible NB” had significantly higher number of both *Borrelia*-specific IL-4 and IFN-γ secreting cells as compared to controls (Figure 14). In blood, the cytokine response showed a similar pattern, but was not as pronounced as in CSF. When analyzing cytokine responses within diagnostic groups, patients in “Confirmed NB” had higher *Borrelia*-specific IFN-γ than IL-4 secretion in both CSF ($p<0.05$) and blood ($p<0.001$). Variables such as duration of symptoms at admission, occurrence of EM or time to recovery did not seem to influence cytokine secretion in CSF or blood.

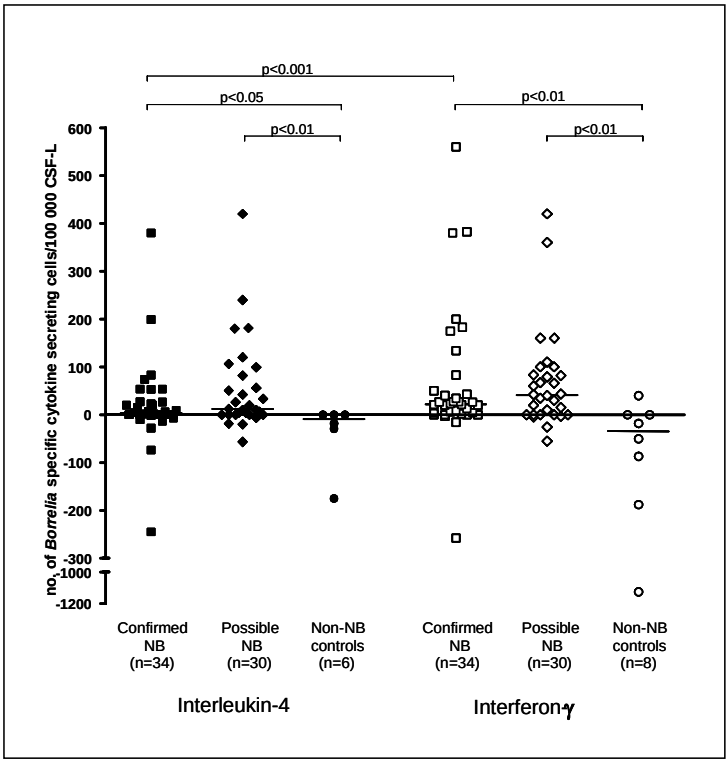


Figure 14. Results of ELISPOT in Paper II

Children as compared to adults

In general, the immune responses in early childhood are known to be skewed towards type 2 reponses and gradually balanced towards type 1 in adulthood (Holt 1995). We therefore considered it of interest to take a closer look at immune responses in children and adults with NB. Reference material on adult patients with “Confirmed NB” or “Possible NB” was compared to the *Borrelia*-specific cytokine pattern of children with “Confirmed NB” or “Possible NB”. Children were found to have equally high *Borrelia*-specific secretion of IFN-γ but significantly higher *Borrelia*-specific IL-4 secretion in CSF as compared to adults ($P<0.05$). Since repeated lumbar punctures are not ethically correct in children, evaluation of cytokine secretion changes over time, as made in previous studies on adult patients with NB (Widhe et al. 2004), was not feasible.

The role of type 1 / type 2 responses

Our results demonstrate that children, in addition to the type 1 response earlier reported in adults, also show *Borrelia*-specific type 2 responses in both "Possible NB" and "Confirmed NB". The type 1 immune response with high production of IFN-γ would appear to be the optimal response to all infections caused by intracellular or phagocytosable microbes (Spellberg & Edwards 2001). By clearing these pathogens, the type 1 response diminishes further antigenic stimulation, allowing a switch to a type 2 response, resulting in the re-establishment of homeostasis. The relative lack of type 2 responses may predispose for development of persistent disease in adults (Widhe et al. 2004).

Our finding strongly suggests that children with NB have a balanced type 1/type 2 immune response which might contribute to the more benign and rapid course of disease seen.

Clinical implications of ELISPOT results

Immune responses in CSF in patients classified as "Confirmed NB" and "Possible NB" were very similar and both differed significantly from the controls, with an up-regulation of both IFN-γ and IL-4 secreting cells in CSF. In the ELISPOT method, cells are stimulated with *Borrelia* specific OF antigen and results indicate a strong *Borrelia* specific reaction in both "Confirmed NB" and "Possible NB". This strongly supports the notion that "Possible NB" patients are most probably true NB cases where the antibody response with the flagella antigen cannot yet be visualized. This further strengthens arguments that antibiotic treatment in this "Possible NB" group of patients is adequate. Results from new antibody tests are also in line with these results and will be further described below.

The ELISPOT method could not only be used to evaluate and understand different immune responses in NB, but possibly also as a diagnostic test. This issue will be discussed in "Additional diagnostic aspects".

Concluding remarks on immune responses in children with Neuroborreliosis

We can herby show differences in *Borrelia*-specific IL-4 and IFN-γ secretion in CSF in children as compared to adults with NB, indicating that children have a more balanced type 1/type 2 immune response. Since IL-4 is known to down-regulate the pro-inflammatory and possibly harmful effects of prolonged IFN-γ responses, our hypothesis is that the observed prominent IL-4 response in the CNS-compartment might contribute to the less severe course of the disease seen in children as compared to adults with NB.

New *Borrelia* antibody tests in Neuroborreliosis (Paper III)

Plausible diagnostic insufficences of the flagella antigen

About one third of children with signs and symptoms indicative of NB, present with pleocytosis in CSF but with no *Borrelia* antibodies in CSF (flagella antigen, routine ELISA) (Paper IV). One suspects shortcomings in this diagnostic method based on the flagella antigen which also has been discussed in earlier studies (Wilske 2003). Hence, new diagnostic methods are needed and recent studies have been encouraging on new *Borrelia* antigens in CSF in mainly mixed-age study material, as well as in serum in children, (Heikkila et al. 2005; Panelius 2002). In our study, we wanted to evaluate three new recombinant DbpA, BBK32 and OspC antigens and the peptide antigen IR6 in different pediatric patient groups and controls, in order to find improved diagnostic tests for NB in children.

Reactivity to four new Borrelia antigens

CSF and serum from patients were tested for IgG antibodies to DbpA, BBK32, OspC and IR6 antigens with a standard ELISA method. Each of the four antigens performed well and reactivity was generally higher in CSF than in serum. No antigen was superior and variants of antigens originating from *B. garinii* dominated. Results in CSF are shown in Figure 15 and Table 15.

Table 15. Antibodies to different new antigens in CSF *

Diagnosis	**DbpA** n (%)	**BBK32** n (%)	**OspC** n (%)	**IR6** n (%)
Confirmed NB (n=24**)**	22 (92)	20 (83)	15 (62)	22 (92)
Possible NB (n=16)	11 (69)	8 (50)	8 (50)	6 (38)
Not determined (n=17)	7 (41)	1 (6)	3 (12)	0 (0)
Controls (n=36)	2 (6)	0 (0)	1 (3)	1 (3)

* Shown as number (n) and percentage (%) of positive IgG ELISAs in CSF

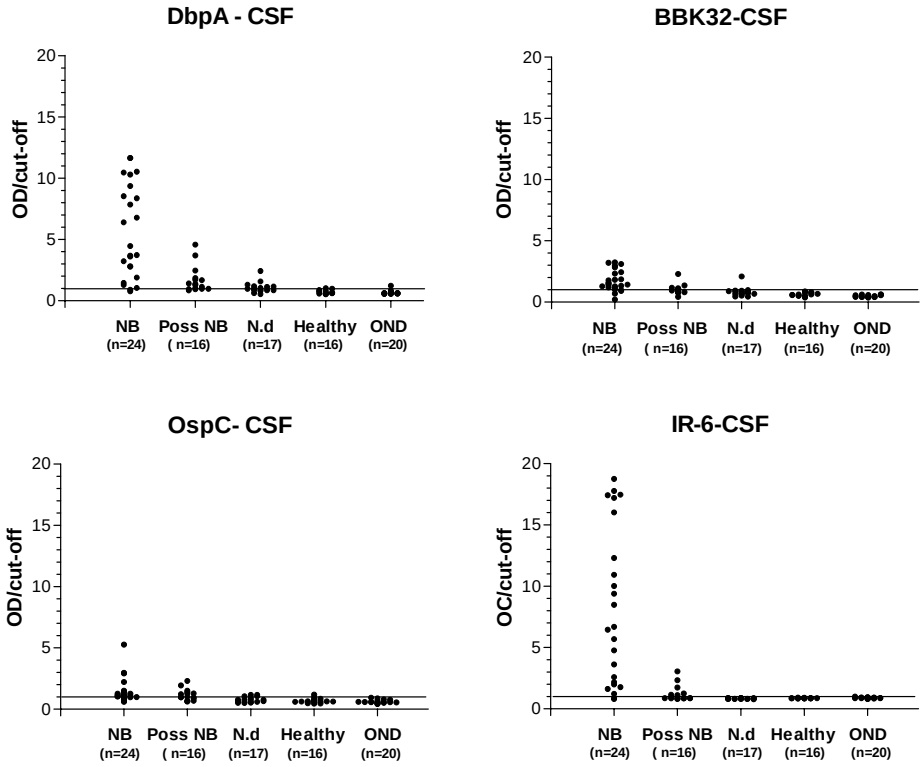


Figure 15. Reactivity in CSF to four new Borrelia antigens

Our results are in concordance with earlier studies on CSF in mixed-age patients with NB (Panelius 2002). The BBK32 antigen though, was more prominent among adult patients with early NB, defined as neurological symptoms less than 3 months (Heikkila et al. 2002b; Panelius 2002), than in our pediatric patients. This could possibly be explained by the fact that the majority of our pediatric patients (82%) presented with duration of symptoms less than 1 month and probably represented NB at an earlier stage than in adult patients.

Antibodies in CSF in correlation to clinical data

There were no significant differences in antibody reactivity in CSF when comparing each of the four new antigens with clinical data such as previous tick bites, EM, facial nerve palsy, duration of symptoms or clinical outcome.

Diagnostic performance

Sensitivity for DbpA was 82%, for BBK32 70%, for OspC 58% and for IR6 70%. Specificities were 94%, 100%, 97% and 97%, respectively. If new antigens were combined in the Panel A, sensitivity was 80%, provided that reactivity to ≥ 2 different antigens was considered as a positive test (Table 16). Specificity was 100%, provided that reactivity to ≤ 1 antigen was considered as a negative test. When adding the flagella antigen to the panel B, the sensitivity improved from 80% to 82% and the specificity stayed at 100% (Table 16). Finally, as a comparison, sensitivity was 60% for the routine flagella antigen alone and specificity was 100% as shown in Table 16.

Table 16. Diagnostic performance of different diagnostic tests

	DbpA	BBK32	OspC	IR6	Panel A	Panel B	Flagella	ELISPOT
Paper III								
Sensitivity	82 %	70 %	58 %	70 %	80 %	82 %	60 %	-
Specificity	94 %	100 %	97 %	97 %	100 %	100 %	100 %	-
Paper II								
Sensitivity	-	-	-	-	-	-	47 %	84 %
Specificity	-	-	-	-	-	-	100 %	80 %

Panel A: DpbA, BBK32, OspC, IR6
Panel B: DpbA, BBK32, OspC, IR6 and Flagella

Concluding remarks on new Borrelia antibody tests

We hereby show that new antigens improve diagnostic performance as compared to the routine flagella antigen. This new finding is in concordance with results from previous Finnish studies on CSF antibodies in material from age-mixed studies (Panelius 2002) and on serum antibodies in children with NB (Heikkila et al. 2005). If different antigens are combined in a panel in order to cover the antigenic diversity, sensitivity further improves and a specificity of 100% can be maintained. Our finding that reactivity to *B. garinii* related antigens is dominant in children with NB is in line with the well known neurotropism of *B. garinii* (Balmelli et al. 1995; Ornstein et al. 2002) and with previous findings on cellmediated immunity in the central nervous system (Ekerfelt et al. 1998).

In a situation were there is no approved ‘golden standard’, there will inevitably be difficulties in classifying patients as well as performing unobjectionable calculations of diagnostic accuracy of new diagnostic tests. We found it justifiable to divide our patients into diagnostic groups based on clinical signs and symptoms and routine laboratory findings at presentation as described earlier (Table 5). In the group of patients with “Confirmed NB” we found a high reactivity (92%) to DbpA and IR6 (Table 15). Diagnostically more challenging is the “Possible NB” group, children with neurological signs and symptoms indicative of NB with pleocytosis but no anti-flagella antibodies in the CSF. New antigens showed reactivity in 38% - 69% of patients with “Possible NB” (Table 15), indicating that more than half of these children may be missed in the diagnostic procedures for NB, based on anti-flagella antibody testing only.

Additional diagnostic aspects (unpublished)

ELISPOT as a diagnostic test

We wanted to look at results from Paper II (same number of patients and controls, shown in Table 4) with another perspective, in order to evaluate ELISPOT as a diagnostic test.
In Paper II we found that children with “Confirmed NB” and “Possible NB” had a significantly higher number of *Borrelia*-specific IL-4 and IFN-γ secreting cells in CSF as compared to controls (Figure 14). Given a cut-off at 0, sensitivity for IFN-γ reactivity would be 75% and specificity 80 %. For IL-4, sensitivity would be 59% and specificity 80%. If combined IFN-γ and IL-4 reactivity, sensitivity would improve to 84% and specificity stay at 80% (Table 16). We have also looked at diagnostic performance with a given cut-off at 5 (in order to achieve higher specificity) and then sensitivity was 39% and specificity 90 %.
We can conclude that, with a cut-off at 0, sensitivity is high but specificity could have been more favourable. However, one must remember that since the number of controls was limited, these preliminary data need to be evaluated with causion and diagnostic performances of the ELISPOT method, as a diagnostic test, need to be studied in larger materials.
Finally, diagnostic performance for ELISPOT as compared to the routine flagella test and new *Borrelia* antibody tests are shown in Table 16.

Viral PCR diagnostics

CSF samples from patients being evaluated for NB but not meeting the criteria for "Confirmed NB", as described under "Additional patients and materials" (page 45), were investigated for neurothrop viruses; HSV 1+2, VZV and Enterovirus. PCR methods were used to enable detection of small amounts of viral proteins and our patient CSF samples were run together with routine patient samples at the laboratory, assuring the reliability of the method, e.g. including accurate positive and negative controls. No positive PCR findings for any of the viruses were found (unpublished data). These negative findings were perhaps a bit surprising and raise a question on whether there could have been circumstances explaining the results or if they are true negatives. Handling of patient samples was correct and centrifugation of CSF should not have been a problem. To detect viral RNA or DNA when re-activation occurs, in facial nerve palsy for example, is known to be a difficulty due to low sensitivities in certain PCR methods (Cinque et al. 2003; Furuta et al. 2005; Furuta et al. 2001; Kawada et al. 2004; Larsson et al. 1998) which could partly explain the negative results. Another possibility is that these patients represent undiagnosed Lyme NB, Bell's palsy, unspecific conditions or other infections not tested for.

TBE serology

Even though the clinical picture often varies between TBE viral infection and Lyme NB (Arnez et al. 2003a; Lesnicar et al. 2003; Logina et al. 2006), they could possibly be confused since both are transmitted by ticks. Thus, it was of interest to investigated serum samples for TBE-antibodies (IgM and IgG) in patients not meeting the criteria fro "Confirmed NB".
We found one out of 99 patients with positive TBE IgG and IgM in serum. In this patient, an acute TBE infection could possibly have been mistaken for "Possible NB". She presented with headache, fatigue, fever and loss of appetite for the previous 2-4 weeks and had pleocytosis (24 mononuclear cells x 10^6/ L) in CSF on admission. She received antibiotic treatment and recovered but later reported persistent headaches. Positive TBE serology should always be confirmed by a neutralization test (Vene et al. 1998) as will henceforth be performed in this patient.

Concluding remarks on additional diagnostics

We can notice from our calculations that the ELISPOT method is sensitive but not highly specific (Table 16). However, if the ELISPOT should be useful as a confirmatory test for NB, diagnostic performance with a strong specificity would be preferable. The ELISPOT method

is a time consuming method most useful in immunology studies, but whether it is suitable for diagnostic routine testing of NB needs to be further investigated.
Furthermore, no signs of re-activation of herpes viruses or missed enteroviral meningitis were found, but one possible TBE case was identified by serology.
In conclusion, we are prone to believe that missed viral infections among patients being evaluated for NB is not a substantial problem. This is further supported by the fact that all children in the "Possible NB" group presented with mononuclear dominance (> 90%) in CSF, which is strongly supportive for Borrelial, not viral, etiology according to earlier studies (Eppes et al. 1999; Shah et al. 2005; Tuerlinckx et al. 2003).

Summary of results and discussion

In clinical practice and in this study sample, about one third of children being evaluated for NB present with pleocytosis in CSF but no *Borrelia* antibodies in CSF, here classified as "Possible NB". This is unsatisfactory and one can suspect shortcomings in the flagella routine ELISA method used. Diagnostic performances of new *Borrelia* antibody tests (DbpA, BK32, OspC and IR6) were evaluated in Paper III and found to perform well. Furthermore, they seemed superior to the routine flagella antibody test. When new antigens were combined with the flagella antigen in a panel, sensitivity was 82% and specificity 100%. Thus, such a panel would improve diagnostic accuracy and be beneficial for pediatric patients being evaluated for NB.

In our prospective study in a Lyme endemic area in southeast Sweden, the NB diagnosis could be confirmed by intrathecal production of *Borrelia* specific antibodies in 41% of patients being evaluated for NB. Early antibiotic treatment was given to 69% of patients but many patients ended up with an uncertain diagnosis. Most patients responded well to treatment and surprisingly few were diagnosed as having other infectious or neurological diseases. No patient was found to have recurrent or progressive neurologic symptoms which is in line with earlier studies (Berglund 1999; Christen et al. 1993; Wang et al. 1998; Vazquez et al. 2003). Recovery was satisfactory in the majority of patients, since symptoms at follow-up were not more frequently reported in patients than in a matched control group. However, 11% of patients with acute facial nerve palsy, reported persistent symptoms at follow-up. When an objective oto-neurologic re-examination was performed 2 years after the acute facial nerve palsy, 22% of patients were found to have a mild to moderate permanent nerve

dysfunction. This is slightly more than in earlier studies (Peltomaa et al. 1998). Problems such as eye closing impairement, extensive tear secretion, pronunciation difficulties and cosmetic complaints were reported. We could not identify any prognostic factors, thus, it is still unknown why some patients get persistent symptoms after facial nerve palsy.

Children in "Confirmed NB" and "Possible NB" were very similar in clinical picture as compared to the "Not determined" group. Thirty-five patients (76%) in the "Possible NB" group presented with the combination of neurological symptoms, pleocytosis, EM/lymphocytoma and/or *Borrelia* antibodies in serum, strongly indicative of early NB. Recovery rate after antibiotic treatment was the same in "Confirmed NB" and "Possible NB". Furthermore, shorter duration of symptoms on admission was seen in "Possible NB" as compared with "Confirmed NB", which further strengthens the impression of "Possible NB" being NB patients where *Borrelia* antibodies in CSF are not yet produced. Results on immune responses are in line with this notion since both "Confirmed NB" and "Possible NB" had significantly higher number of *Borrelia* specific IFN-γ and IL-4 secreting cells in CSF than controls. Performance of new *Borrelia* antibody tests, in addition, showed high reactivity in CSF from children in the "Possible NB" group (Table 17).
With the NB prediction score test, all patients in the "Possible NB" were positive (Table 17) which further supports the clinical routine that children with neurological symptoms and pleocytosis in CSF should receive early antibiotic treatment despite the lack of *Borrelia* antibodies in CSF.

Table 17. Summery of positive test results in different diagnostic groups.

Diagnosis	**Paper I-IV** **Flagella in CSF** (n=250)	**Paper II** [*] **ELISPOT in CSF** (n=64)	**Paper III** [#] **New antigens in CSF** (n=57)	**Paper IV** **NB prediction score test** (n=177)	**Unpublished** **Viral PCR in CSF** (n=99)
Confirmed NB	100 %	85 %	92 %	100 %	-
Possible NB	0 %	80 %	62 %	100 %	0 %
Not determined	0 %	-	12 %	3 %	0 %

[*] Reactivity with *Borrelia* specific IFN-γ or IL-4 secreting cells in CSF
[#] Antibody detection in CSF to ≥ 2 of new antigens in a panel (DbpA, BBK32, OspC and IR6)

However, since three children in the “Possible NB” group were excluded from the study due to documented viral meningitis, it is justified to question whether there could have been further patients with undiagnosed viral meningitis hidden in the “Possible NB” group? We consider this unlikely, since all children in the “Possible NB” presented with mononuclear dominance (> 90%) in CSF, which is strongly supportive for *Borrelia*, not viral, etiology (Eppes et al. 1999; Shah et al. 2005; Tuerlinckx et al. 2003). In the material of additional patients investigated for different viral infections, we found no case with positive PCR regarding HSV 1 or 2, VZV or Enterovirus, (unpublished data, Table 17).
Furthermore, in our clinical setting, children with symptoms indicative of viral meningitis were often followed clinically, without a lumbar puncture, in order not to impose further stress on the child, and were therefore not included in the study.

A male majority in NB in childhood is seen in our “Comfirmed NB” group as well as in earlier studies in children and adults (Berglund et al. 1995; Berglund et al. 2002; Christen et al. 1993). Differences between gender could possibly be explained by different exposure behavior in nature (Bennet et al. 2007; Stjernberg et al. 2005b) but also possibly by differences in immune responses between sexes in LB (Jarefors et al. 2006).
When looking closer at EM in our study, girls and boys were equally represented, in contrast to the female predominance earlier reported in age mixed materials (Bennet et al. 2006b). However, when separating different age groups in that specific study, occurrence of EM were similar in females and males in the lower age groups. This is in line with earlier EM findings in children (Arnez et al. 2003b). It has also been shown that children with multiple EM (early disseminated LB) were in male predominance, similar to our early disseminated NB children, and one might speculate in that males are more susceptible to the development of disseminated LB?

In atopic disease, sex-related differences in immune responses are seen in early childhood (Uekert et al. 2006) as well as in various infectious diseases (Aulock et al. 2006; Nagayama et al. 2006; Schroder et al. 1998; Travi et al. 2002; Walker et al. 1997). Studies on immune responses with a female-male perspective in children with LB have not, to my knowledge, been carried out. When we looked closer at results in Paper II and compared INF-γ and IL-4 immune responses in boys and girls with NB, no major differences in cytokine profile between the sexes was found.

In NB, when looking at cellmediated immune responses in CSF and serum, we found that children with NB show a higher number of *Borrelia* specific INF-γ and IL-4 secreting cells in CSF as compared to controls, but also more IL-4 as compared to adults. Since this is believed to represent an effective and balanced immune response in a relevant compartment, it is suggested that this finding contributes to the more benign course of the disease seen in children as compared to adults with NB. Among children with NB, no specific cytokine was correlated to a less favorable outcome.

Correlations between humoral immune responses and cellular immune reactivity are important in understanding both pathogenesis and diagnostics of NB (Ekerfelt et al. 1998; Forsberg et al. 1995a; Widhe et al. 1998). Antibody production and cellular immune responses to OspC and Osp17 (DbpA) in children with NB has been characterized (Pohl-Koppe et al. 2001). Interestingly, OspC elicited both humoral and cellular immune responses whereas DbpA elicited mainly a humoral response, suggesting that OspC is predominantly expressed during the early acute phase of LB, and DbpA mainly during late stage disease. These results are not in concordance with our results in Paper III, showing antibody reactivity to both DbpA and OspC in rather early stages of NB. However, since we did not follow antibody reactivity during the course of the disease, a comparison between results needs to be made with caution. Furthermore, in Paper II we showed cellmediated immune responses when stimulated with OF (OspA and OspB) which seems to be similar to cellular responses to OspC (Pohl-Koppe et al. 2001).

In our study, the CSF samples were collected on admission to hospital but no follow-up samples were available. It would have been interesting to investigate the antibody response NB as well as the immune responses at different time points in NB, for diagnostic purposes as well as for improved understanding of the importance of different immune responses during the corse of the disease. Repeated lumbar punctures in children are, in my opinion, not ethically feasible while repeated blood samples would have been desirable. However, since CNS is a privileged site and the immune resposes are shown to be compartmentalized (Ekerfelt et al. 1997a), there is an explaination to why no serological test showed a better diagnostic performance in blood samples than in CSF (Paper III). In addition, immune responses in Paper II are shown to be stronger in CSF than in serum. Consequently, a lumbar puncture will still be needed when investigating patients with clinically suspected NB.

IMPLICATIONS FOR PEDIATRIC PRACTICE

This study clearly shows why pediatricians find difficulties in evaluating children with clinically suspected NB. A large number of patients (59 %) with signs and symptoms indicative of NB do not meet the criteria for “Confirmed NB”, because lack of intrathecal synthesis of *Borrelia* specific antibodies. Results in this thesis support the notion that the majority of patients with mononuclear pleocytosis in CSF are NB cases where *Borrelia* antibodies cannot yet be visualized. Hopefully, in the a near future, improved diagnostic antibody tests in NB will come into use in clinical practice to simplify matters for the pediatrician and to reduce anxiety for patients and parents.

In children with NB younger than 8 years of age, antibiotic treatment needs to be administered i.v. because of unwanted side effects from oral doxycycline (Grossman et al. 1971). Therefore, no pediatrician wants to prescribe antibiotics to young children on vague grounds. Unfortunately, there is a 1-2 week delay in confirming the NB diagnosis with results from *Borrelia* antibody tests. Pleocytosis with mononuclear dominance in CSF on admission has been the rationale for decision-making on early antibiotic treatment, together with information of EM and/or previous tick bites. Our study shows that mononuclear pleocytosis is the strongest predictor for “Confirmed NB”, whereas information on tick bites, for example, is of no use in predicting NB. In addition, viral etiology does not seem to be a substantial problem in differential diagnostics. Thus, our results support the clinical routine that children with neurological symptoms and mononuclear pleocytosis in CSF should receive early antibiotic treatment, despite the lack of *Borrelia* antibodies in CSF. Hopefully, with the NB prediction score test, pediatricians can find further support in decision-making about early antibiotic treatment in children being evaluated for NB.

No child reported recurrent or progressive neurologic symptoms and clinical outcome was good. Furthermore, the pediatrician can rely on prompt clinical response to antibiotic treatment in most cases. Ten days antibiotic treatment in NB seemed as sufficient as 14 days, according to our results, but admittedly, the study was not designed as a comparative study of different antibiotic strategies.

There has been a discussion about treatment of EM and whether it is preferable to choose an antibiotic agent with penetration to CNS or not. Bennet et al has, in an extensive study on EM, argued for phenoxymethyl penicillin as the drug of choice, since they did not find any patients that developed neurologic manifestations after oral penicillin treatment of EM (Bennet et al. 2003). The question is controversial (Arnez 2007; Wahlberg et al. 2006), but our findings, with some children developing neurologic symptoms despite oral penicillin as EM treatment, seem to support a revision of treatment recomendations of EM in Sweden.

Bilateral facial nerve palsy has been considered pathognomonic for Lyme NB in children (Christen et al. 1993). We found bilateral facial nerve palsy in one patient without signs or laboratory findings indicative of NB. He had a suspected viral infection in combination with a media otitis. Consequently, we suggest that all children with facial nerve palsy should be referred to an ENT-specialist in order to find differential diagnoses and to evaluate recovery.

Adolescents have the lowest incidence of LB among all age groups (Berglund et al. 1995) and in our material they were over represented in the “Not determined” group. They often presented with few neurological signs but high rate of nonspecific neurological symptoms of long duration. Headache and fatigue were reported at follow-up, but not more frequently than controls. Among pediatricians, it is well known that symptomatology in this age group is difficult to interpret and that the incidence of headache and nonspecific symptoms is high in schoolchildren (Laurell et al. 2006; Rastad et al. 2006). In our study, there was no evidence that prolonged headache or fatigue was attributable to NB or connected to severe neurologic disease in any age group.

CONCLUSIONS

The NB diagnosis could be confirmed by production of *Borrelia* specific antibodies in CSF in less than half of the children being evaluated for NB and consequently, many patients ended up with an uncertain diagnosis. Two thirds of all patients reveived early antibiotic treatment and responded well. Surprisingly few patients were diagnosed as having other infectious or neurologic diseases.

Clinical recovery at follow-up was generally good and nonspecific symptoms, such as headache and fatigue, were not more frequently reported in patients than in controls. No patient was found to have recurrent or progressive neurologic symptoms.

Permanent facial nerve palsy was found in 22% of patients at the 2-year follow-up. The sequelae were mild to moderate but patients reported eye-closing problems, excessive tear secretion, pronunciation difficulties but also cosmetic complaints.

No specific prognostic factors were found in patients with facial nerve palsy or children with "Confirmed NB". Nor was any specific cytokine profile or antibody response to new *Borrelia* antigens in CSF correlated to a less favorable clinical outcome.

New *Borrelia* antigens (DbpA, BBK32, OspC, IR6) performed well in laboratory diagnostics of NB in children. If new antigens were combined with the routine flagella antigen in a panel, sensitivity was 82% and specificity 100%, which was superior to the flagella antigen alone.

Borrelia-specific IL-4 and IFN-γ secretion in CSF was prominent in children with NB, as compared to controls. Furthermore, children showed a much stronger IL-4 respons in CSF than adults with NB. This immune profile is believed to represent an effective and balanced type1/type2 response in a relevant compartment, and could possibly contribute to the less severe course of the disease seen in children as compared to adults with NB.

An NB prediction score test, based on clinical features on admission, is suggested to help physicians to determine whether to start early antibiotic treatment, before results from *Borrelia* antibody tests are available.

FUTURE RESEARCH

In this thesis, clinical outcome in patients with suspected NB has been studied prospectively and retrospectively. To get further valid data on long-term clinical outcome in NB patients, we have performed a 5-year follow-up study, including a questionnaire and a standardized neurologic and oto-neurologic examination. A matched control group was included. Preliminary results are in line with Paper I and IV, but need to be analyzed in more detail.

We have studied different *Borrelia* antigens in laboratory diagnostics of NB. An additional recombinant *Borrelia* antigen, decorin binding protein B (DbpB), has shown promising results in adults with early NB. Since children often represent early NB, we plan to include pediatric NB patients in future investigations of DbpB as a diagnostic test in NB.

As a small part of the large prospective ABIS-project ("Alla Barn I syd-östra Sverige"), we have studied *Borrelia* seroprevalence in 2000 healthy 5-year old children. Those children with *Borrelia* antibodies in serum, but no previously known *Borrelia* infection, will be studied with focus on cellmediated immune responses and compared to seronegative controls and seropositive adults. We want to further investigate which is the optimal response for protective immunity, and to bring some further understanding on why some individuals get an asymptomatic *Borrelia* infection while others get persistent symptoms.

Further studies on immune responses in children and adults will be undertaken, to elucidate the puzzling immunologic network that might influence clinical outcome in NB. Important players that would be of interest in future studies include complement factors and chemokines, as well as the balance in T cell populations like Th17 and regulatory T cells. In the long-term perspective, it would be desirable to find a way of diverging immune responses during infection into a beneficial direction. The rapid and efficient anti-*Borrelia* immune response in children could then serve as a model.

Further evaluation of other tick-borne co-infections, such as HGA or TBE, would be of interest in children being evaluated for NB. *Rickettsia helvetica* is another tick-borne infection hardly studied in children and we are planning a project with serology and PCR diagnostics in serum and CSF in children with tick bites, fever and pleocytosis in CSF.

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